

SOLVING THE BIOLOGY LITERATURE a primer

Robert P Bolender

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PREFACE

Why do we want to solve the biology literature? Currently it suffers two shortcomings: (1) meta-analyses report that all or most of our published data are incorrect (Ioannidis, 2005) and (2) our current reductionist approach to detecting changes experimentally does not allow a first principles approach. Solving the biology literature addresses both issues by updating published data. The central challenges of the primer include (1) discovering biology's rules and principles by copying the way cells change and (2) updating data - originally published as parts - to recover the missing connections and complexities by minimizing the distractions created by the methods. In cells, connections define rules as ratios, complexities appear as patterns based on equations, and phenotypes, which allow forward and reverse engineering, organize published data into well-ordered relationships of structure to function. Starting with the original data as published, the beginner can follow changes occurring within the current research model and in the reality of a rule-based model copied from cells.

We have a problem. Meta-analysis repeatedly assigns a failing grade to the biology literature because of inadequate statistics. Our published p values typically pass the theoretical part of the statistical test ($p \leq 0.05$), but not the practical part (effect size). Statistics is especially important in biology because we use it to detect biological changes by comparing two mean values (usually an experimental to a control). If the two values differ significantly, we conclude that a change occurred. However, this statistical approach to a biological change creates the problem. Implicit in such a conclusion is the assumption that a biological change occurred. Updating the biology literature will challenge this assumption by uncovering three inconvenient truths: (1) it appears that cells never figured out how to squeeze the complexity of a change into a single experimental data point, (2) the mean values we routinely compare statistically can contain two equally important sources of change (biology and methods), and (3) the consequence of mixing biological and methodological changes turn most data references (normally constants) into variables.

Since detecting biological changes appears to be the most important yet least understood method, writing a primer created an opportunity to rethink the change problem by starting from the basics. Recommendations (stated or implied) coming from the Biomatrix group (Morowitz and Smith 1987), which included (1) organize the published data of biology, (2) identify new theory structures, and (3) derive biology from first principles by reverse engineering it empirically suggested a way to proceed.

In truth, cells change one way, but we see the same change differently. That's the basic problem. To avoid our differences, we'll defer to the cells and copy the way they change. This means that updating published data will follow the rules of biology, which we must first discover. In publications, however, biology's changes become mixed with methodological changes but reported as a single mean value. This identifies the second basic problem. Finding solutions to the problems created by the methods required detailed workarounds often involving assumptions that needed testing. Although this will challenge the patience of the reader, it will explain how to design experiments that avoid most of the damage created by the methods.

As a practical application of copying biology, the primer will show that cells change to solve problems by changing relationships of structure to function often in curious and unsuspected ways. A biological change is far from a simple event. When cells change, they become the physical solution by literally transforming from the complexity of one phenotype into that of another. By simply applying a rule-based

approach based on first principles, we will use published data to replay snippets of a biological change as it originally occurred.

How to use this Book: This book – the third in the series - continues the solving biology story. It assumes that the reader is familiar with the underlying theme, which consists of moving experimental biology from its current theory structure based on simplifying biology to one that embraces complexity.

Background: Physics and chemistry qualify as basic sciences because they operate according to theoretical structures based mathematically on rules and first principles. As a result, they can routinely detect, predict, reproduce, and verify changes. Biology combines the theoretical foundations of physics and chemistry with those of living systems to define complex adaptive systems consisting of cells that can change, detect, predict, reproduce, verify, and do truly remarkable things.

The Biomatrix group assumed that organizing published data would lead to a first principles approach for biology empirically. Both primers (Bolender, 2019, 2025) could accept this assumption because the basic requirements were already in place. The postulates of biochemical homogeneity defined the relationship of cell structure to function (de Duve, 1964, 1974) and stereological theory became a practical application because Weibel and Paumgartner (1978) and R. Miles found solutions to the section thickness problems. By combining current theory and practice, published data offered a simple, cost-effective way to approach cell changes as complex events. The primers simply unpack the complexities of the changes by applying rules and principles copied from the changing cells. Although cells cannot detect changes statistically by comparing single data points, they excel at changing parts and connections as complexities.

Managing Complexity: The complexity of living systems first required simplification, which investigators accomplished by taking organisms apart to characterize the properties of the parts. Consequently, the structure of the biology literature resembles a catalogue of parts subdivided into structural and functional disciplines. Since each discipline had its own set of rules and methods, attempting to explore a biological change by reconstructing cells with data from different disciplines becomes a daunting task for the beginner. By studying a small part of a much larger change, a given discipline misses the overall mechanism and strategy of a cell change. Since a change occurs only as a complexity, it must be studied and understood as a complexity.

When something causes a cell to change, for example, it must first diagnose the problem and then become the solution by physically altering many relationships of structure to function. Since a cell cannot define a change as an isolated biochemical or morphological event, it must undergo a complex remodeling of its parts and connections to produce successful outcomes quickly, efficiently, and reproducibly. To detect and explain the change experimentally, we must reconstruct (duplicate) what the cells did.

The primer reworked published data to figure out how cells orchestrated a change that occurs as a complex set of rule-based events. The updating strategy consists of using an eclectic collection of published parts to put cells back together (forward engineer) so that we can take them apart (reverse engineer) to discover how they changed to solve problems by applying rules and first principles. For the beginner, the difficult part of the exercise involves figuring out how to integrate data collected by different disciplines that report

results with different data types and references. To exist as a standalone entity, however, each discipline must resort to making assumptions (knowingly or unwittingly) to account for the missing information that normally would have come from other disciplines. By combining data from several disciplines, such assumptions typically become unnecessary. As we work through the case studies, the reader will follow the updating process and become privy to the fine points of designing and executing more powerful laboratory experiments.

Understanding the Point of The Books: Biology has figured out how to survive and prosper by implementing strategies and methods that have become largely foreign to the way we run our research programs. Under the umbrella of a research model based on simplification, the biomedical literature has become largely a statistical interpretation of biology, wherein population data replace those of individuals and significant differences substitute for verifiable changes. If, as reported by meta-analyses (Ioannidis, 2005), all or most of the results published in the biomedical literature are incorrect, then understanding why this is the case and what we can do to repair this deficiency of the literature becomes a rewarding exercise.

Figuring Out How to Detect a Change: The updating process begins by allowing biology to set the rules by example. In turn, we can (1) apply these rules to experimental models, (2) discover how reproducibility, which serves to verify a biological change, contributes to the robustness of our experimental designs, and (3) use the strategies described herein to minimize the unbridled ability of methods to distort experimental results. When encountering unfathomable results in the lab, consider the possibility that you may have encountered the presence of a different theory structure operating under a different set of rules. Try to capture the properties of an unrecognized theory with equations and use analogies and paradoxes to look for clues and explanations.

Minimizing Assumptions: Skillful investigators eliminate or minimize assumptions. When detecting a cell change statistically, three assumptions automatically come into play: (1) the sampling was unbiased, (2) the data point(s) tested for significance represented a biological change, and (3) the associated methodological disturbances were not important. By reproducing the rule-based strategies used by cells to change, one puts such generous assumptions to the test. Once we know how to reproduce a cell change, we also know how to reproduce experimental results.

Communication: When approaching cells as complexities, a conversation begins because cells and investigators can speak the same language (mathematics). The experimental design asks the question(s) that the cells answer by changing patterns and relationships, which one can read from the rule-based data, connections, phenotypes, and recipes. Instead of being content with just asking cells statistically if they change (yes? or no?), an enterprising investigator can ask for the algorithms, the times and locations of major events, and the reasons why the cells chose one solution over another.

Training AI Models to Explore Biology as a Complexity: Since AI is likely to become the major interface to the biology literature, we can expect that answers to our questions about complex biological events will reflect a reductionist view of biology if the AI was trained with the original literature. If the

algorithms and strategies used by cells to solve problems become of interest to the reader, then the updated data tables appended to the figures of the case studies can serve as training sets.

Acknowledgments¹: The decision to approach biology as a complexity came from a month-long workshop held in Santa Fe, NM (1987) under the auspices of the Santa Fe Institute and funded by the NIH, NSF, and Simon Foundation. It was held in response to a recommendation of the National Research Council (1985). The attendees were charged with the task of figuring out how to organize all the published data of biology in such a way as to reveal generalizations, connections, and new theory structures. The effort produced a strategic plan accompanied by a list of recommendations (Morowitz and Smith, 1987).

In turn, the insights, guidance, and enthusiasm provided by this workshop led to a pilot study (Bolender and Bluhm, 1992) and then to a grant from the National Science Foundation (NSF). The goal of the NSF proposal was to organize the published data of biological stereology within the framework of a relational database. This grant along with helpful suggestions from the NSF provided the foundation for the Enterprise Biology Software Project (2001-2025), which included yearly progress reports, books, and widely distributed software packages (2001 to 2018). The mission of this project was simple, but critical: provide rigorous and objective support to students entering research disciplines in the biomedical sciences.

We can attribute the success of this project to the guidance coming from the Biomatrix Workshop, the generosity of the stereology community for supporting the project and supplying reprints for the stereology literature database, and to the Internet Brain Volume Database (Kennedy, et al., 2012) for providing on line access to the MRI data of patients. For this book, special thanks go to the Journal of Cell Biology for making many of the key publications used for the literature updates and case studies freely available on line.

¹Reprinted in part from Solving Biology – A Primer (Bolender, 2019).

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CHAPTER 1

INTRODUCTION

SUMMARY

The primer follows recommendations included in the Report of the Biomatrix Workshop (Morowitz and Smith, 1987) - the purpose of which was to find new ways to (1) store published data in relational databases, (2) identify new models and theory structures, and (3) organize all the published data of biology. Fortunately, the recommendations in the report served as clues, of which there were three. ***First clue: By storing published data in relational databases, rules and principles will appear in the patterns derived therefrom.*** It worked. Generating a blueprint from a parts database revealed that biology ordered its parts and connections using ratios of small whole numbers (Bolender, 2016). Extensive reproducibility verified the ratios of parts as a fundamental rule-based principle of design. ***Second Clue: Using the insights coming from the first clue, construct a new theory structure for biology.*** Since such a recommendation seemed well-beyond the current state of our understanding, a workaround became necessary. If one assumes that the best theory structure for biology must be the one biology currently uses, then copying the way biology operates should produce plausible results. It worked. Cells solve problems reproducibly by changing relationships of structure to function (Bolender, 2019). ***Third Clue: Organize all the published data of biology.*** Since the previous clue led to an understanding of the way cells changed in a single experiment, scaling the literature from one experiment to many involved using data normalized from many papers to reconstruct global phenotypes. Consequently, the phenotype became the candidate model for organizing published data. It worked. Moreover, forward and reverse engineering phenotypes revealed the rules and first principles of a cellular change (current book). Curiously, a change emerged not as a single problem, but instead as a collection of smaller problems that the cells solved as they literally grew into the solutions. ***The Big Clue: In cells, the essential features – rules, principles, changes, reproducibility, relationships of structure to function, and complexity – all come from ratios.*** This means that solving the biology literature becomes an exercise in updating published data by expressing them as ratios. Moreover, by learning how to copy the way cells change with ratios, cells can show us how to use complexity and offer insights into to what becomes possible by applying the properties of quantum entanglement to parts larger than subatomic particles. In short, the recommendations of the Biomatrix Report led to a strategy for using forward and reverse engineering to derive biology from first principles empirically.

The absence of a first principles approach to biology as a science has created a host of problems. We reduce a complex biology to a collection of isolated parts, believe that a significant difference between two parts detects a biological change, and struggle to reproduce results routinely (Collins and Tabak, (2014). Although we have chosen to detect biological changes statistically, meta-analyses continue to remind us that we failed to apply the rules of statistics correctly (Ioannidis, 2005, Begley and Ioannidis, (2015). Some believe that fixing our statistics will solve our problems without first fixing our methods for detecting biological changes.

Curiously, we give surprisingly little attention to ferreting out the sources of our experimental problems. Why, for example, can't we reproduce experimental results? Since reproducing a result means detecting the same change a second time, could this mean that we didn't detect the change correctly the first time? If a biological change occurs as a rule-based event, how can we expect to get the same result twice when we don't know what rules to apply? Even our definition of a change is at odds with the one of biology. We believe that we can detect a change by demonstrating a significant difference between two isolated data points. In contrast, the biological cells charged with the task of changing must treat a change as a complex problem-solving event, wherein the solution typically involves a major remodeling of their phenotype.

While biology with its complexity and rules is not the problem, the damage and confusion created by our experimental methods is. By taking a reductionist approach to biology, we avoided the anxieties of complexity but lost the protection of biology. The "biological changes" we presume to measure come from data taken out of the context of a complex information management system, which when absent can no longer alert us that our methods routinely contribute as much to a change as biology.

This chapter previews the primer, introduces the experimental models and theories, explains the problems, and includes examples of updating published data. For some, the introduction will seem overwhelming because complexity comes with many parts, connections, relationships, and methods – all of which can change in different directions at the same time. Approaching biology as a complexity turns on a fire hose of details, which become less daunting once the reader sees how everything fits neatly together. By following the strategies and problems accompanying the updating process, for example, one learns how to optimize experimental designs by first understanding how cells solve problems. Bear in mind, however, that this is a work in progress and many questions remain unanswered.

1.1 Solving the Biology Literature

Solving the literature is akin to solving biology (Bolender, 2019) but on a larger scale. Experimentally we take biology apart, study the parts, and publish the results. However, the parts alone can't tell us how a change occurred unless we first put the parts back together (forward engineer) and then take them apart a second time (reverse engineer) to discover how biology originally put them together. Such a task begins by updating published data.

The updating process includes recovering the original biological parts and connections, reconstituting complexity, and exploring changes according to biology's rules and principles. A biological change occurs as a problem-solving event wherein a new cell phenotype replaces the original. In effect, the reconfigured cell becomes the solution. To detect changes and discover solutions, we'll reconstruct cell phenotypes from parts (membrane organelles and enzymes) and connections (data pair ratios). By reverse engineering the reconstructed phenotype, one can see that data pair ratios form subgroups, which can interconnect with other subgroups (bridging data pairs). The key insights to come from reverse engineering cell phenotypes is that a change begins with shifting relationships of structure to function, continues with changes occurring in data pair ratios across multiple subgroups, and concludes with the solution (a new cell recipe).

By documenting the changes in the data pair ratios that occurred between input and output, the phenotype model provides new insights into the complexity of a change and the importance of knowing where and

when to look when detecting, interpreting, and reproducing a change. It short, it takes an army of changes for hepatocytes to find a solution.

When updating the biology literature, the major source of our problems come not from biology with its well-defined complexity and rules but from the ambiguities created by our experimental methods. By taking a reductionist approach to biology, we avoided the anxieties of complexity but lost biology's protection. We missed a critical piece of information. The changes we measure and publish represent a mixed bag in that they come from biology and our methods – often in equal amounts. By simply transferring published data from a reductionist platform to one based on biological complexity, most of the methodological distractors become apparent and fixable. Examples provided in the case studies pursue a strategy that allows us to generate new information from old by simply learning to play by biology's rules. Although we lost the convenience of a simple statistical change, we gained access to biological changes and reproducibility.

1.2 The Data

Solving the biology literature begins by solving the data problem. In keeping with reductionist theory, publications included simplified data with connections and complexity largely removed or ignored. In turn, authors tested the remaining parts for significant differences and statistics became the arbiter of a biological change. To wit: detecting a significant difference between two data points became synonymous with detecting a biological change. By conflating significance with change, the bond between a biological change and biology disappeared and as a result biology became a largely methods-driven science. Updating the literature, however, forced a return to the original data-driven setting of biology, which appear in Table 1.1.

Table 1.1 Goals of updating simple data to complex.

SIMPLE DATA (Methods-Driven)	T0	COMPLEX DATA (Data-Driven)
Statistical Change Data	→	Biological Change Data
Biased Data	→	Unbiased Data
Biased Reference Data	→	Unbiased Reference Data
Published Data	→	Phenotype Data
Simple Data	→	Complex Data
Isolated Data	→	Connected Data
Population Data	→	Individual + Population Data
Unshared Data	→	Shared Data
Discontinuous Data	→	Continuous Data
Eclectic Data	→	Normalized Data
Unreproducible Data	→	Reproducible Data
Unreproducible Pattern Data	→	Reproducible Pattern Data
No Structure-Function Data	→	Structure-Function Data
No Enzyme Density Data	→	Enzyme Density Data
No Symmetrical Data	→	Symmetrical Data
No Recipe Data	→	Recipe Data
Statistical Interpretation of Data	→	Biological Interpretation of Data

1.3 The Puzzles

The biomedical literature exists as a giant puzzle consisting of named parts (pieces of data), typically many steps removed from the intact originals. In biology, these pieces fit together perfectly. In the biological literature, this is no longer the case. Why not? Because the literature adheres to a simplifying theory structure (reductionism) curiously inconsistent with the task of exploring living systems that we know to exist only as complexities. Consequently, the data – as published – require updating before we can begin to copy biology’s rules and discover the many ways cells can change.

Our theory structure allows us to read biology’s stories of complexity and change at a given level of understanding. To expand that understanding, the sciences routinely use theories as steppingstones to read the same stories in greater detail. Our goal here is to update published data from its current reductionist setting to a theory structure that more closely resembles the way biology operates as a complexity. By deliberately copying biology’s approach to change, the likelihood of accessing the rules and principles basic to change improves substantially because a biological change represents a highly structured and rule-based event. Shifting from simple to complex involves translating something old and often limited into something new and more informative.

In truth, complexity puts biology as a science in a favorable position because living systems have already figured out how to translate many universal laws and principles into practical applications. Biology can operate within and across multiple theory structures including those we associate with classical and quantum mechanics. Although the book adheres largely to a strategy based on copying the way biology changes within the familiar framework of classical mechanics, we’ll also be looking for clues that might tell us something about how biology interacts with the rules and principles of quantum mechanics. Perhaps a stratagem based on copying biology might continue to work beyond the boundaries of classical complexity theory.

1.4 The Solutions

If one organizes published data within the framework of relational databases, and if such databases lead to new theory structures, then it should be possible to organize published data and use them to derive biology from first principles via reverse engineering. This was the working hypothesis. However, it soon became apparent that everything depended on detecting biological changes correctly (Bolender, 2019). The most verifiable way of assuring correctness was to copy the way biology changes. Ordinarily this would be a reasonably straightforward task (biology follows rules and operates mathematically) were it not for the fact that the methods create just as much change as biology. Figures 1.1 and 1.2 summarize the strategy the primer used to detect changes in cells.

DETECTING A BIOLOGICAL CHANGE (PART 1)

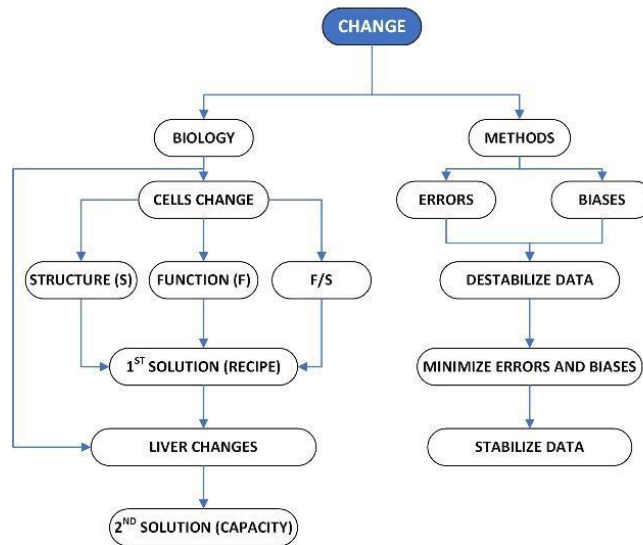


Figure 1.1 To solve a problem, cells change (1) the amounts of parts, (2) the relationships among the parts, (3) the structure-function recipes, and (4) the capacity of the liver to solve the problem. At the same time, methods alter the cell changes by adding errors and biases to the results. Unless corrected, a biological change becomes the product of two competing changes – one from biology and the other from the methods.

DETECTING A BIOLOGICAL CHANGE (PART 2)

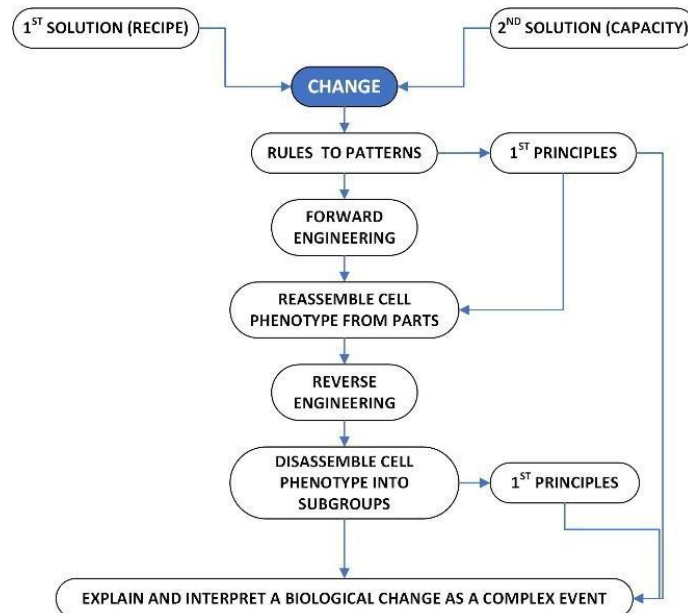


Figure 1.2 Updating published data involves switching the underlying theory structure of the literature from simple to complex. Explaining a change involves reconstructing the cell from its parts and then taking it apart to discover how the changes occurred. When copying the way biology changes, additional steps come into play: Update data → form data pairs → forward engineer a phenotype → reverse engineer the phenotype → identify subgroups → identify patterns → explain results → test results → make predictions → use reproducibility to verify the results.

1.5 Theories Used to Detect Biological Changes

In the sciences, theories create realities that define the rules for detecting, analyzing, and understanding change. They specify what we can see and do by creating “bubbles” of consensus wherein we can work together toward common goals. However, theories inevitably bump into inconsistencies that question their reliability and usefulness. When discrepancies remain unresolved for too long a time, bubbles burst, new bubbles appear, and the cycle repeats. Like most enterprises, a science ultimately survives by adapting to the ever-changing realities of its current circumstances. Figure 1.3 introduces the limitations of our current theory structure called reductionism.

1.5.1 Reductionist Theory

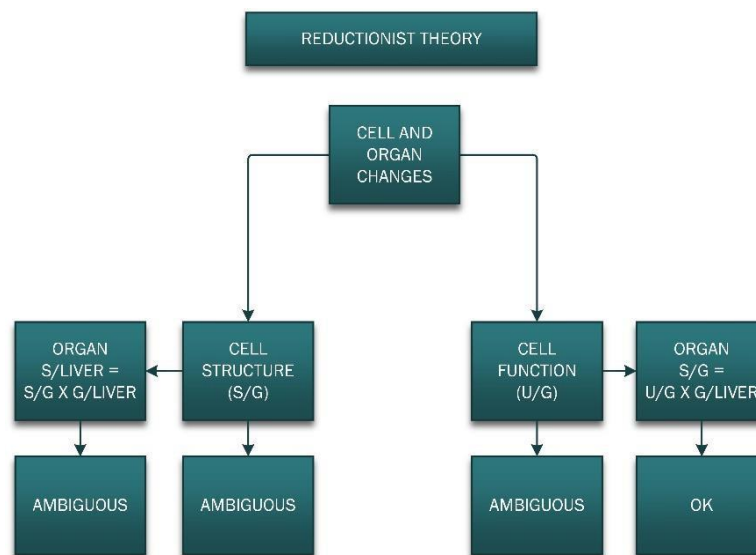


Figure 1.3 Typically, simplifying biology with reductionism misses or ignores many of the confounding changes attributed to biology and methods. These include: (1) errors introduced by changes in data references (changes in the number of cells being compared – the cell packing problem), which routinely affect both morphological and biochemical results, but remain largely unknown and uncorrected, (2) changes based on microsomal fractions that assume representative sampling without checking results by calculating recoveries, and (3) morphological results published without corrections for section thickness and compression tacitly assume that such errors do not affect the results importantly. With living systems, simplification leads to ambiguity.

Classical biology, chemistry, and physics all share reductionism as their primary theory structure (Mazzocchi, 2008). While all three sciences use similar physical data references (e.g., cm³, gram), biology runs into a problem because the contents of a standard unit of volume or weight continually changes in both control and experimental settings. As cells change their volumes, the number cells that can fit into in a physical unit of reference also changes. If cells lose volume, more will fit into the unit or conversely fewer when they gain volume. This means that we cannot assume that control and experimental data points come from the same number of cells. Under such conditions, wherein both the numerator and denominator of an experimental data point behave as variables, detecting and interpreting a biological change becomes a bewildering and unrewarding task.

The cell packing problem, for example, triggers a downward spiral.

- When both the data and data references change at the same time, detecting a biological change becomes problematic.
- When a change is problematic, reproducing the change becomes problematic.
- When reproducing a change is problematic, verifying results becomes problematic.
- When the results are problematic, the literature becomes a source of uncertainty.

Data references create compatibility problems.

- Biochemical studies report data as concentrations with results usually appearing as percentages or related to a mg protein or gram of tissue.
- Morphological (stereological) studies also report data as concentrations but prefer to express results relative to a cm^3 of cytoplasm or to an average cell.
- Since cells respond to problems by changing – quantitatively - relationships of structure to function, data compatibility becomes an important issue when updating published data.

1.5.2 Complexity Theory (Classical Mechanics)

Figure 1.4 summarizes the way complexity theory approaches a solution to the change problem. Note that the biochemical results of the model assume that the data came from homogenates.

In living organisms, wherein changes occurs continuously, copying the way cells change requires access to at least one constant. Instead of the usual weight or volume, we'll use a constant surface area (one square meter). We can do this by introducing a constant into the dataset algebraically by dividing units (U) of enzyme activity by a membrane surface area (S) to generate an enzyme density ($ED = U/S$). For this integrated approach to work, however, we become players in a single point of error (SPOF) game. To win (i.e., to detect a biological change correctly), each data point must have access to the five pieces of data shown in the Figure 1.4 along with necessary correction factors. The point? Detecting and interpreting a biological change requires access to both morphological and biochemical data because cells solve problems using structure-function recipes.

Theoretically, the model works. In practice, the hierarchy equation used to relate cytoplasmic surface densities (S/V) to the liver remains vulnerable to distortions in volumes (V) attributed to the experimental methods. Although workarounds exist for this problem, a correction continues to be elusive. When calculating enzyme densities [$ED = (U/G)/(S/G) = U/S$], cancelling out the data references algebraically eliminates the problem related to changes in cell number per gram (the cell packing problem), but the differences between a gram of fresh and fixed tissue remain. Tests included with the case studies will suggest the tissue fixation bias may amount to a minor error.

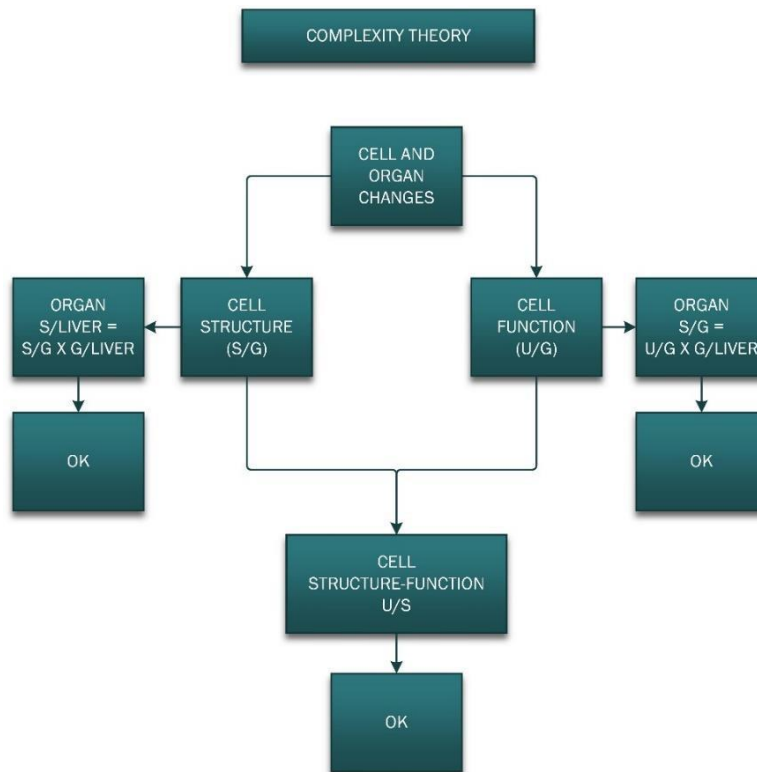


Figure 1.4 Complexity theory combines biochemical and morphological data into relationships of structure to function. When biology changes, these relationships change by rule. Under this theory, both change and reproducibility operate under the same rules.

1.5.3 Complexity Theory Displaying Quantum-Like Properties

Biology seems to have solved many if not all the problems that we encounter experimentally. It appears capable of operating in two distinctly different realities – determinate (classical mechanics) and indeterminant (quantum mechanics). Living systems may accomplish this feat by operating with parts ranging from in size from small (microscopic) to large (macroscopic), wherein the smallest parts operate within a quantum reality while and the larger ones appear in a classical reality. By creating opportunities to copy biology experimentally, complexity theory takes us close enough to the first principles of biological change to find events that one cannot explain by classical mechanics alone (Figure 1.5).

Enter quantum theory. Since electron microscopy creates access to many of the smallest parts of cells, we can postulate that changes in organelles and molecules carry the fingerprints of quantum theory. Reasoning by analogy, for example, allows us to attribute quantum-like properties to classical parts. This assumes that complexity theory extends our ability to copy biological changes occurring within the bounds of complexity theory to events previously linked in some unknown way to quantum theory. Since we already know that living systems have solved the problem of merging the realities of classical and quantum mechanics (living systems, photosynthesis, vision, etcetera), the postulate already enjoys empirical proof.

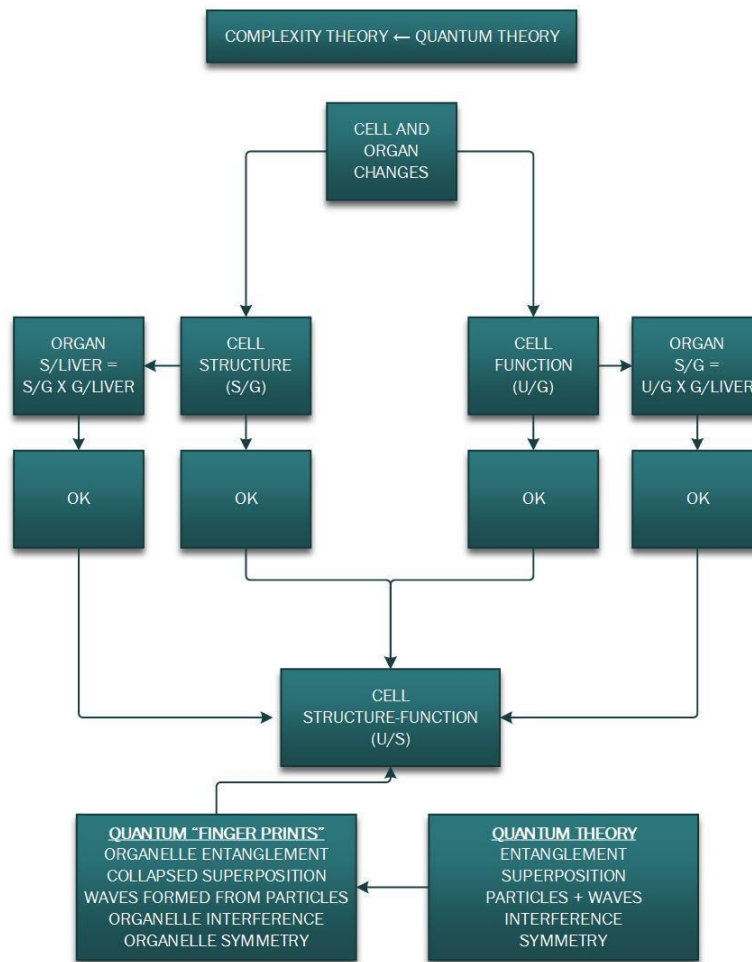


Figure 1.5 When detecting changes, “fingerprints” of quantum mechanics appear in classical settings.

1.6 Basic Assumptions Applied to Biological Changes

When updating our approach to detecting biological changes, new assumptions come into play that require attention first empirically and then theoretically. We start with the premise that copying biology’s approach to change means playing by biology’s rules. This introduces consequences - seen and unseen. By using updated publications to generate phenotypes, for example, the single point of failure constraint becomes activated. Since the biology literature contains simplified data, failures often occur, and workarounds may or may not succeed. Consequently, the primer becomes an exercise wherein both the author and reader practice complexity by making and fixing mistakes.

Listing the assumptions reminds us of where we need to be careful.

1. When mammalian species share similar hepatic genes, they share similar hepatic changes.
2. Similar changes suggest similar rules.
3. A biological change defines a robust approach to complex problem solving.
4. Cells interpret change as a problem-solving event to which they respond by growing into the solution.
5. A change occurs when one cell phenotype becomes another.
6. A cell recipe identifies a phenotype, wherein the recipe defines relationships of structure to function.
7. Different data types detect the same changes differently.

8. Reproducibility requires biological and experimental accuracy; significant differences require precision.
9. Published results can be statistically correct but biologically incorrect.
10. Published results can be statistically correct if biologically correct.
11. Copying biology means copying biology's rules and first principles.
12. Biology has mastered the art of translating universal laws and principles into practical applications.
13. Biology can tell us practically everything we want to know.
14. Everything we know or could ever understand either comes from or through our biology.
15. Science is the art of storytelling with mathematics.

1.7 A Brief Introduction to Updating Published Data

Going from a simplified interpretation of a change (a significant difference between two points) to a complex one (phenotype A → phenotype B), requires (1) attention to the details, (2) fewer assumptions, and (3) a willingness to play by biology's rules. In most cases, fixing problems would have required little more than access to data that the investigators collected during the experiment but did not publish.

Although publishers might not be keen to add the extra pages needed, authors could store raw data in database files designed specifically for future updating of the literature. Since biology is already well on its way to becoming a big data science, turning the biological literature into a renewable resource makes sense because individual publications contribute only brief chapters to much larger stories. To go after biology's mathematics, algorithms, computational coding, and information systems, one needs large amounts of well-connected published data. Biology is highly likely to become a breakthrough science because living systems already know how to bridge the gap between classical and quantum mechanics. Currently, cells know many things we already want to know.

Objective: Explain how organs, cells, organelles, enzymes, and other parts solve problems by changing their phenotypes (recipes).

Strategy: Identify experimental approaches that duplicate the ways the liver and its cells change.

1.7.1 Problems

- Copying the way biology changes, changes the traditional rules because biology treats change as a series complex problem-solving events occurring over time. Such an approach allows us (1) to follow a biological change empirically as the cells work out a solution and (2) to interpret the underlying events with forward and reverse engineering.
- When detecting biological changes experimentally, our methods add a second set of changes roughly equivalent to those of biology. In combination, biology and methods turn practically everything into a variable, including all the data references. Without a reliable data anchor, experimental results collapse into uncertainty. A solution to the change problem becomes tricky because we bundle the biological and methodological changes together in a black box (mean value) and then optimistically apply a biological label.

1.7.2 Methods

- **Structure:** By collecting stereological data from sections with thickness and compression, the resulting data carry biases because the theory behind the methods assumes no thickness and no compression. Fortunately, we can fix this problem by applying correction factors readily available from Weibel and Paumgartner (1978). In addition, preparing sections from living tissues can result in tissue shrinkage and swelling (Bertram et al., 1986, West, 2013), but workarounds exist. By limiting our use of stereological data to surface densities [$S/V = (\text{surface}(S)/\text{volume}(V))$], we can avoid these volume distortions by forming membrane ratios, which cancel out the reference volumes ($S_a/S_b = [(S_a/V)/(S_b/V)]$) and thereby eliminate the bias.

Stereology provides a solid theoretical foundation for estimating morphological parameters (volume, surface, length, number, etcetera) from measurements made on two-dimensional sections. Experimentally, this creates two problems. When applying these methods to electron micrographs of liver tissue, we use three-dimensional sections (instead of two) compressed in the direction of the sectioning. Consequently, specimen preparation breaks the link between theory and practice. Because of the tissue sectioning problem, stereological estimates carry biases based on the relationship of the section thickness to the sizes and shapes of the structures estimated. Accordingly, the literature updates include corrections for the section thickness and compression biases as specified by Weibel and Paumgartner (1978) and Weibel, (1980).

- **Function:** When relating changes in enzyme activities (assayed in tissue homogenates and fractions) to a gram of liver, the data become biased when the number of hepatocytes filling a gram of liver changes. Why? Because the number of cells that can fit into a gram of liver ($\approx 1.07 \text{ cm}^3$) depends on the average volume of the hepatocyte, which typically changes in experimental settings. Morphological data likewise suffer the cell packing problem. Multiplying units of enzyme activity (U) per gram by the weight of the liver removes the bias ($U/\text{liver} = U/g \cdot W_{\text{liver}}$) by cancelling out the weights. Tissue fractionation, which we use to collect the ER membranes in the microsomal (P) fraction, typically recovers about 50% of the total tissue ER. Microsomal results reported without the ER recoveries tacitly assume that the recovery remains constant – across all data points. When this is not the case, the results become ambiguous. A related but largely ignored problem exists when we relate biochemical results to a mg of protein based on the Lowry method for estimating protein. Since this protein assay estimates the amounts of only two amino acids (serine and hydroxyproline), it assumes that two amino acids faithfully represent all the amino acids (Lowry et al., 1951). If not, the method introduces a bias.
- **Structure-Function:** A biological change involves three quantitative changes - structural, functional, and structure-function. We identify a structure-function change as an enzyme density (ED) and estimate it by dividing units of enzyme activity per gram (U/g) by a membrane surface area (M^2/G): $ED = [(U/g)/(\text{m}^2/g)] = U/\text{m}^2$. The enzyme density identifies a complex data type capable of being a variable (U) in the numerator and a constant (1 m^2) in the denominator during a change. As such, the enzyme density becomes the essential anchor of a biological change. It plays a fundamental role in solving the biology literature in that it allows us to detect, interpret, and predict biological changes using change models.

1.7.3 The Updating Process

The biology literature sits on a reductionist foundation that relies fundamentally on a statistical interpretation of biological change. Fortunately, statisticians have challenged this simplified approach to complexity by reporting that most published results tend to be incorrect, at least statistically (Ioannidis, 2005). Specifically, what went wrong? Our definition of a biological change and the one biology uses are incompatible. By updating published data to work in concert with biology's definition, we can begin to do things that in the past only biology could do. Moreover, by allowing the data of a given publication to become part of an interconnected network of information, the power of the literature as a problem-solving tool becomes apparent. The strategy? Learn the rules, play by them, and look for hard problems to solve.

The updating protocol follows a set of steps designed to accommodate a wide range of data and applications. When updating the original data, for example, we'll often apply a mashup model based on data-sharing to expand the amount of information available to a publication. Using case studies, we'll work through each step of the updating process while asking questions about how different configurations of the same data can provide entirely new types of information.

The updating process includes the following steps.

- Collect original data: Apply corrections as needed.
- Expand the original data: Fit the original data to polynomial regressions ($R^2 = 1$) and then use the resulting equations to extend the data at equally spaced time intervals.
- Apply a mashup model: When possible, borrow results from other publications to compensate for missing data.
- Calculate enzyme densities using data from the adaptability layer (original data corrected).
- Normalize the expanded datasets: This applies to organ weights, enzyme activities, and membrane surface areas.
- Calculate enzyme densities from original (NORM1) and normalized data (NORM2).
- Express the normalized data as ratios (data pairs) in the rules layer. Plot the expanded datasets from the adaptability and rules layers to view the patterns of the changes.
- Forward engineer the phenotype (put it back together) – by rule – using normalized data pair ratios.
- Reverse engineer the phenotype (take it apart) to identify the changes occurring in multiple subgroups, which combine to produce the enzyme-membrane recipes – the ongoing solutions to the cell's problem(s).
- Repeat the process one publication at a time.

1.7.4 Case Studies (Individual and Aggregated)

Case studies, which include worked examples of updating publications, fall into four groups including development, drug-induced, miscellaneous, and lobular hepatocytes. When aggregated, each group becomes a global phenotype. By organizing the literature with phenotypes, one can scale information from simple to complex, aggregate data within and across publications, enable diagnosis and prediction, and support large scale simulations.

1.8 Data Layers

By copying biology's rules and principles, complexity theory provides insights into the way cells solves problems, which can first appear as paradoxes. When biology changes, for example, how does it reconcile the problem of allowing for local variability in the individual while at the same time maintaining the constancy of rules? In other words, how can the same data exist as variables and constants at the same time? Biology solved this puzzle by processing the same data two different ways. We'll do the same by putting the variable data in an **adaptability layer** and the consistent (and readily reproducible) data in a **rules layer**. Such a strategy worked for us as well because detecting and figuring out how biology changes requires two different interpretations of the same data.

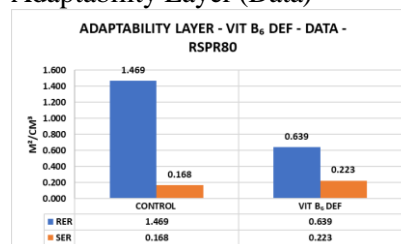
Adaptability Layer: The original published data become the adaptability layer. Subjects belonging to the same population typically display different amounts of the same parts in the adaptability layer. When testing a change statistically, outcomes improve by minimizing the variability among individuals.

Rules Layer: When calculated as ratios, data from the adaptability layer become the **data pairs ratios** of the rules layer. The rules – expressed as ratios - determine the relative amounts of two parts. Subjects belonging to the same population and displaying different amounts of the same parts in the adaptability layer routinely share the same ratios in the rules layer. When testing for representative sampling, applying reproducibility, copying biology, and working with phenotypes, data from the rules layer supply the results.

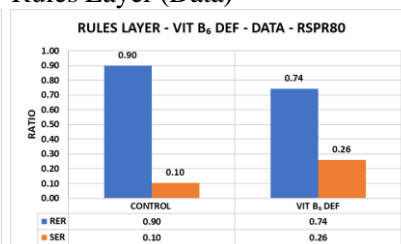
Note that results often include three plots, one from the adaptability layer and two from the rules layer (one before and the other after rounding the ratio data to a single digit). Rounded ratio data ease the task of identifying trends, duplications, and patterns.

An example will help. Riede et al., (1980) reported the effect of vitamin B₆ deficiency on the surface area of hepatocytic organelles. The histograms in Figure 1.6 show that vitamin B₆ deficiency viewed in the adaptability layer decreased the surface area of the RER, increased the SER, decreased the OMIM, and had no effect on the IMIM. In the rules layer, the RER to SER ratio went from 0.9:1.0 (control) to 0.7:0.3 (vitamin B₆ deficient) and the OMIM to IMIM ratio changed from 0.4:0.6 (control) to 0.2:0.8 (vitamin B₆ deficient). The vitamin deficiency changed both the amounts and ratios of the membrane compartments.

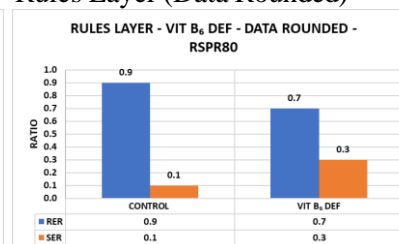
Adaptability Layer (Data)



Rules Layer (Data)



Rules Layer (Data Rounded)



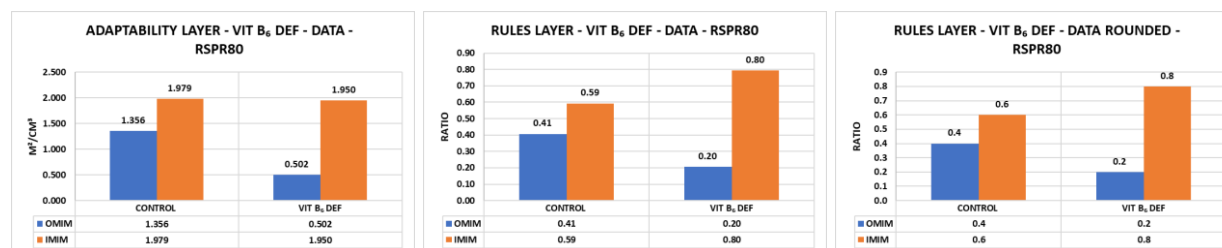


Figure 1.6 By forming ratios of the RER to SER and OMIM to IMIM with data from the adaptability layer, we obtain the rules – the quantitative relationship of one part to the other data in the rules layer (Original data adapted from Riede et al., 1980). [Note that figures carry a publication code (e.g., RSPR80), which identifies the authors (first letter of last name) and the year of publication (1980).]

In biology, we often find complexity nested in complexity, changes nested in changes, and rules nested in rules. If we combine the RER and SER into the ER and the OMIM and IMIM into the mitochondrial membranes (MIM), then we get a second set of results. Now both the ER and MIM showed little or no response to the vitamin deficiency (Figure 1.7).

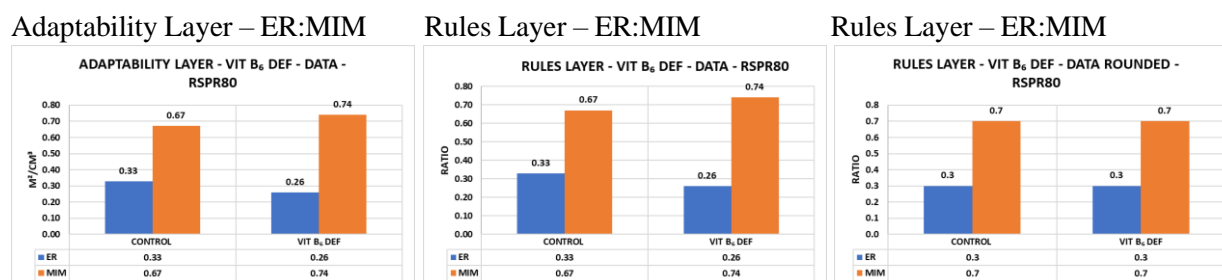


Figure 1.7 Control and Experimental ratios remain unchanged (Original data adapted from Riede et al., 1980).

Can we explain how the hepatocytes solved this vitamin deficiency problem? No. Why not? Because we don't know how the activities of the membrane bound marker enzymes of the ER and mitochondrial membranes changed or how such changes influenced the recipes of the enzyme densities. Moreover, given just a single experimental data point, we don't know whether the hepatocytes solved the problem or if they were still in the process of trying to solve it. What else don't we know? Without access to the liver weights, we don't know if the number of cells filling a cm³ of the reference space (cm³) changed.

Does this mean that we may never know if the hepatocytes solved the problem produced by the vitamin B₆ deficiency? Not at all. By searching the literature, it's possible to find the missing data and use them to generate answers to the questions above. Updating the literature within the framework of a mashup model actively encourages such data sharing. The case studies in chapters five to nine illustrate the effectiveness of this approach.

1.9 The Updating Strategy

Purpose: Updating the biology literature serves two purposes. First, it attempts to extract new information from published data by moving them from the simplified setting of reductionism to the complex setting of biology. Second, it treats the literature as a renewable resource wherein published research data benefit from what we have learned since the original publication. By adding the latest data types and methods, the updating process generates new information and in so doing increases the value

and longevity of publications. Basically, the primer addresses the cost effectiveness of biomedical research by treating publications as a renewable resource.

Assumption: Since mammalian species share remarkably similar liver genomes, the biological rules underlying their structures and functions and how they change are likely to be similar as well. Accordingly, the updating process includes the assumption of sharing, reproducing, and verifying data within and across species and publications. The mashup model becomes the vehicle for the sharing.

Process: Updating published data involves both data and methods. The process consists of applying corrections, relating data to common references, expanding datasets, forming data ratios, and normalizing data. The updating procedures also take a closer look at the data references, mean values, methodological errors, and corrections.

Training Set: Transitioning data from simple to complex involves updating a largely heterogeneous literature into a more homogeneous one. Since step-by-step directions for such exercise were unavailable, a strategy based on machine learning seemed a logical choice. The training set used for the update included fifty-five publications describing biochemical (enzyme assays) and or morphological (stereological estimates) changes in the hepatocytes of mammalian livers. The initial goal of the exercise was to figure out how to update data so that they could detect, interpret, and predict changes within the framework of a complexity model copied from biology (Bolender, 2019).

In machine learning, the software repeatedly cycles through a training dataset, each time adjusting values until it becomes optimized to a given task. Doing it manually involved revisiting each publication and reworking its dataset until the data, calculations, patterns, and results stabilized. After completing multiple cycles through the entire set of publications, most of the problems became identified, mitigated, or solved. In effect, an approach based on repetition proved to be the effective strategy for organizing published data into an equivalent of a biological change – the normalized phenotype. Perhaps of greater interest to the reader is the fact that such an exercise offers the beginner a hands-on approach to designing experiments that can deliver reproducible answers to the basic questions most studies try to ask. To wit: how, why, when, and where did the cells change to solve a given problem and to what extent were they successful?

The first time through the training set, the updating began by copying data from of each paper into an Excel worksheet, continued by applying corrections (when needed), and concluded by reformatting the data types (when possible). At the end of the first cycle, it became apparent that updating the biology literature might benefit from an approach based on copying and applying biology's rules and principles. However, there was a problem. When related to a wide array of time points, data types, references, species, ages, methods, exposures, and so forth, published data offered little if any basis for comparison. The second time through the stack of papers resulted in moving data from the adaptability layer to the rules layer by expressing the results as data pairs ratios. Although reproducible patterns – as rules - began to appear across publications, the scarcity of data still prevented paper to paper comparisons. The third time through produced a solution to the data scarcity problem by expanding the dataset with polynomial regressions, but still most papers still lacked critical data. The next cycle addressed the critical data problem by sharing data across publications. Additional cycles worked out solutions using normalizing datasets, and so forth. In short, each new idea became rigorously tested paper-by-paper by adding it to the cycle. Although time consuming, repeatedly cycling through the papers proved to be an effective way of recovering complex information from otherwise sparse, fragmented, and incompatible data sources.

Data References: In physics, chemistry, and biology, we routinely express experimental data as a concentration by relating a variable in the numerator to a constant reference value in the denominator

(usually, a standard unit of volume or weight). When detecting a change, for example, dividing the experimental value by the control gives the expected result because the reference value cancels out. While this approach works most of the time for physics and chemistry, it almost never works for biology because the numerator and the denominator of the concentrations become variables when biology changes. But, why? In biology, chemistry, and physics, a gram is still a gram and a cubic centimeter a cubic centimeter, but biology turns these references into variables by continually changing the cellular contents of the references. When the volumes of cells change, for example, the number of cells that can fit into the data reference changes. Consequently, the concentration data become ambiguous because they come from changes occurring in two variables – the numerator (data) and the denominator (data reference). For investigators, this creates an experimental nightmare because reproducing such a result independently often becomes technically impossible. Instead of reproducing just two data values in the numerator (one control, one experimental), one must reproduce four values: one value for the numerator (data) and one for the denominator (data reference) for both the control and experimental time points. For biology, comparing concentrations becomes a lethal trap for the unsuspecting. Consequently, most studies tacitly assume that the number of cells in the data reference remains constant. When compared to absolute data, concentrations will detect the same change only about fifty percent of the time (Bolender, 2019). And a practical solution? Move adaptability data into the rules layer where biology defines change and reproducibility with dimensionless ratios. Alternatively, there's a simpler solution.

Consider this. If data references worked as expected in biology, then we would expect them to give interpretable results because the denominator of a concentration would be acting as a constant. In phenobarbital treated animals, for example, hepatocytes respond to the drug by synthesizing new ER membranes enriched with drug-metabolizing enzymes. As the treatment continues, the hepatocytes produce ever increasing amounts of these cytoplasmic membranes thereby increasing their mean volumes. This means that over time, it takes fewer and fewer hepatocytes to fill a gram (or ml) of liver. In turn, this results in an increasing underestimate for both morphological and biochemical results when related to a mg of protein or to a gram of liver. We can recover these “lost” hepatocytes along with their membrane surface areas and enzyme activities by simply multiplying data related to a gram of liver by the weight of the liver. Unfortunately, investigators rarely apply this simple fix because reductionism assures us that we can collect parts, study them in isolation, and use them to generate useful information.

Reductionism and statistics work beautifully together when the goal is to show a significant difference between two parts. Likening a difference to a biological change, however, depends entirely on using data previously shown to have changed biologically. Otherwise, a significant difference tests for a difference, but not necessarily for a biological change.

1.10 Examples Taken from Updated Publications

In this section, we'll work through the basics of a change by observing the same change related to different references (original data adapted from Orrenius et al., 1965 – OEE65). In the first two panels of Figure 1.8, for example, the gram and liver references showed parallel changes for glucose-6-phosphatase (G6PASE) and demethylase (DEM) activities for the first four days of phenobarbital (PB) treatment but then diverged at day five. At day five the spread of the changes differed by almost 100%. In effect, we're seeing the result of multiple changes occurring simultaneously across all the enzymes. Changes occurred in the (1) enzyme activities, in the (2) number of hepatocytes in a gram of liver, in the (3) protein content of the hepatocytes, and in the (4) weight of the liver (not shown).

Original Data

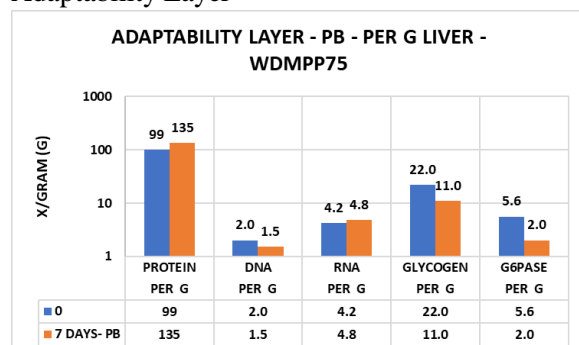


Figure 1.8 Different data references produce different results (Original data adapted from Orrenius et al., 1965).

The next example (Figure 1.9) shows the effect of comparing data collected from different numbers of hepatocytes (original data adapted from Wanson et al., 1975-WDMPP75). Note that hepatocytes exposed to phenobarbital (PB) for seven days became larger, which meant that compared to the controls it took fewer of them to fill a gram of liver. Consequently, the PB data point underestimated the amount of

change reported in the adaptability layer because of the cell packing bias. Moreover, this bias compromised the validity of the ratios (rules).

Adaptability Layer



Rules Layer

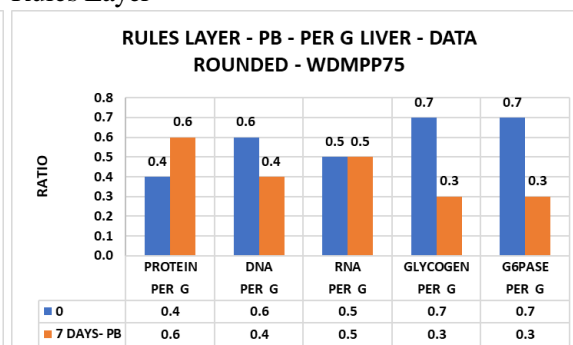


Figure 1.9 Unstable data references introduce methodological errors into experimental results (Original data adapted from Wanson et al., 1975).

The concluding example (Figure 1.10) shows changes in two enzyme activities related to three references (MG PROTEIN, GRAM OF LIVER, and LIVER) during a 48-hour period of phenobarbital treatment (Original data adapted from Noguchi et al., 1994 - NFHK94). Notice – as seen earlier - that the liver reference once again detected the greatest amount of change. Although the hepatocytes solved the problem posed by the experiment at the cellular level, the liver capacity ultimately decides the success or failure of the change. Ideally, the strength of the solution matches the strength of the problem.

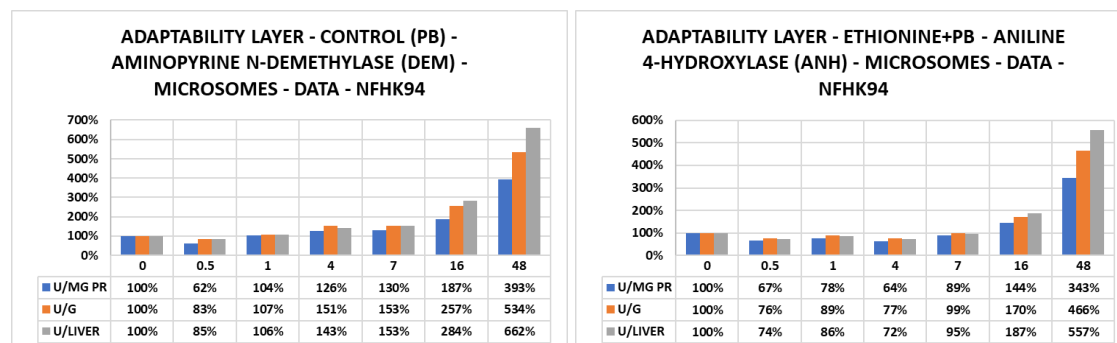
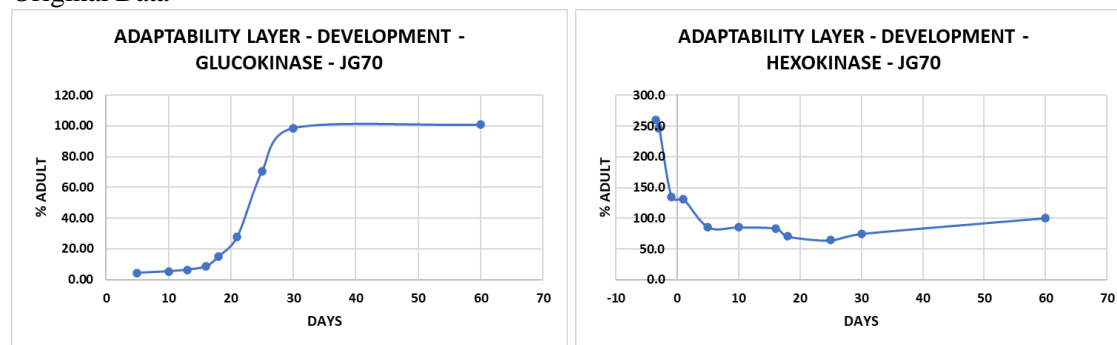


Figure 1.10 Sometimes different reference systems produce similar results, other times not. When both the enzyme activity and the number of cells producing the activity change at the same time, the results become ambiguous and consequently unreliable (Original data adapted from Noguchi et al., 1994). Here the liver serves as the gold standard because it includes data from all the cells (hepatocytes).

Original Data Expanded: During an early cycle through the training set, it became clear that the change model needed numerous and uniformly distributed data. The solution? Expand the original dataset to include a set of equally spaced data points. Since polynomial regressions allow one to fit the original data points to equations, solving such equations for equally spaced time points along the x axis solved the problem. Moreover, the expanded data mitigated the scarcity of data issue, supplied the uniform datasets needed to assemble phenotypes from multiple publications, and eventually led to workarounds for the incompatibility of data problems. Figure 1.11 shows data as published (Jamdar and Greengard, 1970; JG70) before and after expanding the data.

Original Data



Expanded Data

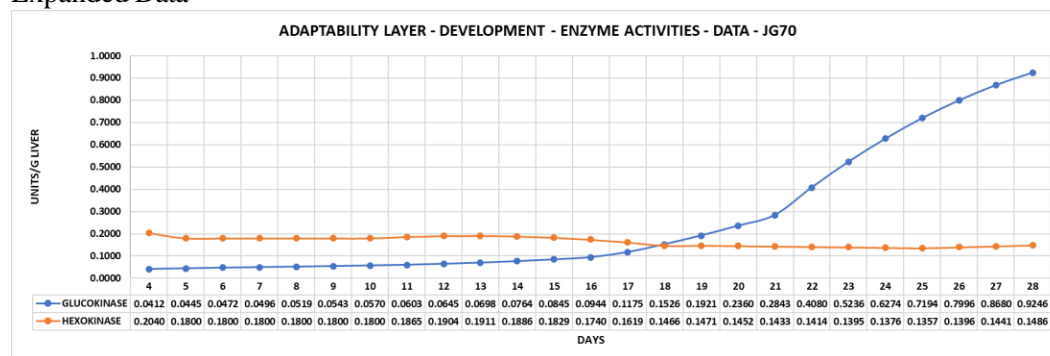


Figure 1.11 Regression analysis provides the equation, solving the equation at daily intervals expands the dataset (Original data adapted from Jamdar and Greengard, 1970). [Note that calculators for solving complex polynomials exist online.]

Complexity Layers (Adaptability and Rules): For biology, and for us as well, the same biological data occur on two different layers and exhibit different properties. The adaptability layer displays the original data collected experimentally while the rules layer shows the same data expressed as data pair ratios. The adaptability layer remains open-ended to accommodate the variability of individuals, whereas the rules layer limits the number of rules to ratios of whole small numbers (1 to 9). As shown earlier by compiling a biological blueprint of ratios (Bolender, 2016), biology prefers to use relatively few rule-based ratios.

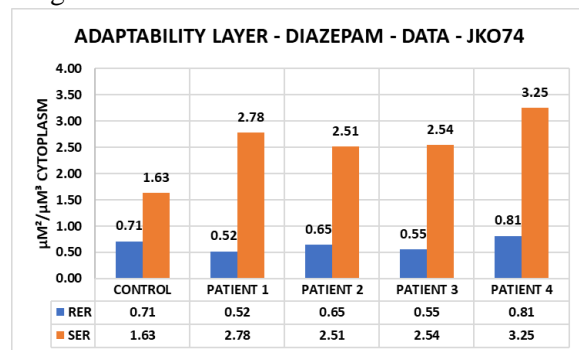
Since the rules define the individual and individuals define the population, it follows that the same rules define both the individual and the population. If true, then the rules layer becomes a built-in test for reproducibility for individuals and by extension for the population. This highlights two key properties. First, the ratios of the rules layer tend to agree numerically, whereas the original data in the adaptability layer do not. Second, checking ratios for reproducibility across the individuals in a sample provides a way to determine if the dataset in play comes from a homogeneous or heterogeneous population. Indirectly, it also serves as a check on the reproducibility of the methods. This means that we can inspect the properties of the population before we begin to assemble phenotypes or attempt to pass more challenging statistical tests. To sum up, reproducibility applied at the level of individuals becomes a quick test of representative sampling. Now let's put this idea to the test.

The point? Enforcing reproducibility locally with individuals takes a step toward solving the problem globally with population data. Recall that unbiased sampling assumes that each member of a population has an equal chance of being part of the sample. However, such a statement does not distinguish between homogeneous and heterogeneous populations. This raises several thought-provoking questions. If we use local tests of reproducibility to identify a homogeneous population, will that improve the precision and accuracy of our experimental results? Does a sampling protocol remain representative if it includes

individuals that display rules different from those of the population we wish to characterize? Would collecting data from a known heterogeneous population result in a sampling bias? What, for example, would be the advantage of studying or comparing groups of individuals changing differently because they were applying different rules? Would the effect size calculation be the same for both homogeneous and heterogeneous populations? In short, by running on rules, biology changes our rules.

Figure 1.12 shows the effect of diazepam on the hepatocytic membranes (ER) sampled from four patients (original data adapted from Jezequel et al., 1974 – JKO74). In the adaptability layer, the original data – corrected for section thickness and compression – displayed three different experimental values for the RER and SER membranes. When expressed as ratios in the rules layer, however, all four patients in the drug-treated group displayed the same rules (RER 0.2: SER 0.8). This would seem to be telling us that the results were reproducible across four patients and that they all belonged to the same rule-based population.

Original Data



Rules Layer (Data Rounded)

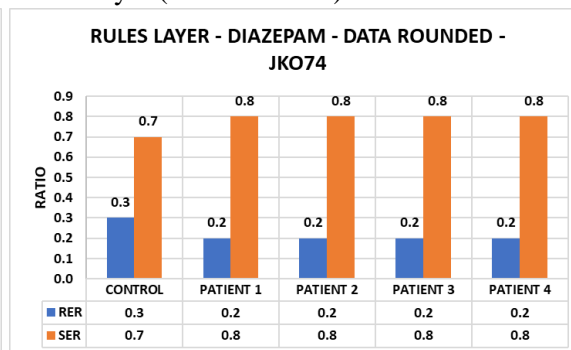


Figure 1.12 The adaptability layer showed the expected variability, whereas the same data viewed the rules layer detected a uniform response (Original data adapted from Jezequel et al., 1974). Four different patients solved the same problem by applying the same rule. Reproducibility across four individuals (0.2:0.8) suggests that cells can arrive at the same solution by applying the same algorithm (set of rules).

With the addition of biochemical data, we could have calculated the enzyme densities to see how the cells modified their ER in response to diazepam. When a cell changes, it changes enzyme activities, membrane surface areas, and enzyme-membrane recipes. This means that the results explaining a change come from a set of rules defined by the cell.

Structure-Function Changes: To calculate an enzyme density (ED), we divide units of enzyme activity by a membrane surface area. It represents a construct wherein the calculation generates a constant reference (one meter squared), which allows us to detect both morphological and biochemical changes. The constant is the one-by-one meter frame surrounding a membrane that can continually change structurally and functionally. In effect, the reference exists both as a constant and a variable (a paradox). With three variables in play, rearranging the enzyme density equation ($ED = U/S$; $U = ED \cdot S$; $S = U/ED$) allows us to predict an unknown variable when the other two are known. When we know the enzyme density, morphology predicts biochemistry, and biochemistry predicts morphology. This is how a science based on rules and first principles uses equations to get answers.

Recall that detecting a biological change experimentally becomes challenging because practically everything in the adaptability layer is changing. By evaluating the enzyme density equation, we create a solution to the problem of everything changing by introducing a virtual membrane window (one meter squared) into the cell that maintains a constant surface area while everything else within the window – the

enzymes and membrane - can change. In turn, we can extrapolate the changes occurring within the window to enzyme and membrane compartments, cells, and organs. Moreover, each step of the process becomes an opportunity to run a reproducibility test. When working with a complex information processing system such as a cell, knowing what's happening locally and globally increases the strength of the results.

The Enzyme Density Model: Figure 1.13 and Table 1.2 identify the cast of variables in play, noting that they can increase, decrease, or not change as the cell works its way toward a solution. The first thing to notice is that when biology changes more than one variable is in play. For example, 15 variables taken 2 at a time gives 105 possible outcomes. The point being that biology succeeds as well as it does because the solution extends across its hierarchy of size.

The table also shows that that many variables contribute to a biological change in unexpected ways and that both the change and the solution focus specifically on forming new relationships of structure to function. In effect, the enzyme density allows us to put biology back together at a time when practically everything – except the size of the reference window - is changing. Moreover, it repeatedly confirms the theory of biochemical homogeneity as postulated by de Duve (1974). Table 1.2 summarizes hierarchically the minimum dataset required to copy a biological change.

So far, the enzyme density is the one data type that can change and not change at the same time.

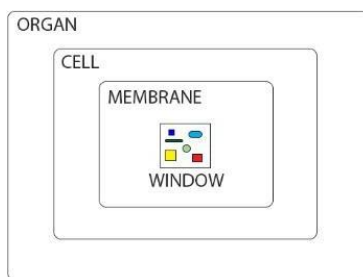


Figure 1.13 Biological changes occur hierarchically. Changes occur by changing enzyme-membrane recipes (shown above as a collection of colored bits) on membranes changing their surface areas, in cells changing their organelles and volumes, and in organs changing their volumes and weights. It's a group effort.

Table 1.2 A biological change taken apart. Since each part (except the frame) can increase, decrease, or remain the same, a change becomes a complex event.

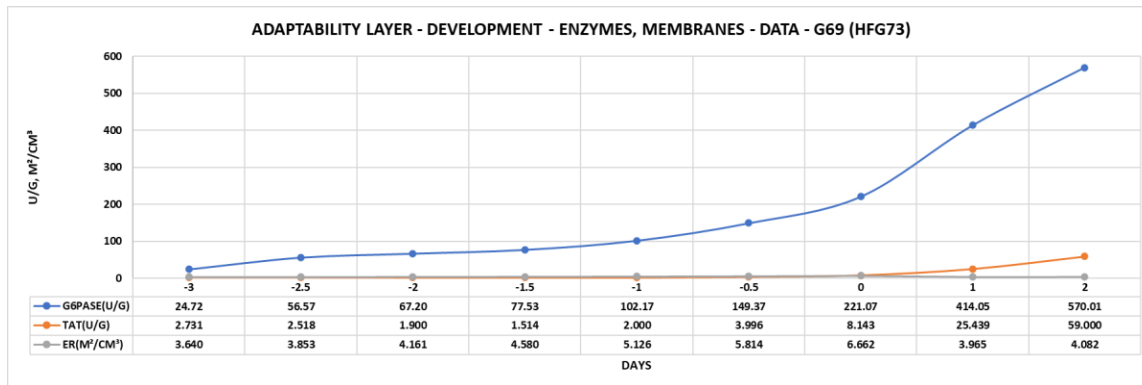
References	Variables	Range of Changes		
Organ	weight	increase	decrease	no change
	volume	increase	decrease	no change
	enzyme activity	increase	decrease	no change
	membrane area	increase	decrease	no change
Cell	volume	increase	decrease	no change
	enzyme activity	increase	decrease	no change
	membrane area	increase	decrease	no change
	enzyme density (U/M ²)	increase	decrease	no change
	enzyme ratios	increase	decrease	no change
	membrane ratios	increase	decrease	no change

	enzyme density ratios	increase	decrease	no change
Membrane	area	increase	decrease	no change
Window	enzyme density (U/M ²)	increase	decrease	no change
	enzyme density ratios	increase	decrease	no change
Frame	Constant	no change	no change	no change

Recall that we can relate changes to the liver by multiplying changes related to a gram of liver by the weight of the liver ($U_{\text{LIVER}} = U/G \cdot W_{\text{LIVER}}$; $S_{\text{LIVER}} = S/G \cdot W_{\text{LIVER}}$). Unfortunately, when we use the hierarchy equations of stereology to estimate liver values, the results may carry a bias related to the volume distortions produced by preparing the tissue for microscopy. Moreover, such a bias might vary from one experimental data point to the next.

Normalized Changes: Normalization allows us to generate literature-based datasets large enough to reconstitute phenotypes from data pair ratios. Starting with a column of data, the min-max normalizing equation converts the data into dimensionless values ranging from zero to one, wherein zero represents the smallest value and one the largest.

Figure 1.14 shows the same dataset before and after normalization (original data adapted from Greengard, 1969; G69). Notice how different the changes appear after normalization. Since we want to follow changes in enzymes and membranes displaying a wide range of experimental values, normalization conveniently puts all the changes within the same frame of reference (0 to 1). By removing data units, normalization allow us to represent a change as a polynomial curve defined by an equation. In turn, we can generate many such curves from many published studies to forward and reverse engineer phenotypes to discover the rules and first principles of a cell change.



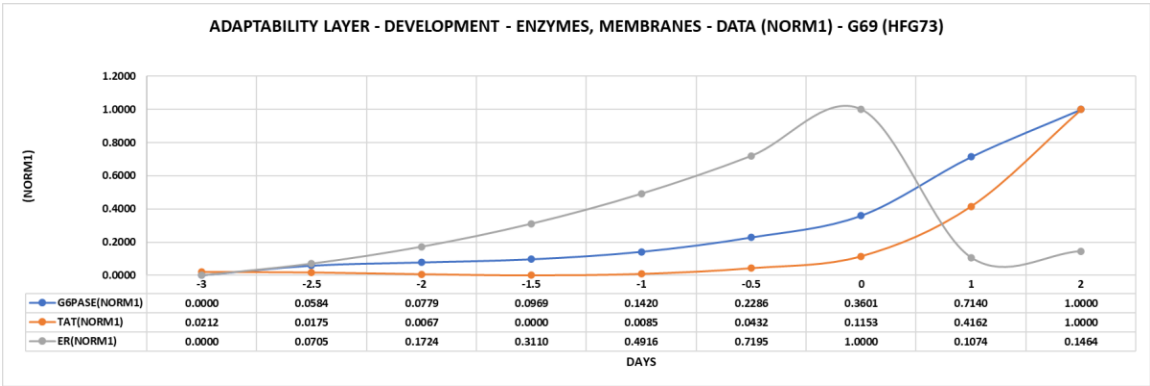
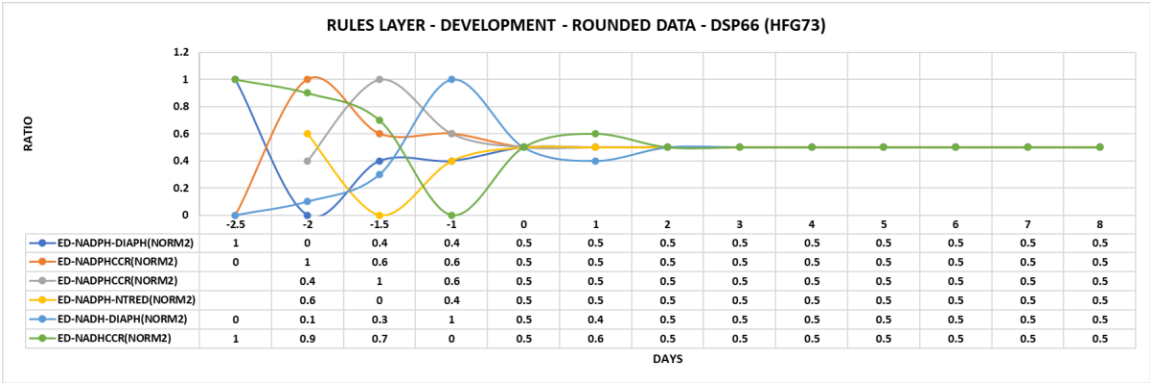


Figure 1.14 Normalized data (NORM1) share a single data range (0 to 1), wherein zero represents the smallest value and one the largest (Original data adapted from Greengard, 1969). The NORM1 plot reveals the details of all the changes.

The point? By delivering local and global pictures of a biological change, normalized datasets provide access to the basic details of a complex biological change. Moreover, using normalized changes in biochemistry, morphology, and in the relationships of biochemistry to morphology, creates an opportunity to predict gene expression merely by changing the names of enzymes to their corresponding genes. One of the unresolved questions in biology is what is doing what, where, when, how, and why. When hepatocytes change, are they supplied with the instructions for synthesizing proteins (mRNA) but left with the task of engineering and running the cytoplasmic phenotype that solves the problem, or do the genes largely micromanage the entire process? If organelles compute the solutions (microtubules?), do they also read and write the code (algorithms)?

Change as a Problem-Solving Event: Figure 1.15 illustrates changes in enzyme densities (expressed as data pair ratios) during pre and postnatal development (original data adapted from Dallner et al., 1966 – DSP66; Herzfeld et al., 1973 - HFG73). When viewed as a continuous event, a biological change displays distinct structure-function patterns, which when unfolded reveal the roles of parts and their relationships in problem solving. Before birth, the hepatocytes used the enzyme densities to search for a developmental solution, which appeared after birth on day 2 when the membrane ratios (recipes) remained constant (0.5:0.5) – by changing in parallel. Notice that order began with single data pairs (picture entangled) and progressed by adding additional data pairs.



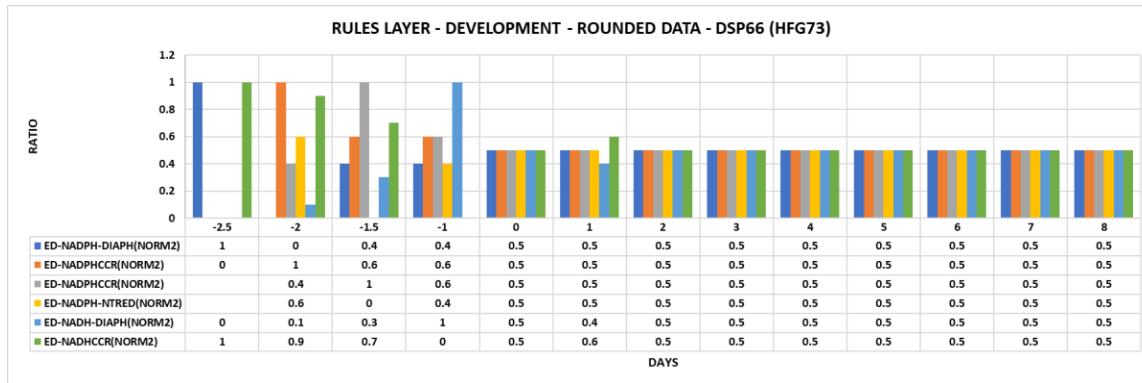


Figure 1.15 By combining structure and function quantitatively, enzyme densities tell us how long it takes the membrane-enzyme relationship to solve a problem. The group of three data pairs found the solution to their part of the developmental problem by postnatal day 2 (Original data adapted from Dallner et al., 1966 and Herzfeld et al., 1973).

1.11 Head Winds

In attempting to copy the way biology changes, one repeatedly bumps into long-held assumptions and practices. This tension comes from the fact that we have learned to approach a biological change as a simplicity, whereas copying biology requires unwavering attention to details, rules, and complexity. Since evidence continues to accumulate that we are failing to detect biological changes correctly (e.g., meta-analyses and problems with reproducibility), copying biology's approach to change offers a welcome and promising alternative.

The update routinely tests widely held assumptions and brings forward alternative solutions in keeping with a first principles approach.

1. **Assumption:** We believe we can take biology apart, study the parts, and understand how biology works.
In Practice: Biology – as an organism and a science – is an interface to complexity not to simplicity.
2. **Assumption:** We believe that studying biological parts alone avoids complexity.
In Practice: We can take the parts out of biology, but not the complexity out of the parts.
3. **Assumption:** Statisticians believe that all or most of our published results are incorrect.
In Practice: Fixing our statistical results will not guarantee a solution to the problem of detecting biological changes correctly.
4. **Assumption:** We believe that stereological results can provide meaningful results without applying corrections for section thickness and compression.
In Practice: Theoretical (Weibel, 1980) and empirical evidence suggest otherwise (Weibel and Paumgartner, 1978).
5. **Assumption:** We believe that mean values faithfully represent biological results and provide the most effective way to detect and report biological changes.
In Practice: Biological data come from individuals that represent the primary source of first principles and reproducibility. Mean values come from biology and methods.
6. **Assumption:** We believe that a mean value is true to its label.
In Practice: A mean value defines a black box containing one named variable plus an undetermined number of competing methodological variables and biases.

CHAPTER 2

EXPERIMENTAL METHODS BECOME VARIABLES

SUMMARY

Methods increase the difficulty of experimental biology because they partner with biology to produce “biological changes” with the split between biology and methods often around fifty percent. In turn, we package these combined results together as mean values and label them biological. This means that our task of identifying and removing the unwanted contributions of the methods from published mean values defines a central challenge of the literature update. In this chapter, we’ll introduce the major troublemakers and suggest workarounds. In short, experimentally induced changes can come from section thickness and compression, specimen preparation, concentration data, data references, tissue fractionation, assumptions, and faulty data analysis. An estimated mean value (morphological), for example, can include as many as thirteen experimental variables but only one from biology. Moreover, mixing biological and methodological variables produce unsuspected results. Data references - the constants basic to detecting a change - typically become variables. This begins to explain why detecting and reproducing biological changes correctly has become such an elusive goal. The solution? Return to the basics (complexity) and find workarounds.

Using methods to interact with biology changes the information we collect, our perception of the way cells change, and the quality of the data we publish. Since the effectiveness of a literature update depends on our ability to represent biology faithfully, knowing what can go wrong experimentally at the outset becomes an essential first step.

In this chapter, we consider how experimental methods influence our results and what we can do to mitigate the damage they create. Problems are persistent and pervasive. Variables assumed to represent biology unfortunately contain distracting variables produced by our experimental methods. When both biological and methodological variables contribute to a change, published results become ambiguous. Moreover, we amplify our methodological problems by the way we report our findings. Even though our results include two distinct sets of variables (biological and methodological), we combine them into a single mean value but assign just a biological name. The methodological variables remain nameless and often tacitly assumed not to exist. As a result, we now have a literature populated with mean values of largely unknown origin with no easy way to check for validity. Such a situation is the consequence of operating under a theory structure that prefers simplicity over complexity. We publish chiefly parts data, largely ignore the underlying complexities, and then struggle to understand why others fail to reproduce our results (Baker, 2016).

The point? In truth, we can take the parts out of biology, but not the biology out of the parts. Biological variables treated as simple objects turn out to be remarkably complex combinations of biological and methodological variables. This means that updating the literature requires a strategy based on minimizing the effects of the methodological variables currently populating our published mean values.

A second point. When different investigators estimate the same part, they typically publish different results. In effect, similar methods in different hands produce different results. How, for example, do we

deal with control values when they display differences with ranges usually reserved for experimental changes? Do our methods contribute to “biological” variation?

Chapter 2 considers five methodological problems we inadvertently create when collecting experimental data from biology. Once again, we start with the basics.

1. Confounding Variables: Biological data contain multiple variables.
2. Concentrations: Both the numerator and denominator of a concentration can behave as variables.
3. Data References: Different data references produce different results.
4. Mean Values: Mean values decrease reliability by introducing variability.
5. Biological Changes: Changes become defined by the methods used to detect them.

2.1 Problem 1 – Confounding Variables

Laboratory experiments collect two categories of variables: biological and methodological. Since the effect of the methodological variables on an experimental result can be equal to or greater than that of the biological, they contribute a major source of uncertainty. Consequently, the updating process makes every effort to identify and minimize the uncertainties produced by our experimental methods. Such a probing level of inquiry becomes indispensable when attempting a first principles approach to biological change.

Recall the familiar routine. When looking for biological changes, we compare experimental to control values wherein the control serves as the “constant” of the experiment. The literature, however, tells a somewhat different story. Although a given control works for a given experiment or publication, similar control values seldom occur across studies. This leads us to the unavoidable conclusion that published control values apply locally but not necessarily globally. But is this possible? If, for example, mammalian livers share nearly identical genetic coding, shouldn’t the parts from the same cell type in a similar control setting be largely the same? Theoretically yes, but in practice no because the confounding variables of the methods (M_i) routinely influence the biological results. But what are these disrupting variables and how do they end up in our results?

If we take control values from eleven of the papers used in our training set, we can begin to look for answers by generating patterns (1. Blouin et al., 1977, 2. Bolender and Weibel, 1973, 3. Weibel et al., 1969, 4. Bolender et al., 1978, 5. Krahenbuhl et al., 1992, 6. Krahenbuhl et al., 1990, 7. Denk et al., 1977, 8. De la Iglesia et al., 1975, 9. De la Iglesia et al., 1976, 10. Rohr et al., 1976, and 11. Pieri et al., 1975).

Figure 2.1, which summarizes the control values of the eleven publications identified above, shows what we’re dealing with. In estimating the surface areas of the endoplasmic reticulum (ER) in adult hepatocytes, the studies generated control values ranging from 1.8 to 9.2 m^2 /gram liver - a five-fold spread. Such a result leads us to one of two possible explanations. Either the observed variability is real, or it’s not. If it’s not, then our methods must be compromising our ability to reproduce experimental results.

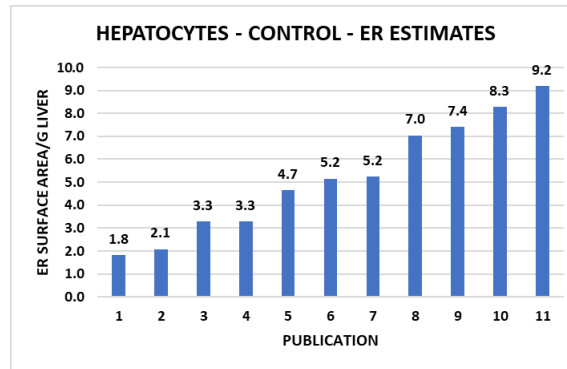


Figure 2.1 Published control values of the adaptability layer can display a remarkably broad range (Original data adapted from the authors cited above). Missing ER values were predicted from mitochondrial membranes using a prediction model based on Blouin et al., (1977).

The point? By updating this set of eleven publications, we can ask if the differences in the above surface area estimates resulted from the way we (1) prepare tissues for microscopy, (2) collect data, (3) interpret results, or all three. Although all the sciences experience methods related problems, our interest in reverse engineering biological changes requires a brief return to the basics to look for solutions to our methods related problems.

As the approach to the literature update developed, the initial idea of making corrections for the methods related problems evolved from thinking about specific problems and disciplines to looking for general solutions. This shift in strategy occurred once it became apparent that data from two disciplines - biochemistry and morphology – working together ended up solving more methodological problems than when working alone. Since most publications consisted of taking either structures or functions apart separately, a primary job of the update became one of reconnecting all the parts. A key insight to come from the reconnecting exercise was that biochemical data could fix the shortcomings of morphology and the other way around. But why did this work? Because cells connect morphology to biochemistry by rule mathematically. It's a basic principle of living systems.

Methodological Variables (M_i): By observing the relationship of M variables to estimates of organelle surface areas, we can begin to identify the problems we'll be encountering when looking for ways to update the literature. The data used for the ensuing plots came from the eleven papers identified at the outset.

Given the variability of data references, making paper to paper comparisons became problematic. This called for a workaround. By starting with a set of standard values for the organelles of hepatocytes (Blouin et al., 1977) expressed as proportions, entering a seed value (available data) from another publication into a simulator (based on the standard values) generated a collection of organelles that defined a linear curve with a slope M and a $R^2 = 1$. For example, two of eleven papers shown in Figure 2.1 contained mitochondrial data but not ER. This required a workaround. By using the results of one paper (Blouin et al., 1977) as a lookup table, data from one organelle provided access to the data of a missing organelle. In effect, the approach provided the homogeneous sets of organelles needed to make the comparisons. This explains the how and why slopes (M) compared results across the eleven publications.

(M₁) Species: When data come from different species can they display the same organelle recipes (proportions of morphological components)? Yes, but only sometimes (Figure 2.2). While the slopes indicated that both rats and humans produced different slopes, three papers ($\approx 30\%$) also showed that two

rats and one human (yellow bars) can produce the same control slope (1.0). This suggests - albeit tentatively - that different species can share similar values (recipes). The four-fold range in the estimates for the rats (4.2) exceeded the nearly three-fold range (2.8) seen for the humans. The point? Controls display changes with ranges usually associated with experimentally induced changes. Such a finding understandably questioned the possibility of aggregating data across publications.

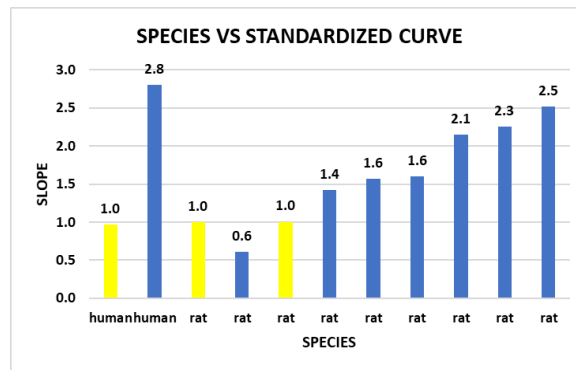


Figure 2.2 Different species can display similar and different values for the ER of controls (Original data adapted from the authors cited above).

(M₂) Glycogen: Why do studies of the liver often use fasted animals? Hepatocytes store glycogen in close association with the smooth endoplasmic reticulum (SER), which can obscure these membranes and lead to underestimates for SER surface areas. Moreover, glycogen can interfere with the separation of membranes into different fractions with differential centrifugation. This explains why both structural and functional studies often fast animals for 24 hours to deplete glycogen stores. For control data, Figure 2.3 shows an overlap between fed and fasted animals.

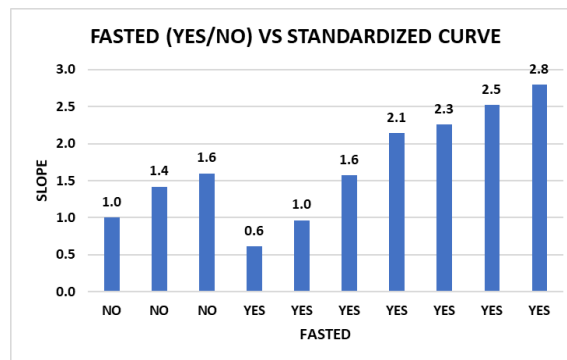


Figure 2.3 Estimates for the surface area of the smooth endoplasmic reticulum (SER) - with and without fasting - showed mixed results (Original data adapted from the authors cited above). Note that the increases in the SER seen for the fasted animals may be a compensatory response of the cells to the falling levels of cytoplasmic glycogen.

(M₆) Tissue Fixation: Is the variability of a stereological estimate independent of the fixation method? Apparently not (Figure 2.4). In tissues fixed by immersion, estimates for the control ER surface areas in the adult liver varied by almost 500%, whereas the same estimates based on perfusion fixation varied by

about 60%. Based on the data presented, immersion fixation produced a greater range of ER estimates than when fixed by perfusion.

Fixation – be it by immersion (tissue blocks held in a fixative solution) or perfusion (fixative pumped through the vascular system) – produced variable results for the ER estimates as shown in the figure below. On average, however, estimates for the ER were smaller for the livers fixed by perfusion than for those fixed by immersion. Note that the larger range in values for the immersion fixed material could have been the result of the fixation method and the condition of the tissue sampled. Tissue blocks fixed by immersion can display a distinct fixation gradient ranging from swelling at the periphery of a tissue block to shrinkage at the center. The results presented thus far point to the difficulty of using data from the adaptability layer to aggregate published data across publications.

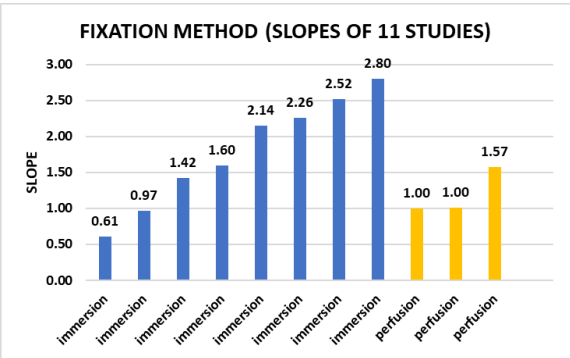


Figure 2.4 Estimates for control surface areas of the ER were more variable when fixed by immersion than by perfusion (Original data adapted from the authors cited above).

(M7) Fixative: When used as the primary fixative, both glutaraldehyde and osmium tetroxide displayed similar ranges for the estimates of ER surface areas (Figure 2.5).

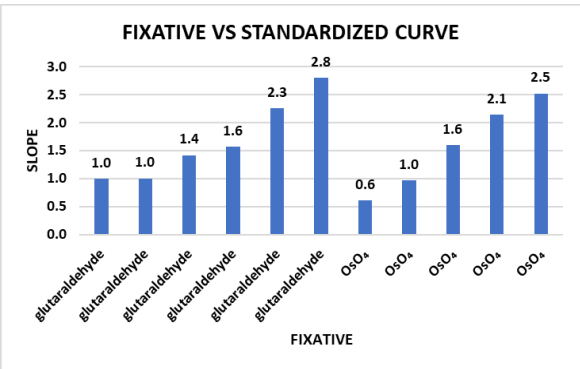


Figure 2.5 Estimates for the surface area of the ER showed similar variability for glutaraldehyde and osmium tetroxide (Original data adapted from the authors cited above).

(M₈) Fixative Buffer: Figure 2.6 shows overlapping ranges for the fixative buffers used by the eleven papers.

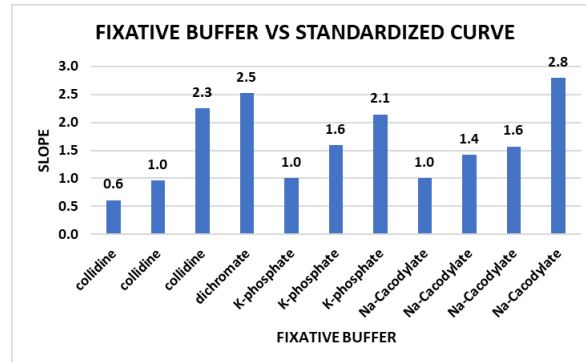


Figure 2.6 Estimates for the different fixative buffers showed similar ranges (Original data adapted from the authors cited above).

Summary: Five experimental variables [M₁ (Species), M₂ (Glycogen), M₆ (Tissue Fixation), M₇ (Fixative), and M₈ (Fixative Buffer)], which can play a role in influencing cell and tissue volumes, showed few similarities across publications. Such inconsistent results suggest that multiple variables come into play during tissue fixation. This information becomes useful because it suggests that our best option for mitigating the volume distortion problem might come from a general correction derived from both morphological and biochemical data.

(M₁₀) Tissue Sections: Figure 2.7 compares estimates for the ER and its subcompartments (RER, SER) - with and without corrections for section thickness and compression. Notice that the stereological estimates for membrane surface areas carry substantial overestimates because the sections have thickness and compression. The theory for estimating surface densities stereologically assumes no section thickness (two-dimensional sections) and no compression. Fortunately, we can deal effectively with this major source of error by applying correction factors (see Blouin et al., 1977 - BBW77; Weibel and Paumgartner, 1978). Notice that the errors produced by the section related biases can become large enough to change the results of an experiment.

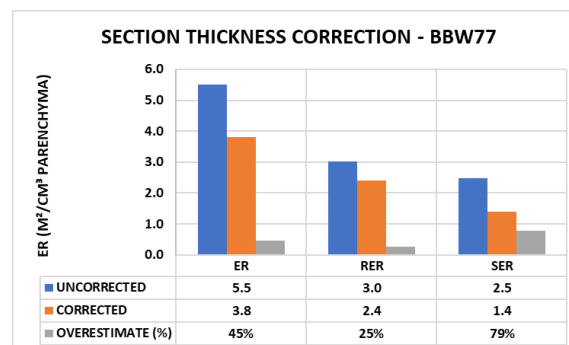


Figure 2.7 Estimates for the ER, RER, and SER displayed substantial errors when not corrected for sections thickness and compression (Original data adapted from Blouin et al., 1977).

(M₁₁) Cell Packing Problem: When the average cell volume changes, the number of cells filling a gram or cm³ of tissue also changes. It's a simple cell packing problem – more little cells fit into a cm³ or gram of liver than big ones. Consequently, detecting biological changes with concentration data carries a substantial load of uncertainty because both the numerator and denominator become variables. Curiously, the cell packing problem (concentration trap) has a quick and easy fix, that practically no one uses.

After relating a concentration to the weight or volume of the liver, change becomes indexed to the total number of hepatocytes and relative changes per liver and per average cell become the same (when cell number remains constant). By applying this basic truth, one can often read a research paper and quickly know whether to believe the results.

The M₇ variable (cell packing problem), which applies to both morphology and biochemistry, frequently represents a major source of error. Fortunately, the problem has a ready solution. When the total number of cells in the liver remains constant (or nearly so), we can avoid the cell packing problem by relating results to the weight or volume of the liver. Simply multiplying a result related to a gram of liver by the weight of the liver solves the problem for biochemistry ($U_{\text{LIVER}} = U/G_{\text{LIVER}} \cdot W_{\text{LIVER}}$) and for morphology ($S_{\text{LIVER}} = S/G_{\text{LIVER}} \cdot W_{\text{LIVER}}$). However, the morphological calculation assumes a similar number of cells in a gram (or cm³) of fixed and fresh tissue.

The usual stereological approach to the cell packing problem, which consists of counting cells to estimate average cell data, doesn't apply to the liver because cell counting involves counting nuclei. A liver hepatocyte doesn't fit the profile of an average cell because it has both mononucleated and binucleated cells as well as cells with polyploid nuclei that can routinely undergo fission and fusion. This means that the total number of hepatocytic nuclei in a liver can change – even when the number of cells remains essentially constant. Such an arrangement became essential for the liver because it can increase its synthetic capacity very quickly when called upon to metabolize harmful substances without resorting to mitosis, which might expose its DNA to damage.

Table 2.1 summarizes the methodological variables (M_i) we add to our mean values when reporting biological changes - with suggested workarounds. The influence of methods on experimental results becomes a major part of the data analysis by introducing uncertainties into the ways cells change. In response to the methodological damage, the updating process applied corrections, avoided errors, and relied heavily on biology's rules and principles. With access to the original raw data of the publications, most of the methodological problems would cease to exist. Regrettably, we don't publish raw data.

Table 2.1 Variables associated with mean values – effects and mitigations.

MEAN VARIABLES = (Biology + Biological Variation + Methodological Biases)			
Variable	M	Effects	Fix
M ₁	Species, Animal	Variable	Standardize data
M ₂	Glycogen	Obscures smooth ER in hepatocytes	Fast (24 hours) removes glycogen
M ₃	Tissue removal	Loss of blood pressure → shrinkage	Use perfusion
M ₄	Tissue Sampling	Unbiased = Design-Based Biased = Model-Based	Favor unbiased sampling methods
M ₅	Multiple Stage Sampling	Different embedding → incompatible reference volumes	Use only one Embedding medium
M ₆	Tissue Fixation	Immersion → uneven fixation Shrinkage (≈ major) Swelling (≈ minor) Perfusion → uniform fixation Shrinkage (≈ minor) Swelling (≈ minor)	Standardize data to perfusion equivalent Adjust to living volumes
M ₇	Fixative	Influences what appears in micrographs	Be consistent
M ₈	Fixative Buffer	Influences what appears in micrographs	Be consistent
M ₉	Tissue Embedding	Changes in tissue volume subject to embedding material	When possible, adjust to living volumes
M ₁₀	Tissue Sections	Section compression → overestimates surface areas Section Thickness → overestimates surface areas and volumes	Apply correction Apply correction
M ₁₁	N _{cells} /G Tissue	Cell number in a gram of tissue changes	Relate data to organ or average cell
M ₁₂	Collecting Image Data	Differences in investigator skill when counting points and intersection	Circulate teaching data sets with ≈ 40 images + results
M ₁₃	Data References	Define relationship of data to biology	Apply first principles

2.2 Problem 2 – Concentrations – Two Variables Acting as One

Since we can detect changes in physics and chemistry by comparing concentrations (wherein the reference space is a constant), it's widely believed that we can do the same for biology. Not so, because biology changes the rules for concentrations. When detecting a cell change, we use the same reference units (e.g., gram or cm³) as physics and chemistry for concentrations but instead of dilute solutions biology fills the reference spaces with cells that continually change their volumes. Using a stereology database, one can discover that changes in the same part expressed as absolute (total amount) and relative (concentrations) values agree only about 50% of the time (Bolender, 2019). Given the importance of detecting experimental changes correctly, the growing body of statistical (meta-analyses) and empirical evidence (absence of reproducibility) suggests that the cost effectiveness of biomedical research could benefit importantly from a return to the basics.

For example, to cancel out the volume distortions (tissue preparation; changes in cell number per unit of volume or weight) associated for estimates of membrane surface densities ($S_v = \text{m}^2/\text{cm}^3$), one can divide

one surface density by another [$S_v(\text{SER})/S_v(\text{RER})$] to get a dimensionless ratio [$S(\text{SER}):S(\text{RER})$]. A similar approach works for biochemical data. Note that cells run most of their business with ratios.

2.3 Problem 3 – Data References Determine Results

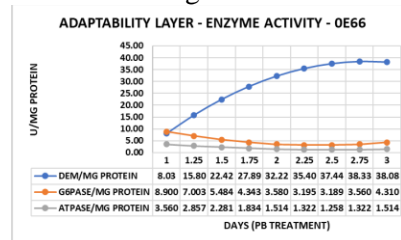
Since the update depends on aggregating data from several publications to form phenotypes, the problem becomes one of figuring out how to reconnect data when related to different data references. This would be a relatively simple task were it not for the fact that most papers don't include enough information to switch to a common reference. Although we can often manage this limitation provisionally by copying missing data from one paper and pasting them in another, simply updating our antiquated experimental designs would eliminate the cumbersome data reference problem altogether. Nonetheless, the fact remains that when encountering data related to mismatched references, we can't use them to solve the standard equations needed to detect changes or to explain how the change occurred. When this occurs, normalizing the data becomes a viable option because it removes all the data units. Now the equations work but one must check and double-check the results. While normalization comes with problems of its own, it allows us to complete the calculations needed to assemble and explore the properties of new models for detecting changes.

Now let's turn to the literature and ask a few specific questions. Can data references affect the magnitude and or direction of a biological change? Yes. Can data references alter statistical outcomes? Yes. Can data references determine the success or failure of a study? Yes.

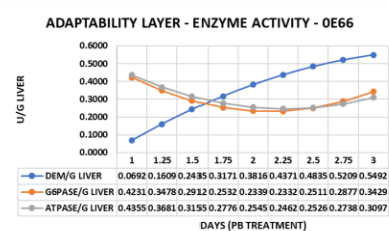
Figure 2.8 returns to the results of a phenobarbital induction study by Orrenius and Ericsson, (1966; OE66) by relating the same data [aminopyrine demethylase activity (DEM), glucose-6-phosphatase (G6PASE), and adenosine triphosphatase (ATPASE)] to three commonly used data references: mg protein, gram of liver, and the liver. We want to see how these enzymes responded when related to these references before and after normalization.

As expected, the three references produced markedly different responses. During the experiment, (1) the protein content of the hepatocytes continued to change, (2) the cells increased their production of enzymes and membranes, grew larger, and continued to exit from the gram of liver. Bear in mind that the plots in figure 2.8 represent results produced by the changes listed above.

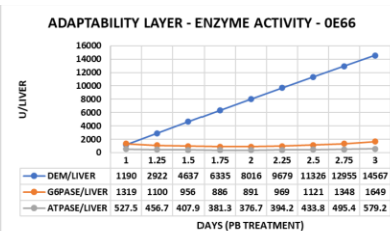
Units Per Milligram Protein



Units Per Gram of Liver



Units Per Liver



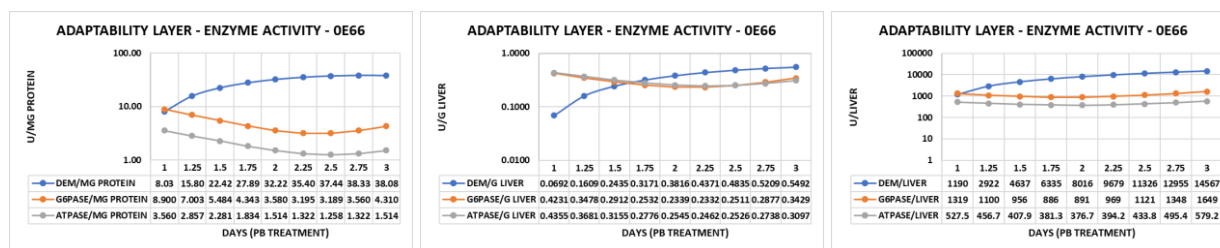


Figure 2.8 Estimates for enzyme activities responding to phenobarbital tell different stories when related to different references. The second row of the figure plotted the results of the first row with a log scale to identify parallel patterns of change for G6PASE and ATPASE (Original data adapted from Orrenius and Ericsson, 1966). When building models to discover rules and principles, knowing the properties of reference systems becomes essential.

Normalizing the data (Figure 2.9) provided the clearest picture of the changes because they all shared the same scale (0 to 1). Recall that zero (0) represents the smallest value and one (1) the largest. Notice that the changes in demethylase (DEM) activity per liver displayed a linear response ($R^2 \approx 1$) for its drug-metabolizing capacity. Why did the liver's linear response become polynomial when related to a mg protein or a gram of liver? Because (1) protein content per hepatocyte increased, (2) the liver experienced a temporary loss of volume (remodeling), and (3) fewer hepatocytes were fitting into a gram of liver (the average cell volume was increasing). Detecting the biological changes required playing two change games, one for biology and another for the methods.

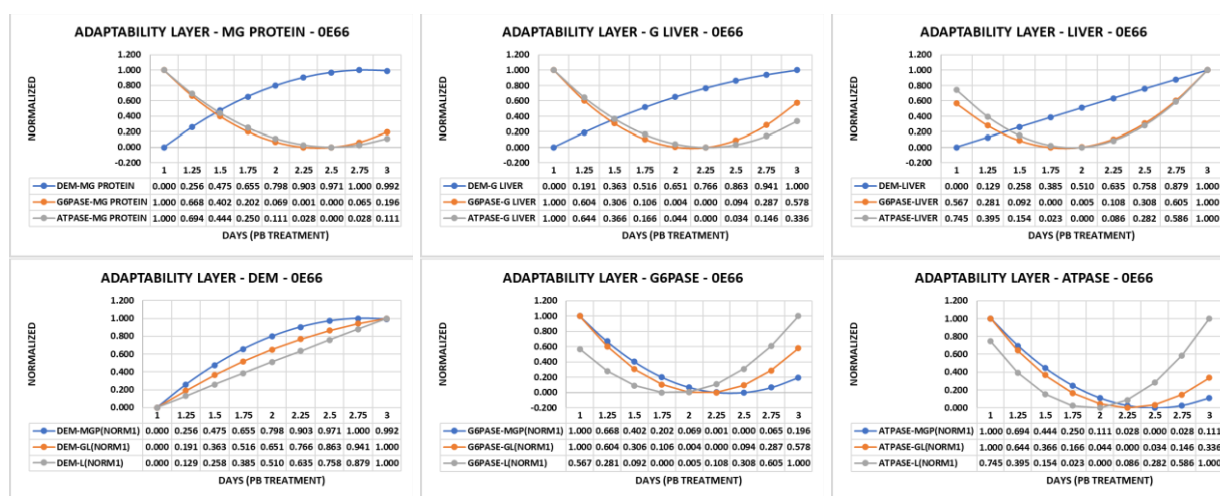


Figure 2.9 Three enzymes generated three different results when related to three different data references and normalized. Now the linear and polynomial curves identify the changes without the data units and references (Original data adapted from Orrenius and Ericsson, 1966). Notice in the second row of the figure that the enzymatic changes and rates of change of each data reference clearly tell different stories. Data references create reproducibility nightmares.

A summary plot becomes instructive (Figure 2.10). Without the raw data we can only imagine what might have happened to the p values and effect sizes when moving from one data reference to the next. The results in the rules layer make the point once again that different data references can lead to different rules. The mg protein produced one result (0.5:0.5), while the liver references another (0.4:0.6). But what did we learn biologically about how enzymes change in response to phenobarbital (PB)? Both the drug-metabolizing enzyme (DEM) and the housekeeping enzymes (G6PASE, ATPASE) used the same rule.

Ratios based on a gram of liver and the liver will always be the same because they differ only by a constant - the weight of the liver.

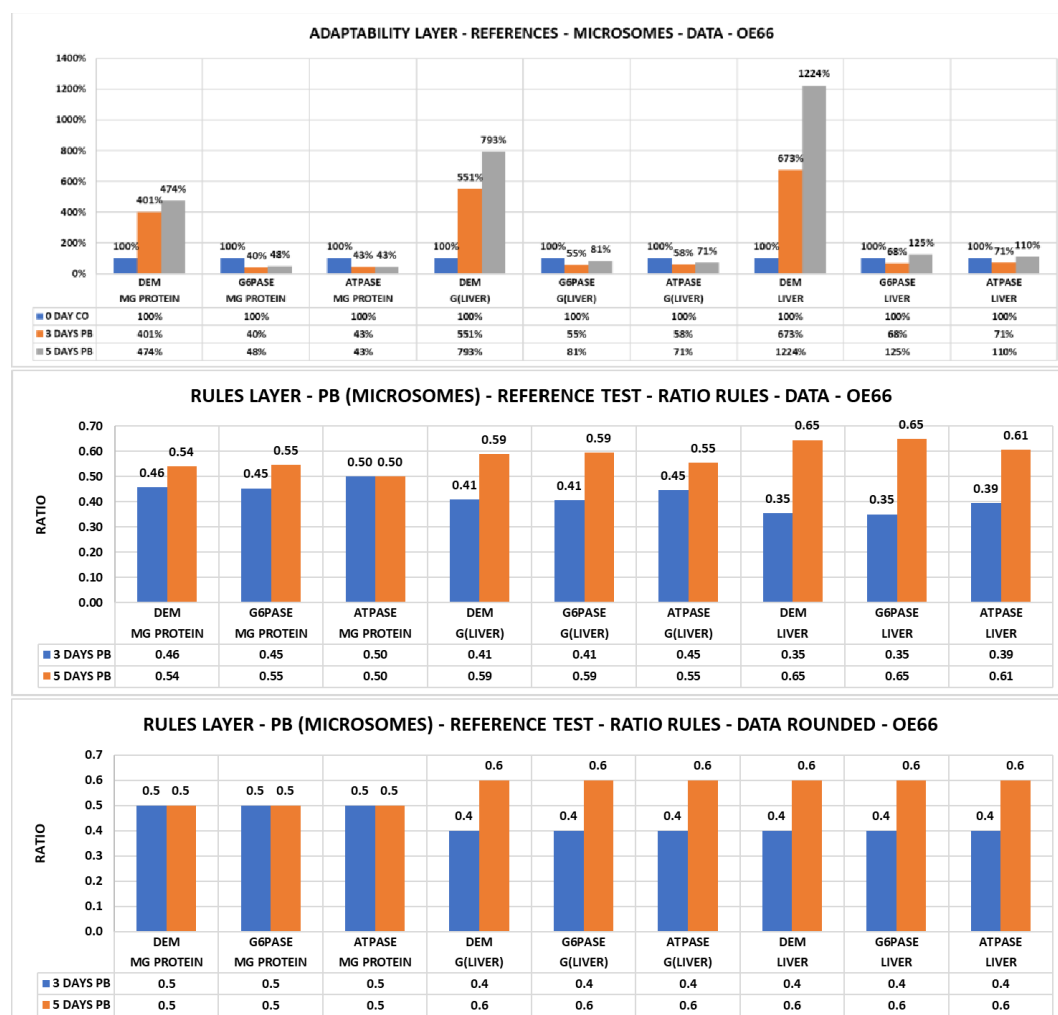


Figure 2.10 By expressing the normalized changes as a percentage of the control, the differences between the data references become easier to compare. Hepatocytes exposed to phenobarbital showed different responses to the references in the adaptability layer, but similar responses in the rules layer. The mg protein reference used one rule (0.5:0.5) and the gram of liver and the liver used a different one (0.4:0.6)(Original data adapted from Orrenius and Ericsson, 1966).

What else can Figure 2.10 tell us? If we want to check the correctness of the results, we now have two options. We can test for reproducibility using data coming from either the adaptability or rules layer. Notice that by calculating the same enzyme data pair ratios from microsomes taken from two different experimental days, one can posit that the consistent ratios indicate similar microsomal recoveries. In the absence of recoveries, one tacitly assumes similar recoveries.

2.4 Problem 4 - The Mean Value is a Black Box

What is a mean value? It represents a shared data space occupied by one biological variable and an unknown number of troublesome variables coming from the experimental methods. For us, a mean value is a number, the averaged sum of different competing variables only one of which came from biology. In effect, a part estimated experimentally and labelled “RER” is often a rough estimate.

When updating the literature, multiple shortcomings of the mean value come into sharp focus. By averaging the data of individuals, we forfeit tests of representative sampling and reproducibility and skip the contributions of the methodological variables. This creates an untenable situation experimentally because reproducing a biological change includes duplicating the biological variable along with its accompanying entourage of methodological variables. AI models, for example, trained on mean values alone lose the ability to discover, interpret, and learn from the unique patterns of individuals. The hard truth is that we’re not skilled at detecting cell changes and demonstrating reproducibility.

2.5 Problem 5 – How, When, and Where to Look for Biological Changes

Since we want to optimize the amount of information coming from an experimental change, we’ll use regression analysis to fit updated data points to curves displaying coefficients of determination (R^2) equal to one. The resulting equations, which typically fit polynomial curves, offer several advantages. As a complex expression, a polynomial equation can serve as a unique marker for an experimental result, generate uniform arrays of data points from sparse datasets, produce a wide array of patterns, and capture complex changes.

By starting with a uniform set of points and translating them into a corresponding set of rules, we can determine when and where the rules change or if they stay the same. Since ratio-based rules routinely produce patterns, they become the unique identifiers of biological changes. As such we can use them to test for reproducibility and representative sampling, and to work out the basics (rules and first principles) of a biological change with forward and reverse engineering.

When a cell changes its phenotype to solve a problem, it’s either receiving instructions from the genome, figuring out what to do, or some combination of the two. Using polynomial equations based on the expanded datasets of published data, we can use changes in enzymes and enzyme densities to predict genetic events upstream by merely switching the name of an enzyme to that of its parental gene. If we link changes in the phenotype to those of the genotype, what can we do?

CHAPTER 3

DATA REFERENCES

SUMMARY

Data references become defined by the theory structure. Reductionism defines data references with parts, whereas complexity theory extends the definition to include parts, connections, and complexity. Since solving the biological literature begins by copying the way cells change, understanding the complex behavior of data references becomes fundamental to the task of updating the literature. Unfortunately, published data contain a double dose of complexity. When running an experiment, we mix changes coming from two sources - methods and biology - and as a result most references lose their status as constants and become variables. Without constant references, detecting a change experimentally can become a high risk, low return undertaking. To make matters worse, different references used to detect the same change return different results but fail to explain why. Since most publications focused on just changes to parts, the data reference problem failed to surface because parts alone cannot gain access to the intricacies of a biological change. Detecting biological changes requires parts, connections, complexity, functioning data references, and a supportive theory structure. In short, retrieving biological changes from publications becomes a challenging problem of data management. The chapter explores the relationship of data references to experimental methods when detecting and interpreting biological changes.

When looking for changes experimentally, our methods change the rules by adding their changes to the biological results. We start with the rule-based complexities of biology, add the complexities produced by our methods, mix both together, and bundle both into a single mean value. By detecting a significant difference between two such mean values, publications assume that they are detecting “biological” changes. The chapter will test that assumption by showing how difficult it is to detect a biological change without inside help from cells.

When updating the literature, the primer will take the mean values back to the time when the mixing of biology and methods occurred to disassociate the biological changes from those of the methods. However, dealing with the convoluted behaviors of data references, which sit at the heart of the change problem, becomes a challenging task for both beginner and expert. Learning how to keep track of a host of changes occurring within changes - without getting lost - takes practice and patience.

A primer promising to update and solve the biological literature, looks for answers to the following questions:

- Why did the current literature become what is today? The literature contains: (1) practically no raw data (the primary data from individuals), (2) mostly mean values carrying a heavy load of methodological biases and errors, (3) too few experimental data points, (4) too many incompatible and/or isolated data references, and (5) largely incorrect results statistically (Ioannides, 2005, Ioannidis and Panagiotou, 2011).

- If we solve the literature and then use it as a user interface to biology, what will happen? By organizing published data into phenotypes, how do we reverse engineer our way into a first principles approach to biology? Will phenotypes predict gene expression and vice versa? Can this lead to simulating changes while at the same time provide “biology-inspired” datasets for training AI programs? Given the presence of unusually narrow windows through which to view biology changing by rule, how realistic is the assumption that AI can use the literature as a training set to make the transition from the simplicity of isolated parts (reductionist theory) to the reality of biology (complexity theories)?
- If we collect enough of biology’s rules and first principles, can we begin to explain how biology works theoretically? Probably yes, at least in terms of classical mechanics. Since biology has already figured out how to design classical solutions around the photons of quantum mechanics (e.g., photosynthesis, vision), will quantum mechanics begin to explain how biology changes in our classical world? Do the first principles of classical biology come from those of quantum mechanics? If we can copy our way to understanding classical changes, will more copying take us to an understanding of quantum-based changes? The point? Everything leads back to the same starting point – our ability to detect complex biological changes. Detecting such changes starts and ends with an understanding of data references.

3.1 Data References (The Basic Issue)

For the most part, we collect biological data as concentrations. This includes a variable of interest (X) in the numerator related to a reference value (Ref) in the denominator, which typically includes a standard unit of weight, surface, or volume. In the simplest case, two concentration models exist – one for physics and chemistry and the other for biology.

Think of it this way. Detecting a change in an enzyme activity is a chemical question dealing with experimental changes occurring in dilute solutions. The reference space (ml or cm^3) exists as an experimental constant in a test tube or cuvette. When working with cell parts isolated from intact tissues, the rules change – not for the biochemical assay but for the reference space (ml or cm^3) used for the interpretation. Interpreting changes in cells, which fill a gram or cm^3 of liver, do not qualify as dilute solutions. This means that changes in the average volume of the cells producing the enzyme activities will determine the number of cells that can fit the reference space (gram or cm^3).

When, for example, both the enzyme activity (per gram) and the number of cells (per gram) producing the enzyme activity change simultaneously, the results become ambiguous. If the reference spaces of the controls and experimentals contain a different number of cells, then one cannot calculate a change because the data references differ and fail to cancel out. In short, methods based on comparing concentrations come with a built-in and extraordinarily successful algebraic trap. In practice, published results based on concentration data will be faulty (based on different number of cells) roughly 50% of the time. To make matters worse, using concentration data to reproduce results creates a near impossible requirement in that one must collect the same data from the same numbers of control and experimental cells a second time. Fortunately, a workaround exists and is far easier to apply. Relate the concentrations to the weight or volume of the organ or gland before calculating the change.

Case A: When detecting a change in X, the data reference (e.g., cm^3) conveniently cancels out when comparing an experimental (ex) to a control (co) data point.

- Physics and Chemistry (dilute solution)

$$\Delta X (\text{Change}) = (X_{\text{ex}}/\text{cm}^3)/(X_{\text{co}}/\text{cm}^3) \text{ and} \quad 3.1$$

$$\Delta X (\text{Change}) = (X_{\text{ex}})/(X_{\text{co}}) \quad 3.2$$

Case B: When detecting a change in X, the data reference (e.g., cm^3) fails to cancel out when the number of cells contained within a cm^3 of tissue changed.

- Biology (not a dilute solution)

$$\Delta X (\text{Change}) = (X_{\text{ex}}/\text{cm}^3)/(X_{\text{co}}/\text{cm}^3), \quad 3.3$$

when the number of cells/ cm^3 of the control (c) \neq number of cells/ cm^3 of the experimental (ex).

$$\Delta X (\text{Change}) \neq (X_{\text{ex}})/(X_{\text{co}}). \quad 3.4$$

When hepatocytes make cytoplasmic changes, the number of cells of interest contained within the standard reference compartment (e.g., a cm^3 or gram of tissue) usually changes because average cell volumes will increase or decrease. To detect a cellular change correctly (biologically), one must relate results to the same number of cells or to an average cell. Bearing in mind that both morphological and biochemical data are subject to this requirement (equal cell number) when detecting biological changes, ignoring it routinely leads to faulty results. Since most published studies in biology traditionally compared concentrations, reproducing results with four concentration variables in play became understandably unlikely. When something is reproducible biologically but not experimentally, then something is wrong with the experimental design. Fortunately, we can avoid this problem of interpretation merely by expressing our results relative to the total weight or volume of the parent organ or gland. Here we'll use the liver.

By focusing on the basics, a primer alerts the beginner to strategies that lead to robust experimental designs. One of the most challenging tasks of updating the literature included making the transition from simple to a complex by avoiding "well-established" assumptions by no longer needing them. The point? Understanding the complex behavior of the data references represents a basic research skill regrettably understood and practiced by few in biomedical sciences.

3.2 Data References (Problems and Solutions)

After summarizing the problems and solutions, examples will put theory into practice.

Problems:

- When viewing a change experimentally, practically everything changes.
- Most of the commonly used data references do not work as expected.
- Mean values combine two competing sources of change (biology + methods), but most published results attribute changes just to biology.
- Morphological data (estimated stereologically) carry limitations related to section thickness/compression, volume distortions, changes in mean cell volumes, and incompatible data references.
- Biochemical data carry limitations related to changes in mean cell volumes, incompatible data references, and the tendency to report results from tissue fractionations (e.g., microsomes) without including recoveries.
- Most publications do not contain enough information to detect a complex biological change.

Solutions:

- Redefine change biologically.
- Expand data sets.
- Detect changes by applying biological rules.
- Respond to the absence of physical constants during a change by introducing virtual constants.
- Update biochemical and morphological references and explain the need for standardization.
- Update published results locally (single paper) and globally (multiple papers).
- Express and interpret complex results with patterns.
- Include prediction and reproducibility in experimental designs and models for change.
- Reconstitute (forward engineer) a cell phenotype from changes in data pair ratios, reverse engineer the changes to understand how the cell solved separate parts of the problem (subgroups), use recipes to summarize the changes needed to deliver the solution.

3.3 Biochemical Data References

To begin, we'll use examples taken from the updated literature to illustrate some of the problems created by data references.

3.3.1 Example 1: The Effect of Phenobarbital on Data References (Original data adapted from Orrenius and Ericsson, 1966; OE66).

The first example uses a phenobarbital study that reported the effects of the drug on the activity of microsomal glucose-6-phosphase (G6PASE). The authors wanted to know why the drug caused the G6PASE activity to decrease. The paper represents a classic example of the “tug of war” that develops between the changes of biology and those of our experimental methods when we try to detect a change. What happened experimentally? By day five of the phenobarbital treatment, the gram of liver reference (G6PASE/G) decreased the amount of G6PASE activity by 50%. How? As the hepatocytes filled up with drug-metabolizing membranes and enzymes, the average hepatocyte became increasingly larger, which meant that fewer of them could fit into a gram of liver. Fewer cells in a gram of liver produced less G6PASE activity per gram of liver. Collecting G6PASE data from fewer hepatocytes diluted the amount of activity per gram of liver. [Initially new synthetic activity favored the drug-metabolizing enzymes (e.g., DEM).]

Next example. When assaying enzyme activity biochemically, we often express results as units of activity (U) relative to a mg of protein. In turn, this result can be related to a gram of liver and then to the liver:

$$U/g \text{ liver} = (U/mg \text{ protein}) \cdot (mg \text{ protein}/g \text{ liver}) \text{ and}$$

$$U/liver = (U/g \text{ liver}) \cdot (W_{\text{liver}}),$$

where W_{liver} is the weight of the liver in grams.

Publications often report results relative to a mg protein or to a gram of liver, but rarely to the liver. By borrowing liver weights from a similar phenobarbital study (Stäubli et al., 1969; SWH69), we can relate the same biochemical data to three different references (mg protein, g liver, and liver) at two levels of information (adaptability and rules layers). Since all three references produced different results, which one should we use? For an investigator, the challenge becomes one of selecting the data reference best

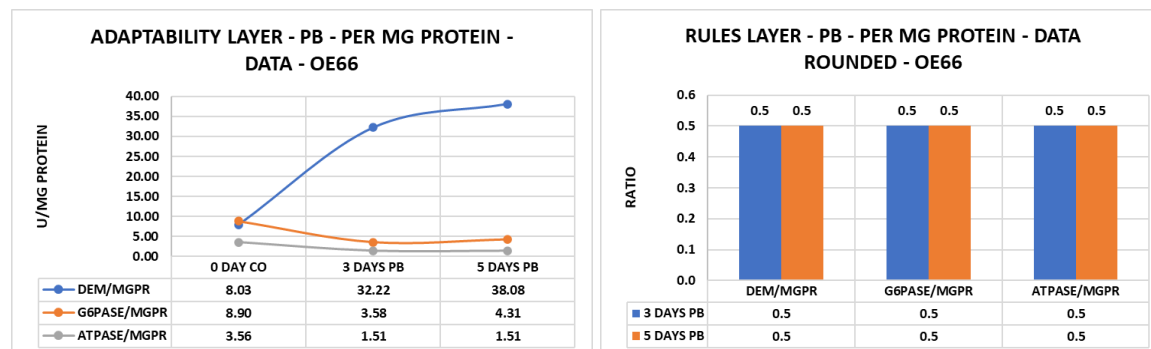
suited to answering the question asked. When detecting a change in a part (enzyme or organelle), the liver becomes the best choice because the result is an absolute value not a concentration.

After treating animals with phenobarbital, the investigators (OE66) removed the livers, which they then homogenized, collected a microsomal fraction, and ran assays for three marker enzymes of the hepatocytic endoplasmic reticulum (ER): Aminopyrine demethylase (DEM), glucose-6-phosphatase (G6PASE), and adenosine-tri-phosphatase (ATPASE).

Adaptability Layer: By expressing the original data (enzyme activities) relative to three different references, the same data gave three different responses to the drug (Figure 3.1). DEM, for example, showed a 4-fold increase in activity when related to a mg of protein, an 8-fold increase when related to a gram of liver, and a 12-fold increase when related to the liver. Compared to the liver reference, the mg protein reference substantially underestimated the change in the enzyme activity.

Rules layer: As shown in the Figure 3.1, the ratios for the mg protein reference (0.5:0.5) differed from those related to a gram of liver and to the total liver (0.4:0.6, 0.4:0.6). Why did the gram of liver and the liver share the same rules? Because multiplying the gram of liver by the weight of the liver, changes the absolute amounts but not the relative ratios. Why did the ratios of the milligram protein reference differ from those of the gram of liver and liver references? The enzyme activities referenced changes occurring in the protein content of the hepatocytes, the gram of liver referenced changes to a changing number of hepatocytes, and the liver referenced changes to the total number of hepatocytes. Point? The same data applied to three different references can give both similar and different results. Look at the assumptions of each data reference. The mg protein reference assumed that the protein content of the hepatocytes remains constant (it did not), the gram of liver reference assumed that the number of hepatocytes contained in a gram of liver remained constant (it did not), and the liver reference assumed that for practical purposes the number of hepatocytes remained constant (it did). Why is this important? When a reading a research paper, identifying the assumptions in play tells one a great deal about the credibility of the results. By allowing significant differences to substitute for biological changes, for example, authors often inadvertently let data indexed to wobbly data references slip by unnoticed. A first principles approach to change quickly catches such mistakes and provides workable solutions.

When forming ratios across time points (e.g., 3 days PB vs 5), the gram of liver at each time point did not contain the same number of hepatocytes. We can see this in Figure 3.1 by comparing the changes in DEM activity at days 3 and 5 in the adaptability layer where the results were related to the gram of liver and to the liver. The rules layer, however, shows that the ratios of these two references remained the same (0.5:0.5) as expected. Enzyme data related to a mg protein also showed a nonlinear change in DEM activity, but the ratio in the rules layer remained unchanged. What happened with the protein? Without access to the original raw data and calculations, we can't explain the results of the protein reference.



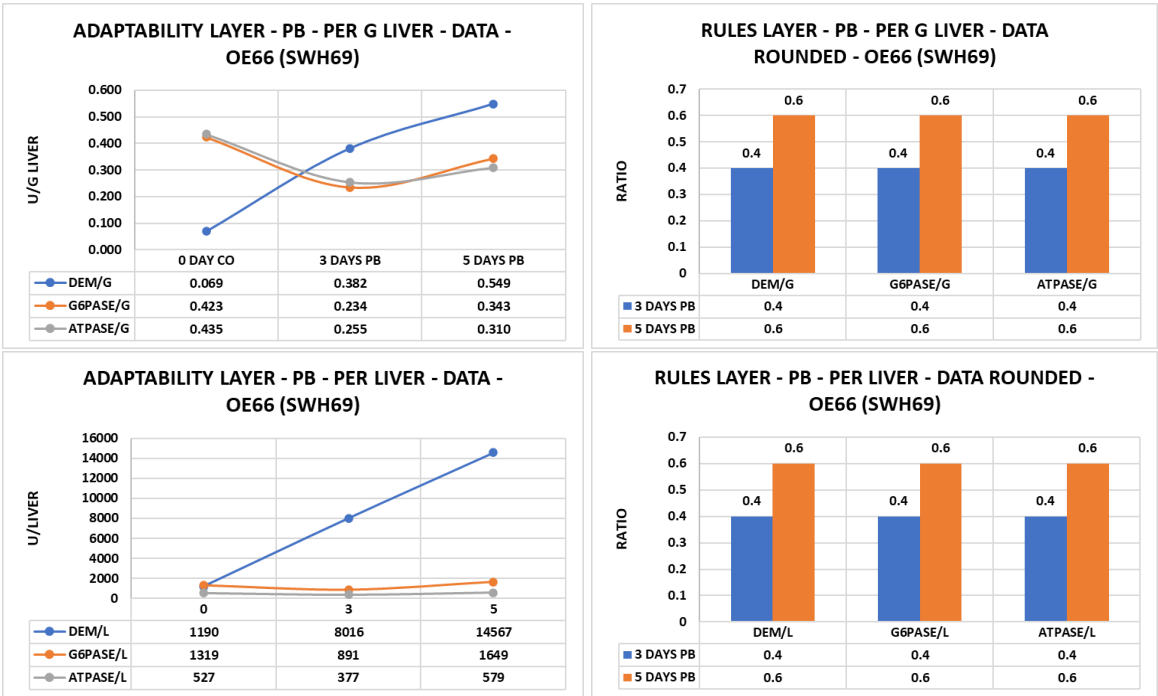


Figure 3.1 Data reference determined the hepatocytic responses to phenobarbital in both the adaptability and rules layers. In the rules layer, for example, the mg protein reference used one rule (0.5:0.5) and the gram of liver and the liver used a different one (0.4:0.6). Notice that DEM/L showed a linear response, whereas the slope of the DEM/G curve decreased because the activity was coming from fewer cells. The DEM/MGPR curve showed the effect of changing the amount of protein per cell (Original data adapted from Orrenius and Ericsson, 1966 and Stäubli et al., 1969).

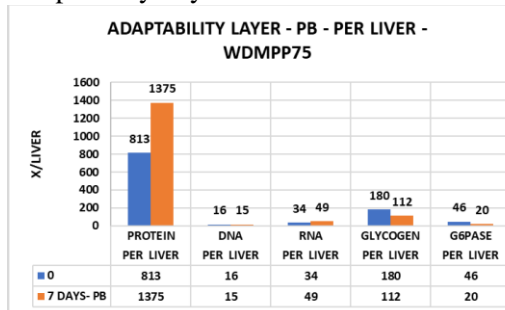
3.3.2 Example 2: Phenobarbital Treatment for 7 Days (original data adapted from Wanson et al., 1975; WDMPP75)

For the next example, we'll use a phenobarbital study that contained a rich collection of biochemical results well-suited to exploring the relationship of experimental change to data reference.

By using the liver reference as the gold standard for detecting a biological change, we can show the extent to which commonly used references stray from the standard (Figure 3.2). We begin by switching the data reference for calculating the results from the liver to several other references (gram of liver, mg protein, mg DNA, mg RNA), expressing the results as ratios, and plotting the results side by side. While discovering that each reference displays a unique pattern of change, the plots show the risks involved when comparing the results per liver (standard) to the results related to other references. Basically, Figure 3.2 becomes a test to see how different references detect the same biological change.

Clue: We have a situation where a gram of liver at day zero (control) contains roughly twice the number of hepatocytes compared to a gram of liver treated with phenobarbital for seven days (experimental).

Adaptability Layer



Rules Layer - Data Rounded

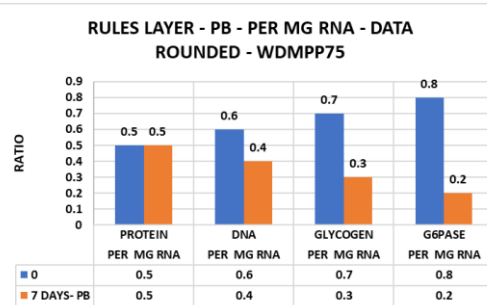
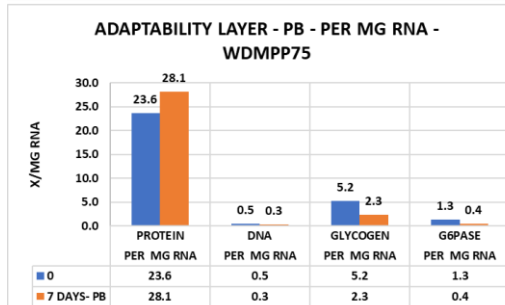
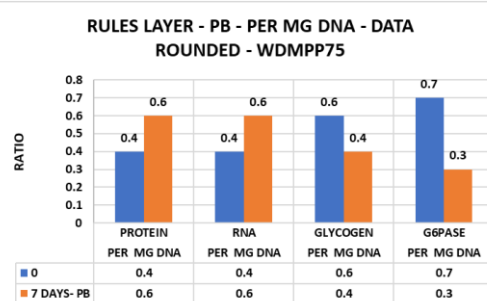
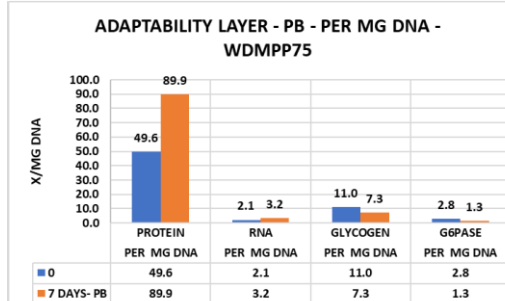
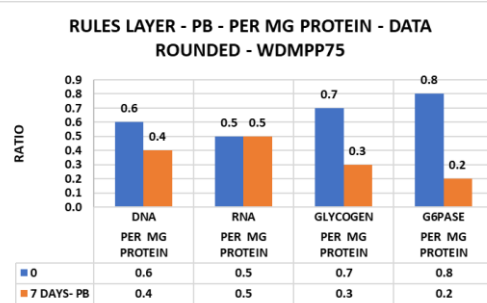
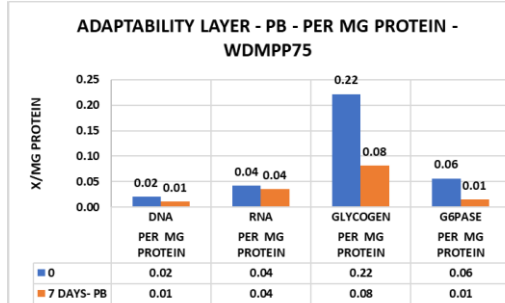
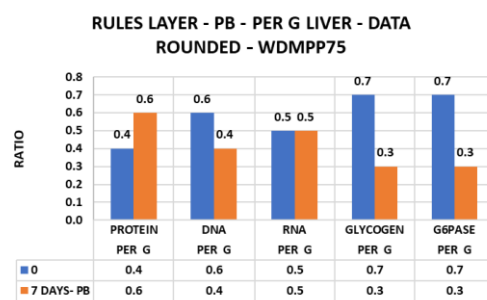
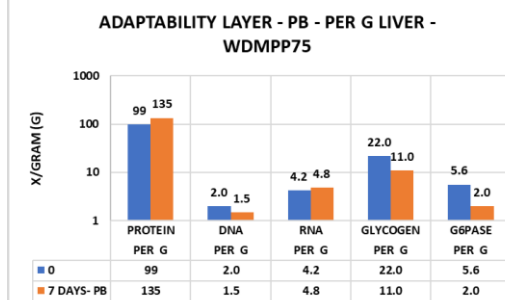
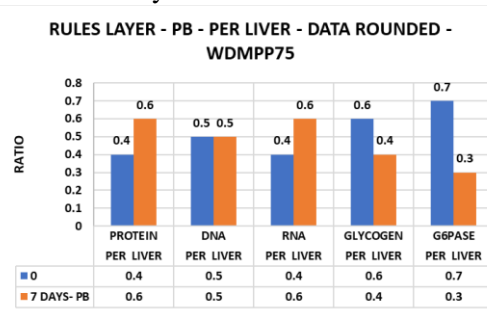


Figure 3.2 Comparing results in the adaptability and rules layers. By interpreting the same data with different references, one can choose from a wide range of results. Reproducing most of these results becomes difficult because the underlying assumptions act as variables that can change from one time point to another (Original data adapted from Wanson et al., 1975).

How do we begin to explain the changes displayed in Figure 3.3? Did, for example, the number of hepatocytes filling a gram of liver change in response to the phenobarbital (PB) treatment? Yes. Except for the liver reference, all the other references compared control data to roughly 50% fewer cells in the experimental data point (7 days PB). [Since hepatocytes can double their volume in response to PB, a gram of liver at day 5 would be expected to have roughly 50% fewer cells]. Not convinced? Good. Okay, let's use the updated results in the figures below to produce a biochemical estimate for the relative number of cells in a gram of liver treated with phenobarbital for seven days.

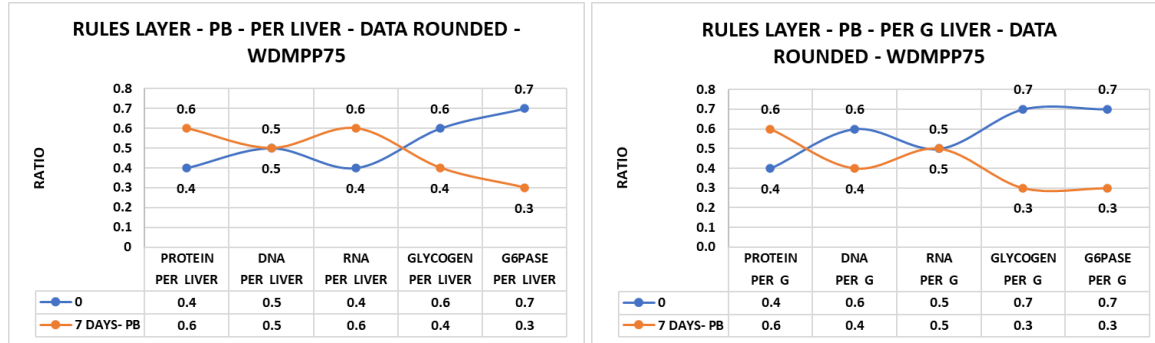


Figure 3.3 Comparing changes in the liver vs a gram of liver before and after treatment with phenobarbital. The proportion of the DNA per liver remained the same before and after exposure to the drug but decreased when related to a gram of liver because fewer cells filled the gram (the hepatocytes enlarged). Notice that the amount of RNA per gram didn't change apparently because the induced hepatocytes became enriched with RNA (e.g., ribosomes, etcetera). To what extent was the hepatocytic RNA enriched per gram? 50%? (Original data adapted from Wanson et al., 1975).

Using the data in Figure 3.3, we can compare the amount of DNA per liver (left plot) to the amount per gram of liver (right plot). If PB had no effect on the total number of hepatocytes in the liver, then the amount of DNA in both the control and PB treated livers should be the same. Finding a control to PB ratio of (0.5:0.5) for the DNA would be consistent with such a result (left plot). Notice, however, in the plot at the right (DNA per g of liver) that the control ratio had relatively more DNA (0.6) than that of the gram of liver exposed to PB (0.4). Such information becomes useful because we can use it to generate a correction factor ($CF-\Delta CV_{\Delta DNA}$) for the change in cell volume (ΔCV) based solely on the change (a dilution) of the ΔDNA : $CF-\Delta CV_{\Delta DNA} = \text{DNA}(\text{control})/\text{DNA}(\text{PB}) = 6/4 = 1.5$.

A correction factor of 1.5 suggests that a gram of liver treated with PB contained only 50% of the cells (by number) found in the control, which means that the PB treatment increased the volume of the cells by 50% (because of the addition of new ER membranes). In effect, reporting the experimental results relative to a gram of liver underestimated the amount of the PB-induced change by roughly 50%.

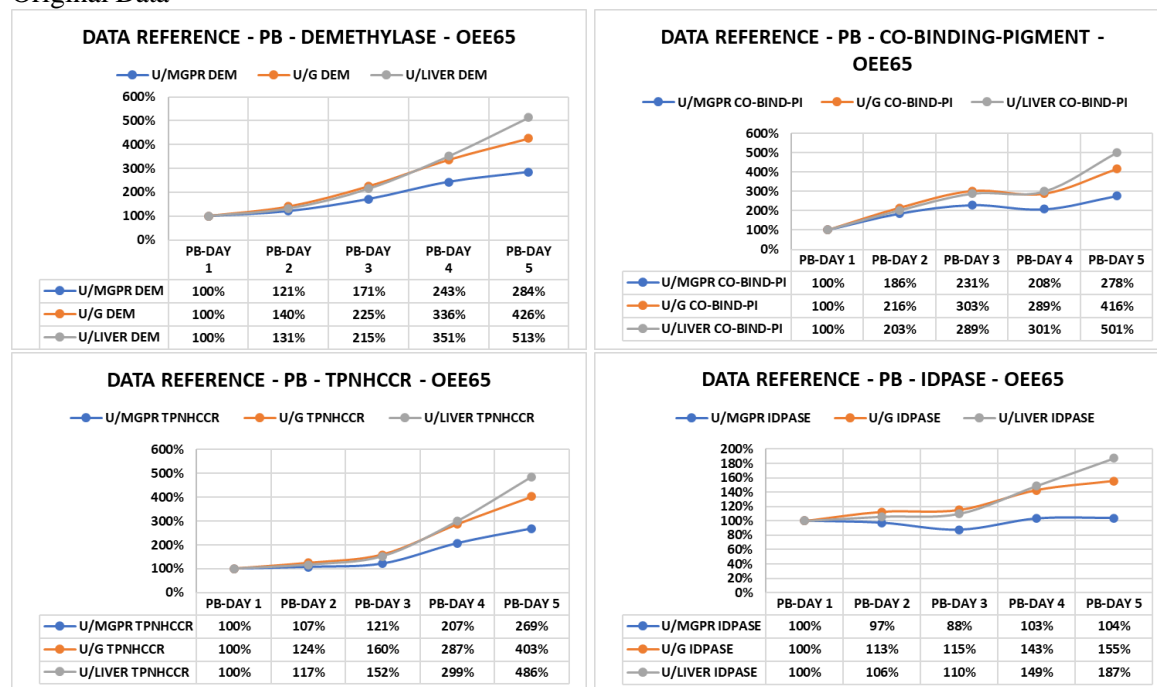
Since biochemists routinely publish results relative to a gram of liver but rarely include liver weights, this DNA correction factor contributes to the updating process by offering an alternative way of converting data related to a gram of liver to the liver. Such insights help because they put us back on the road that leads to reproducing and explaining experimental results. The point? Detecting a change experimentally begins with a basic understanding of biological concentrations and data references. Since experimental designs produce mixtures of changes coming from both biology and methods, understanding the basics of each source of change becomes a challenging but rewarding exercise. The point? By pursuing a first principles approach, we solve the change problem, the reproducibility problem, and parts of the methods problem.

3.3.3 Example 3: Phenobarbital Treatment for 5 Days (original data adapted from Orrenius et al., 1965; OEE65)

Orrenius et al., (1965) used microsomes from phenobarbital treated animals to follow the activities of several enzymes (Figure 3.4). The update of Example 2, however, told us that when used as references the mg protein and gram of liver detected smaller amounts of change than the liver. A similar pattern appeared in this paper. Once again, the liver reference detected the largest amount of change whereas the mg protein the least. At day five of phenobarbital induction, for example, the average increases in microsomal enzyme activities were 285% for the liver, 237% for the gram of liver, and 158% for the mg protein. Such results once again show the extent to which our choice of data reference influences the experimental outcome.

Data References Determine Results: Notice that the enzyme plots show some similarities but overall, the pattern seen at PB day five shows distinct differences (Figure 3.4). Bear in mind that we don't know if the enzyme activities of the earlier fractions (collected before the microsomes) remained relatively constant when compared to those on PB day one. By applying analytical fractionation (de Duve, 1964), this assumption of constancy becomes unnecessary. The point? The chance of reproducing an experimental result may be inversely proportional to the number of assumptions in play. Too many unchecked assumptions forfeits reproducibility.

Original Data



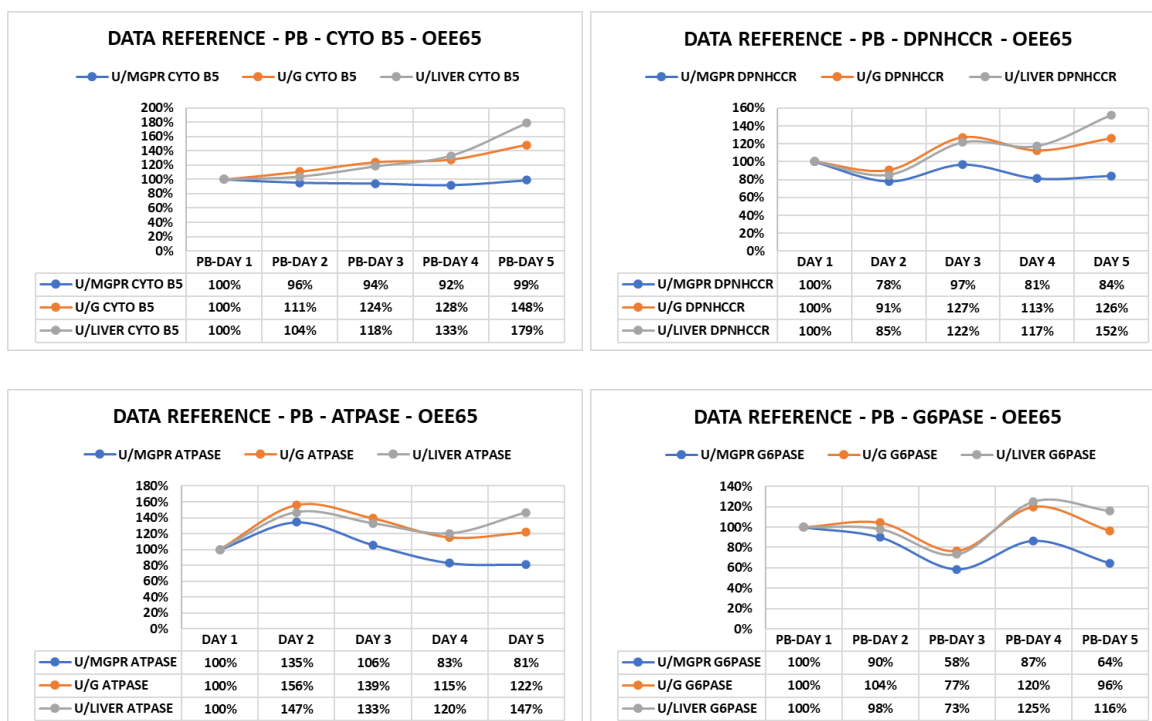


Figure 3.4 As expected, the data references determine the results. Moreover, the range of the differences produced by the references show changes often large enough to detect a significant difference (Original data adapted from Orrenius et al., 1965).

By expanding the data related to a gram of liver (Figure 3.5), we can follow the changes in enzyme activities during the induction with phenobarbital (PB) at a time when hepatocytes are exiting the gram of liver as they enlarge in response to the increased synthesis of drug metabolizing enzymes and membranes (the cell packing problem).

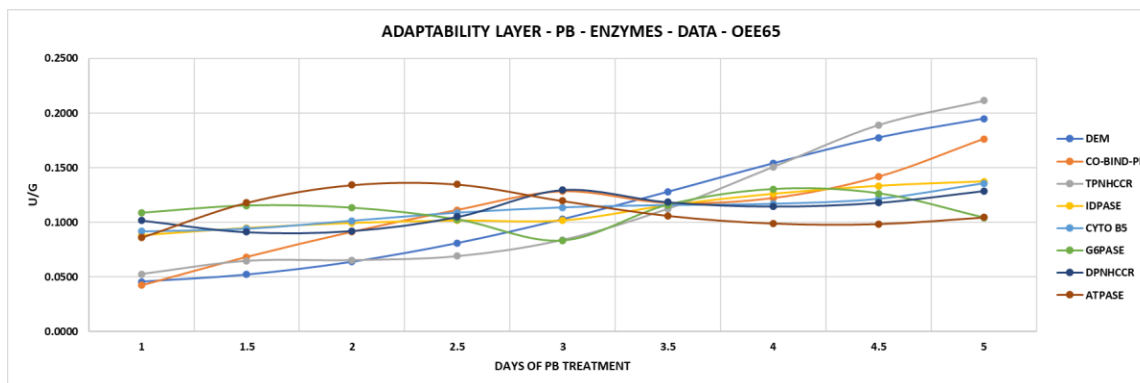


Figure 3.5 Notice that the collection of enzymes responded differently to the phenobarbital treatment. Are these data ready to show us how the hepatocytes are solving their PB problem? No, they can't because all the results tacitly assumed that the enzyme assays were based on the same the number of hepatocytes. Since this was not the case (the number of hepatocytes per gram of liver steadily decreased), the assumption produced biased results. Point? To answer an experiment question correctly, one must connect the data to the right data reference. [Note: multiplying the gram data by the weight of the liver removes the bias]. Original data adapted from Orrenius et al., (1965).

Normalizing Changes: Changes produce highly informative and reproducible patterns when standardized. Normalizing the data produces a new pattern wherein all the changes in enzymes fit within the range of 0 to 1 (Figure 3.6).

Expanded Data Normalized (Norm1) – Adaptability Layer

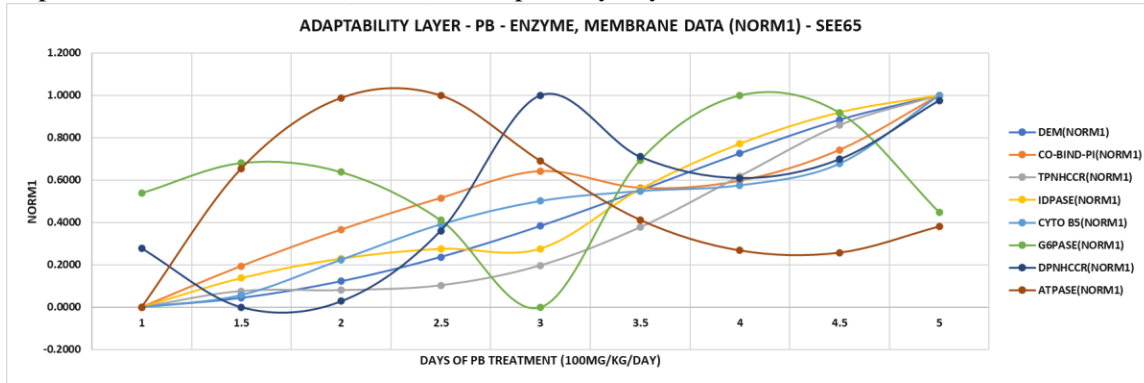


Figure 3.6 When normalized, details of all the changes become apparent. Notice the crossover point at day 3.5, wherein the relationships between most of the data pair ratios reversed. Such crossovers often appear during growth (Original data adapted from Orrenius et al., 1965).

Next, we can use the normalized data in the rules layer to generate data pair ratios, plot them, look for duplicates (data pairs showing similar changes), and finally superimpose the plots that change similarly (Figure 3.7). These plots show how the data pair ratios changed during five days of phenobarbital treatment. The data pair ratios (pairs of enzymes) went from different, to similar, to the same. In short, this summarizes a basic strategy that hepatocytes can use to solve problems.

Notice in the Figure 3.7 that the enzyme pairs in the first plot (subgroup 1) found the solution (0.5:0.5) one day earlier than those in the second plot (subgroup 2). In effect, different roads can lead to the same destination. Most likely, the changes in both groups represent optimizing routines based on local conditions. Such an analysis becomes interesting because changes in the data pair ratios of enzymes predict the ratios, sequences, and timing of the data pair ratios of genes. To check a prediction, change the names of the enzymes to their parental genes and look for confirmation in the literature. In summary, updating publications produces new information that creates new opportunities.

Expanded Data Normalized (Norm1) – Rules Layer

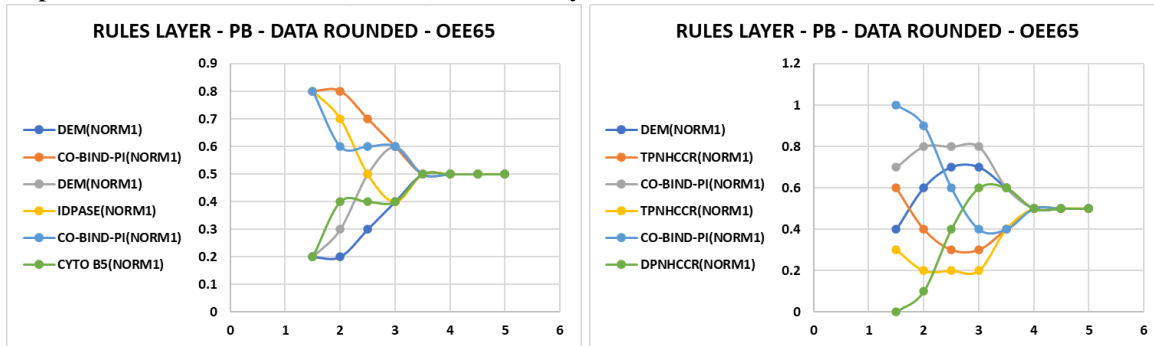
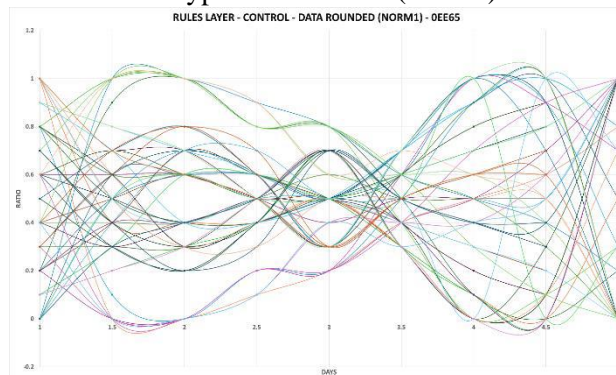


Figure 3.7 Ratios of data pairs (enzymes) responding to phenobarbital. One set of enzymes synchronized their changes at day 3.5 and the other set at day 4 (Original data adapted from Orrenius et al., 1965).

By combining all the patterns of the data pair ratios from the rules layer, we can follow the normalized changes in the enzymes as they occurred in the control and phenobarbital phenotypes over the five days of drug treatment (Figure 3.8). Notice that the control phenotype (no drug treatment) is not the constant control value we imagine it to be. Instead, it's just a different phenotype designed to do the daily housekeeping chores.

In fact, a control phenotype can be reverse engineered to identify subpopulations of parts changing in response to daily requirements, conditions, and rhythms. In comparison to the control, the phenobarbital phenotype focused on finding a solution to the drug problem. Bear in mind, however, that the phenobarbital plot included a mixture of housekeeping and drug-metabolizing data pair ratios. To separate the housekeeping data pair ratios from those involved with the drug-metabolizing, one can reverse engineer the PB phenotype into functional subgroups.

Control Phenotype - Normalized (Norm1)



Phenobarbital Phenotype - Normalized (Norm1)

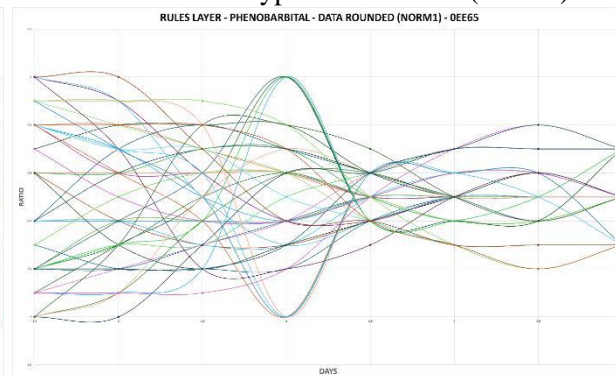
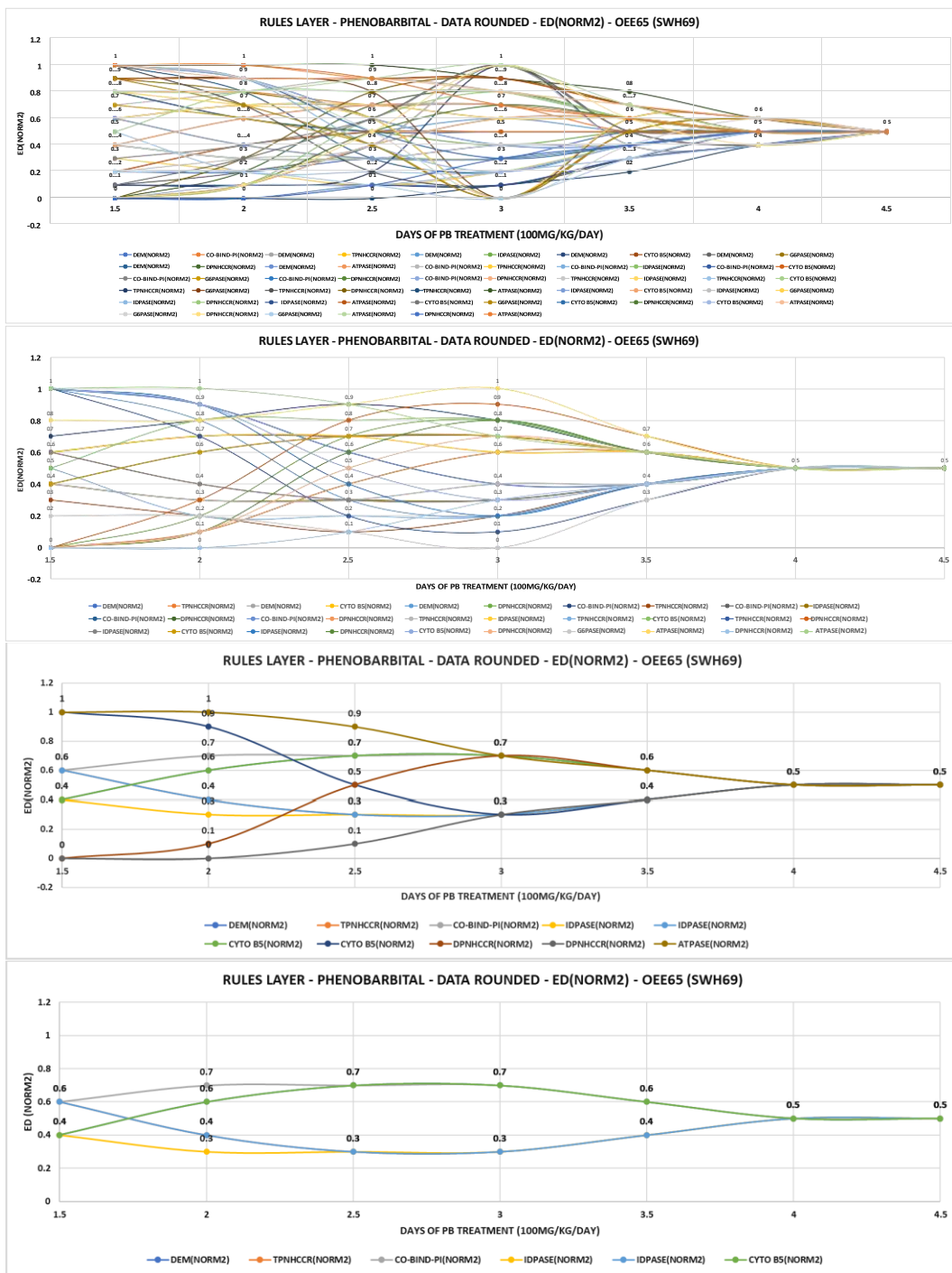


Figure 3.8 Control: The control enzyme phenotype shows changes in the normalized data pairs over 5 days. Such a pattern tells us that a control behaved like a variable in that the relationship between data pairs continually changed. Notice that days 1.5, 2, and 3.5 identified crossover events preparing for the next set of changes. **Phenobarbital:** Hepatocytes exposed to phenobarbital, had a problem to solve. To become the solution, they presumably focused on optimizing enzyme data pairs to create an enzyme recipe sufficient to the task, while at the same time meeting their other obligations. After five days of phenobarbital treatment, the solution to the enzyme dataset required just three data points – two for housekeeping enzymes (0.3:0.7) and one for the drug metabolizing enzymes (0.5:0.5). Although the figure represents a partially reconstructed phenotype, we can still unfold (reverse engineer) it into multiple enzyme subcompartments. However, these two phenotypes represent changes in enzymes taken out of the context of a larger and far more complex result involving relationships of structure to function.

Detecting Changes with Enzyme Densities: While a biochemical interpretation of the results might serve our interests in reverse engineering the production of new enzyme molecules, it wouldn't give us the solutions to the larger problems the hepatocytes are trying to solve. Those solutions come from normalized enzyme densities (NORM2) and include collections of subgroups defining relationships of structure to function. Successful outcomes occur in cells as sets of complex relationships defining new properties extending across the information space defined by the organism.

Figure 3.9 includes enzyme densities (ED) expressed as data pairs ratios displaying patterns characteristic of hepatocytes solving a phenobarbital (PB) problem (0.5:0.5) while maintaining the housekeeping functions (0.4:0.6). By reverse engineering the phenotype, we can separate the two functions into one or more subgroups. The set of six panels shown in Figure 3.9 begins with the complex solution (drug metabolism and housekeeping) and continues by unfolding the multiple part solution to see how the subgroups of enzyme densities formed and changed. The second panel identifies the PB solution (0.5:0.5) as the first subgroup, followed by panels down to two data pairs. The sixth panel shows the housekeeping solution (4:6). Notice how the subgroups remained interconnected by sharing the same enzyme densities. The point? The phenotype (data pair ratio) plots either define or become defined by the algorithms orchestrating the problem-solving process. But can we reproduce this PB solution (0.5:0.5) using similar or different data from other published studies? Yes (see Chapter 6).



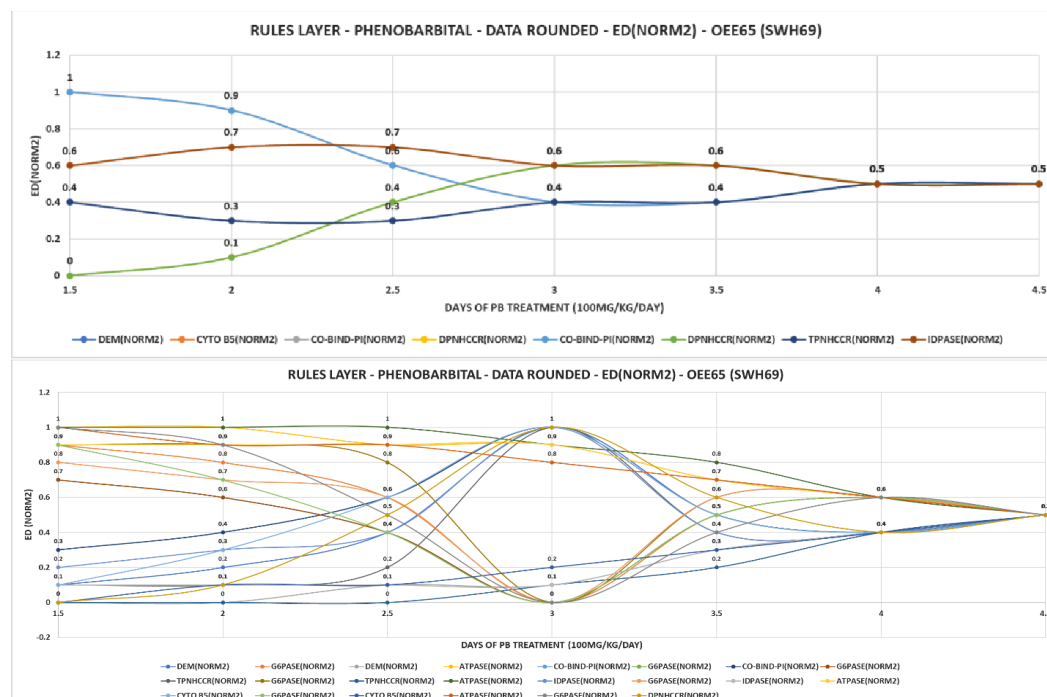


Figure 3.9 Reverse engineering a small part of the phenobarbital phenotype previously forward engineered with enzyme densities (as data pair ratios). The subgroups of PB and housekeeping enzyme density pairs presumably optimizing their rates of change to the 0.5:0.5 and 0.4:0.6 ratios. Notice that enzymes more likely to be members of one subgroup also appear in the other subgroup. Such an arrangement may assure optimal results within and across subgroups.

Phenotypes as Pathways to Change: When using a phenotype model to duplicate a biological change, solutions become expressed as data pair ratios that continue to change until a solution appears. This redefines a change as a complex event based on a fundamental bipartite unit of change – the data pair ratio. When reversed engineered, the complex mix of data pair ratios organize at different times into subgroups of similar and persistent data pair ratios. When the same data pair ratio persists for at least three consecutive time points, they define a subgroup.

The phenotype model applies biology's principle of organization wherein one part is related to another according to ratios of small, whole numbers. However, the mechanisms of connecting the parts and allowing the ratios to change as they proceed toward a solution, remain unknown. If we posit that a change represents an optimizing event, then we can envision a process that begins with an individual data pair ratio, each part of which can also form data pair ratios with many other parts. This would appear to be the simplest way for cells to solve problems of massive complexity. Moreover, it fits with biology's ability to apply the properties of quantum mechanics (i.e., entanglement) to classical events (e.g., a biological change).

Since the complexity of phenotypes becomes a poor fit with the standard statistical model for detecting biological changes, pursuing a first principles approach to change will require updating or at least rethinking our statistical approach to validating outcomes. In the meantime, predicting and reproducing results will serve as a working model for detecting and verifying biological changes.

3.4 Morphological Data References

Morphological references, which also fall victim to the cell packing problem just described for biochemistry, can become further disrupted by the volume distortions that result from preparing specimens for microscopy. If, for example, the distortion results in a net tissue shrinkage, then a cm^3 of fixed liver will contain more cells than an unfixed one. In such cases, the morphological results overestimate the absolute amounts – but not the relative amounts of membrane surface areas of organelles contained within a cm^3 of fresh or fixed tissue. The next point is important because it allows us to calculate data pair ratios from concentrations carrying volume distortions. Notice that by calculating ratios from two surface areas **equal to the same reference** – even with distorted data reference, the data reference cancels out [e.g., $S_{\text{RER}}/S_{\text{SER}} = (S_{\text{RER}}/\text{cm}^3)/(S_{\text{SER}}/\text{cm}^3)$]. This algebraic convenience allows us to generate the ratios of rules layer free from the volume distortions. It's basic to understanding how to detect biological changes. However, when expressing stereological results relative to the total liver, multiplying a cm^3 of fixed liver by the volume of a fresh liver leads to an overestimate when the tissue fixation produced a net shrinkage. We'll return to this problem in the case studies.

3.4.1 Example 4: Liver Regeneration (original data adapted from Pieri et al., (1975; PZMG75)

How can we use the updated data of this study to explore ways of finding solutions to the problems created by our data references? To begin, a basic question. Will updating the results of this study of liver regeneration allow us to explain how the hepatocytes and liver changed in response to a two-thirds hepatectomy? No. Why not? Explaining a change involves reverse engineering it, which means that we must reconstruct – step by step - the way biology produced the change. Since such a change requires both morphological and biochemical data, surface areas working alone cannot explain the solution to the problem. Nonetheless, the updated results provided a clue that might prove helpful in resolving the swelling-shrinkage problem of tissue fixation.

Figure 3.10 shows that increases in the surface areas of regenerating cytoplasmic organelles increased more when related to the liver than to a gram of liver. Presumably, this resulted from the doubling of the average cell volume. Recall that we could check this result if we measured the DNA and repeated the same calculation. If the expected 50% difference didn't appear, what could explain the difference between the expected and observed value? The volume distortion produced by specimen preparation. The point? Biochemical data come from living material, whereas morphological data usually do not. This means that a biochemical and a morphological gram of liver differ by the changes in tissue volume produced by the methods used to prepare the tissue for microscopy. Consequently, the same gram of liver in fresh and fixed liver may or may not qualify as the same data reference. Will situations exist when the difference between the two grams – fresh and fixed - becomes important? Yes.

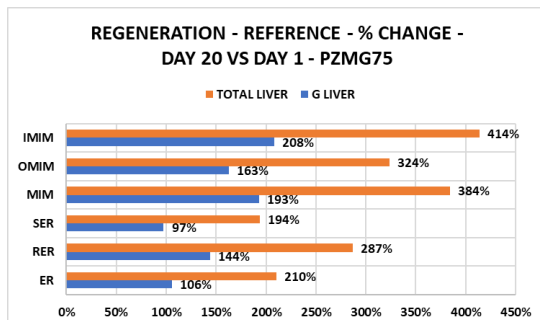


Figure 3.10 Relative changes in ER and mitochondrial membranes during liver regeneration related to two different references (Data adapted from Pieri et al., 1975).

Figure 3.11 shows that the surface area of ER membranes (RER, SER) made it back to the normal ratio by postoperative day twenty, but the mitochondrial membranes did not. Since the proportion of the mitochondrial membranes remained elevated (0.7) above the control (0 Days) at 20 days, we would need the biochemical and enzyme density data to calculate the total liver capacities and the membrane recipes to explain how the hepatocytes solved the problem. The point? Updating results from a significance difference between two data points to explaining how cells solve problems updates our perception of a biological change.

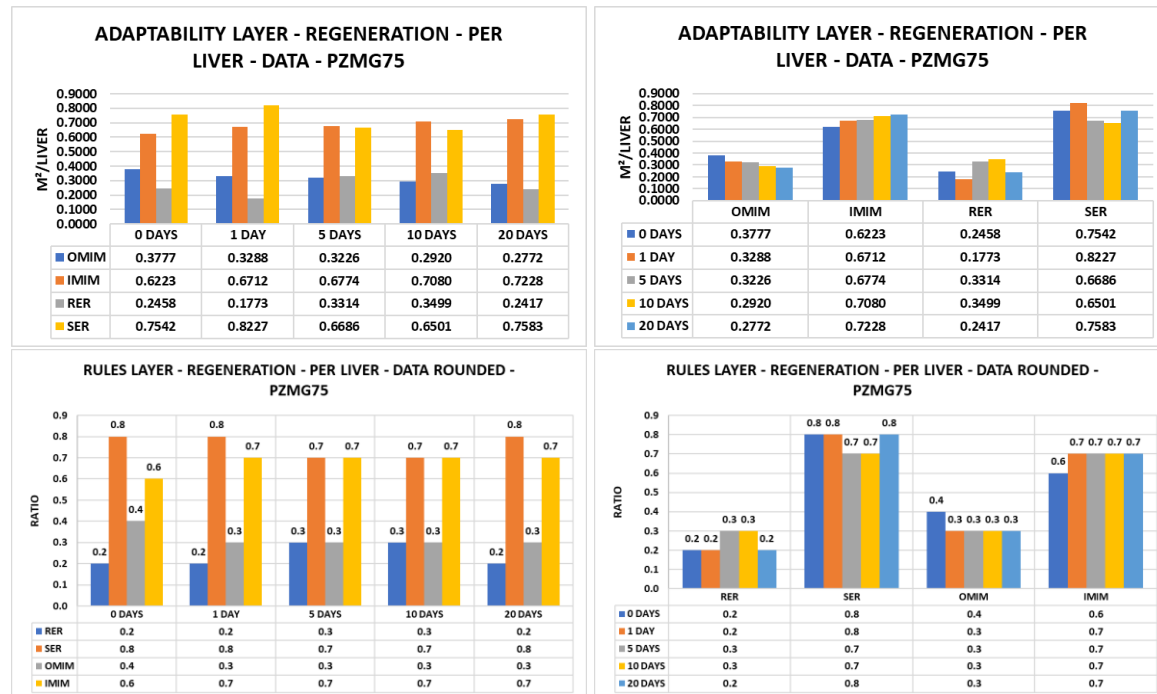


Figure 3.11 Recovering from hepatectomy involves returning to the control values of the cells. While the ER membranes returned, the extra IMIM membranes remained perhaps because the liver still needed to produce more hepatocytes or enrich the existing membranes. Notice that to demonstrate a successful outcome for the hepatocytes statistically, one could show that the 0- and 20-day data points were *not significantly different*.

Complex Data References (Variables + Constants): Detecting a biological change biologically involves paying attention to many changes occurring at the same time. Why? Because multiple complexities come into play wherein biological parts change in magnitude, direction, and in the relationships of structure to function. In effect, practically everything in play becomes a variable including the data references.

How do we solve the data reference problem? We know of two ways. One involves relating results to the liver but the other one, which in our case involves hepatocytic membranes, is part of the larger problem of detecting biological changes biologically. Although the liver reference is straightforward, the one for the cell is not. To detect changes in cells experimentally – at a time when all the parts and references are changing – we needed a solution to a paradox wherein the same data reference can change and not change at the same time. This meant that the same data reference had to operate in two different realities simultaneously.

Question: During a biological change in a cell, how can the same data reference be a constant and a variable at the same time?

Answer: Use a multidimensional reference. When the data reference defines the boundary of a one-dimensional square (unchangeable) enclosing a 2-dimensional plane (changeable), the paradox is satisfied, and we can detect a cellular change biologically.

In practice, implementing the change paradox involves calculating an enzyme density, which relates a variable enzyme activity to a constant square meter of membrane surface area. [Note: By dividing the enzyme activity by a membrane surface area (both related to a gram of liver), the denominator automatically becomes the constant (1 m² of membrane) while the numerator (units of enzyme activity) acts as the variable. It's a simple algebraic solution.

The insight? To detect and explain a biological change, one must reconnect biochemistry to morphology and collect results from both the hepatocytes and the liver. Cell data related to the liver provide the total output of the change (the capacity needed to solve a problem), whereas the cell data expressed as enzyme densities deliver the solution (the recipe). Operating side by side, these two solutions allow us to detect and interpret biological changes. But there's an additional level of complexity. Cells can find more than one solution to the same problem by changing the mix of enzyme activities and membrane surface areas to produce a wide range of enzyme densities (solutions). Since the hepatocytes appear capable of figuring out what to do by using different combinations of the available resources, one can imagine something akin to an intelligence linked to their information management systems. Phenotypes, for example, become the physical expressions of those systems.

3.5 Solving Data Reference Problems

Background: For the biological sciences, detecting a change consists largely of demonstrating a statistically significant difference between at least two data points. Curiously, we require a test for statistical significance but not a comparable test for biological significance. Since rules didn't exist for authenticating a biological change or for demonstrating reproducibility, we transferred the responsibility for detecting a change from biology to statistics. In effect, we put all our research "eggs" into the statistical basket. At the time, this solution was a good fit with our theory structure (reductionism), which favored simplicity over complexity, and we accepted statistical significance as our measure of a biological change. Bear in mind that the primer deals with outcomes related to events occurring in **cells** not to clinical trials with different outcomes.

We now know from reports based on meta-analyses that much of our published results are incorrect – at least statistically (Ioannidis, 2005; Ioannidis and Panagiotou, 2011). We also know that fixing the statistics cannot make the results correct unless we first test our biological results for correctness. But if we fix the results by updating the literature, we can't test them in the usual way statistically. Why not? Because we publish mean values, not the original raw data coming from individuals. Without a way to recalculate statistical tests from mean values, the task of detecting, validating, reproducing, and updating a change defaulted to duplicating the way biology changes.

Although we know how to use the rules layer to test for reproducibility within and across publications for one-to-one comparisons, combining data related to different references with unique outcomes becomes far more challenging. How then do we escape the uncertainties coming from incompatible data units and variable data references? So far, the best answer involves a new type of data reference. If we normalize changes and use change as the data reference, then we can largely avoid the above-mentioned problems. In turn, such a workaround allows us to integrate biological changes within and across publications, thereby creating new opportunities and theory structures. In practice, we can use change itself as a new

data type to assemble phenotypes designed to forward and reverse engineer biology and to test for reproducibility.

Change Produces New Information: Consider the basics. When biology changes, it changes the properties of its cytoplasmic membranes, which we can detect as enzyme densities. In turn, we can use these relationships of structure to function to track the changes in these enzyme densities as the cell works out the fine points of a solution. Moreover, enzyme densities expressed as data pair ratios uncover patterns of parallel changes. When arranged as phenotypes, such information, which would otherwise be unavailable, creates platforms well-suited to the task of predicting the chronological sequences of gene expression. By establishing a quantitative link between changes in cytoplasmic parts and their respective genes, it should be possible to reconstruct equivalents (algorithms) expressed as the large-scale information systems. Such algorithms may become the general solutions needed to construct simulators. With technology moving so quickly, simulations connecting genotypes to their phenotypes may soon become possible.

Phenotypes redefine the literature as an interactive research tool. When, for example, investigators use crisper to modify a genome, they have no way of knowing the unintended consequences. Potentially, this turns crisper into the enigmatic butterfly of chaos theory. Sometimes when wings flap something happens, other times not. The point? If all parts of cells connect and interconnect, changing a single part potentially changes the cell. Often, it's better to know than to assume.

3.6 Change as the Reference

When normalized, change becomes the universal reference for published data. Although such a reference works equally well with morphological and biochemical data, it works most successfully with enzyme densities because they encapsulate the key ingredients cells use to work out the solutions to problems. By interpreting enzyme densities (NORM2) in the rules layer, one can generate patterns that show how changes in one enzyme relates to changes in others. Such patterns, which predict the swings of gene expression and coregulation, can also reveal - in detail - the changes occurring in the relationships of one enzyme to another as they proceed together toward a shared solution. In effect, this new dimensionless reference plays a central role in solving the biology literature because it allows us to forward and reverse engineer changes by combining data collected from large numbers of otherwise incompatible publications.

Throughout the problem-solving process, the primer continually applies the basic principles of a biological change. The glue that holds everything together comes from a decision-making process based on asking the same question repeatedly. Namely, what's important to the living systems? We now know that it's important for cells to change their phenotype by generating new membrane recipes in sufficient quantities to solve specific problems. When trying to solve the biology literature, copying the cell's approach to problem solving becomes the logical choice because we know it works for cells.

CHAPTER 4

THEORIES AS STEPPINGSTONES

SUMMARY

Explaining a biological change from first principles enlists help from three theories. We'll use reductionist theory to take biology apart to study the parts, complexity theory to reconstruct biology from the updated parts, connections, and rules, and quantum theory to consider events beyond the reach of classical complexity theory. Basically, theories solve problems encountered by the primer by allowing us to view the same changes occurring in different realities. Zero-dimensional changes based on columns of data points fit well with a simplified interpretation of change, whereas one-dimensional lines and two-dimensional surfaces become gateways to the complexities of change. Across all the dimensions, however, detecting a change requires a strategy aimed at distinguishing between the reality of biology and the reality created by a theory and its methods. When guided by theories, detecting changes requires paying close attention to the rules.

A science progresses incrementally by exploring the territory of a given theory structure but often explosively when new theories appear. Since a new theory comes with its own set of rules, it triggers entirely new ideas, data, understandings, and unforeseen problems. Few would argue that our current reductionist model with its simplifying assumptions has become inconsistent with an experimental biology defined largely by complexity. For this reason, the primer prefers to use an updated theory structure based largely on a first principles approach, which becomes a better fit with information technologies and the emerging theories of biology, chemistry, and physics.

To this end, we'll continue to copy biology by exploring change not as a significant difference between two data points but instead as a problem-solving event engineered and executed by highly accomplished and brilliantly engineered cells. By updating a wide range of published changes, we'll continue the process of gathering clues to uncover the basic mechanics and principles of complex biological changes. But moving from one theory to another can cause unforeseen outcomes. For example, what happens when we encounter a change so inexplicable that copying biology no longer leads to a familiar rule or principle as an explanation? It reminds us that biology is privy to most if not all the universal laws of nature because of its ability to operate with mathematical prowess across a vast hierarchy of size (recall that it operates with parts ranging in size from subatomic particles to atoms, molecules, organelles, cells, organs, ecosystems, and planets).

Since biology has already demonstrated an understanding of quantum mechanics by creating major applications such as photosynthesis and visual systems by interacting with photons, we have reason to believe that it already figured out how to link the properties of classical and quantum mechanics. If true, then we can expect that our experimental questions will become increasingly difficult to answer but eventually easier to understand. If hepatocytes use quantum-based rules to change, how do they convert the indeterminate events of quantum mechanics into the determinate changes that we observe experimentally in our world of classical mechanics? How will we deal experimentally with the

convergence of two seemingly incompatible theories? Will we continue to find answers our experimental questions by copying them from biology?

A first step toward answering such questions begins by allowing biology to operate as a data driven science based on rules and first principles. This is in line with the goals advanced by the Biomatrix Workshop (Morowitz and Smith, 1987). By providing ready access to experimental data, we encourage the appearance of new theories, data structures, and principles. A second step involves aggregating published data in keeping with the rules, principles, and organizational framework as defined by living systems. Such a strategy enables wide-scale data integration and prediction with an emphasis on understanding the basic mechanisms of biological changes. The third step encourages model building as a strategy for introducing and testing new theory structures as we begin the process of reverse engineering our way to biology's first principles.

Success doesn't happen on its own, it takes time, determination, and resources. Once biology learned how to capture the properties of quantum theory, it brought them into our classical world as practical applications. As it became an expert at using universal laws, biology terraformed our planet, populated every nook and cranny with living things, and created countless opportunities for discovering new solutions. If given the chance, biology will show us how to do what it does so successfully. Moreover, by adopting a first principles approach to our basic science of biology, we undoubtedly improve our own chances for success and survival.

In truth, theory structures define the way we see, interpret, and understand. To illustrate this point, we'll put the above statement to the test by applying a single point of failure (SPOF) analysis to two working theories for detecting biological changes – reductionism and complexity. Recall that a single point of failure test resembles a collection of light bulbs connected in series. To pass the test, all the bulbs must light up. Unscrew just one bulb, and they all go off. In short, it's an all or nothing outcome. We'll apply it to the theories to see how well they measure up as operating systems for a basic science.

4.1 Reductionist Theory: Applying a Statistical Definition of Change

Definition: Under reductionist theory, we routinely define a biological change statistically as the significant difference between two data points. Instead of distinguishing between difference and change, difference becomes the measure of change. Although this allowed us to use statistics to solve our complexity problem temporarily, it unintentionally replaced biology with a statistical interpretation of biology. How? By reducing the complexity of biology to the simplicity of isolated parts, we avoided the unmanageable complexity of the time (mid twentieth century) by focusing only on the parts we wanted to study. Statistical significance became the essential backup needed to “validate” our results. With reductionism, everything seemed to fall neatly into place. Methods, parts, and significance effectively “solved” the problem of demonstrating biological changes.

Our Problem: It now appears that our biological interpretation of reductionist theory was too good to be true. Why? Although reductionism allowed us to take the complexity out of our experimental models, it couldn't take the complexity out of the data. In truth, “simplified data” remain indelibly complex because of what biology and our experimental methods left behind. The portending clue remains today as the widespread absence of reproduceable results. Given our inability to reproduce results independently, the parallelism so carefully constructed between statistical significance and biological change became unglued. And what did the absence of reproducibility tell us? If we can't do reproducibility, we can't do change. The conclusion? Although reductionism as an experimental strategy works reasonably well for

physics and chemistry, it's not a good fit for biology when it comes to detecting changes in complex cells. Why not? Because during a biological change most if not all the data types and data references become variables. This means that reproducing a change involves reproducing not just one variable but a sizable collection of variables. Given the absence of cell complexity, such a task became largely impossible.

Penalty: No one was prepared for what was to come next. Several meta-analyses reported that all or most of the results published in the biomedical literature were incorrect (Ioannidis, 2005; Ioannidis and Panagiotou, 2011). The implication? Attempting to detect biological changes with just statistical p values (plus or minus the effect size test) carries a substantial risk. Why? Because doing so effectively takes biology out of the loop. Unless the biological results were correct to begin with (i.e., a biological change occurred), all the statistics in the world can't demonstrate that a biological change occurred. That power belongs exclusively to biology because it determines how it changes (by rule) and how it changes reproducibly (by rule). This explains why copying the way biology changes solves both our change and reproducibility problems because biology's approach to change works whereas our overly simplified reductionist models applied to biology cannot because they fail to play by biology's rules. But can we still show that a change has occurred by demonstrating a significant difference between a control and an experimental data point? Yes, of course, if we first demonstrate that a biological change occurred.

Background: The primary goal of reductionism in biology is to simplify complexity not to explore solutions to complex problems. We use reductionism to simplify biology by taking it apart so that we can characterize the parts. But do we teach our students that detecting a biological change or demonstrating reproducibility requires solutions to complex problems, which can include taking biology apart to collect data and then putting biology back together to explain the results? No. This explains why we routinely bump into the limitations imposed by our reductionist model when we want to detect a biological change or to reproduce a result. The point? Biology doesn't do simplicity, it does complexity. Reductionism, which remains duty-bound to its theory, does simplicity not complexity.

Reductionist Model for Change: A reductionist approach to detecting a biological change requires just two data points (one control, one experimental) – even though biology needs substantially more information to change or to reproduce a change (Table 4.1). Investigators trying to detect a biological change need even more information because of the chaos created by our experimental methods.

Table 4.1 Detecting a biological change with insufficient information.

REDUCTIONIST THEORY(CHANGE MODEL)		
DATA	SPOF	PROBLEM
Control	Failed	Too many missing parts and connections
Experimental	Failed	Too many missing parts and connections

Limitations of Reductionist Theory When Applied to Detecting Changes in Cells

1. Statistics can detect a significant difference between two data points.
2. Given two data points, statistics cannot test for a biological change unless the change occurred.
3. Given two data points (two values), biology cannot execute or demonstrate a cell change.
4. Biology changes by changing cell phenotypes.

5. Statistical procedures often create mean values with one biological variable accompanied by multiple experimental variables (morphological: section thickness and compression, volume distortions, unstable references (cell packing problem), nonrepresentative sampling, etcetera; biochemical: unstable references (cell packing problem, mg protein), nonrepresentative sampling (e.g., microsomal data without recoveries), etcetera.

Figure 4.1 illustrates a significant difference between control and experimental data points. If we applied the statistical tests correctly, is it fair to assume that the points are different? Yes. Since the points are significantly different, can we also conclude that a biological change occurred? No. Why not? Because to do so we must assume that demonstrating a significant difference is equivalent to demonstrating a biological change. To eliminate the assumption, we must demonstrate that a change occurred biologically and that the change was not the result of methodological errors, biases, or missing information.

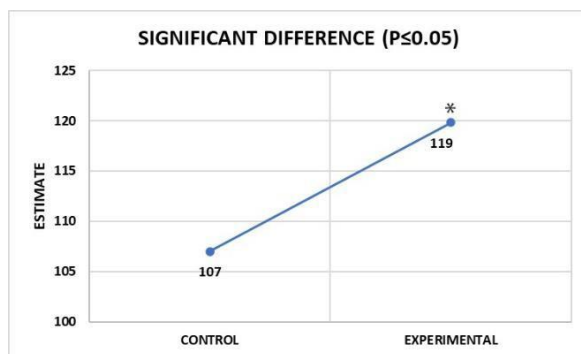


Figure 4.1 Detecting a biological change statistically by comparing two parts begins by assuming the existence of a biological change. No biological change, no statistical interpretation beyond that of a difference. Recall that an experimental change includes two sets of changes, one coming biology and the other from the methods. The asterisk (*) identifies a significant difference not a biological change.

4.2 Complexity Theory: Copying Biology's Definition of Change

Catch-22: The Biomatrix Workshop (1987) suggested that we could derive biology from first principles by reverse engineering biology – if we first organized the published data of biology. Since publications typically include data collected from isolated parts, there's nothing left to take apart (reverse engineer). Herein lies a catch-22. To detect and explain a biological change, we must first put biology back together from the parts (by rule) before we can take it apart to discover the rules responsible for the way the change originally occurred. The point being that the way we put biology back together determines what we get when we take it apart.

Background: The first two books of this series figured out how to (1) find the design principles of biology, (2) reconstruct - by rule - fragments of biology assembled from published parts (expressed as mathematical markers), (3) reverse engineer biology from the parts using cluster analysis, and (4) generate models for diagnosis, prediction, and reproducibility (Bolender, 2016, 2019). This book uses a first principles approach to (1) reconnect, explain, and redefine biological changes, (2) forward and reverse engineer changes, (3) combine changes across publications, (4) use reconstructed phenotypes to characterize, diagnose, and predict biological changes, (5) create phenotypic models suitable for predicting networks of gene expression, and (6) respond to a series of inexplicable changes by juxtaposing the properties of classical and quantum mechanics.

Additions to Complexity Theory (Bolender, 2019): (1) Two interpretations of the same data occur in two distinct layers - adaptability and rules, (2) enzyme densities (ED-NORM2) can be calculated from normalized datasets with or sometimes without matched data references, (3) change is the process, the solution is the cell, (4) transitioning from one cell phenotype to another explains the mechanism of a change, (5) biological changes detected within the reality of classical mechanics can display properties typically attributed to quantum mechanics.

The Problem: Biology treats a change as a problem-solving event. Such solutions have two parts, (1) a cell recipe, and (2) a total organ capacity. The recipe describes relationships of structure to function wherein groups of parts define patterns expressed as ratios. Such patterns represent the response to the need for a change. Capacity involves scaling the individual capability of cells to the magnitude of the problem, which for our purposes here occur at the level of the organ, namely the liver. The primary vehicle of change becomes the phenotype, which generates solutions by rearranging its parts and connections through a process that involves multiple changes occurring simultaneously and hierarchically. Since biological parts interact by rule and display extensive connectivity, a change operates within an information space defined by a mathematically coherent system. The glue that holds everything together comes from the basic relationship of structure to function as defined by the postulate (or perhaps more appropriately the rule) of biochemical homogeneity (deDuve, 1974).

Reverse Engineering Biology: The process of reverse engineering involves taking something apart, understanding the contributions of each part, possibly reengineering the parts, reassembling the parts, and finally turning it on to see if it works. Will repeating such a process allow us to reverse engineer biology from published data? Not directly. Why not? Since the biology literature publishes almost exclusively parts data, there's little left to take apart. This means that using reverse engineering to figure out how to derive biology from first principles will not work unless we first figure out to put biology back together correctly using just the published parts. In effect, this means that we must reconstitute or "forward engineer" biology from its parts and rules (first principles) before discovering the rules of a change by reverse engineering (e.g., a cell). Although the rules were unknown at the outset, we knew how to find them given the exercises described in the first two books (Bolender, 2016, 2019). The rules described earlier as mathematical markers became the data pair ratios of the current rules layer.

By connecting parts two at a time to form data pairs and calculating their ratios over the course of an experiment, we can reassemble (forward engineer) a copy of the original biology identified herein as a phenotype. In turn, reverse engineering a reassembled cell phenotype (or part thereof) identifies a set of subgroups contributing their local solutions to the global problem. When executed with enzyme densities, the change leads to a new enzyme-membrane recipe (mix of enzymes associated with a membrane) capable of solving (or mitigating) the problem. The advantage of the phenotype model comes from its ability to accommodate large amounts of published data. In effect, it suggests a workable solution to a central question of the Biomatrix (Morowitz and Smith, 1987): "How do we organize all the published data of biology?"

Figure 4.2 tracks changes in the recipes (enzyme densities) of ER membranes in developing hepatocytes before and after birth (original data adapted from Dallner et al., 1966; DSP66 with an ER assist from Herzfeld et al., 1973; HFG73). If we had a similar plot for RNA molecules producing these enzymes, would the two sets of changes (curves) superimpose at least in part? If yes, or close to yes, then prediction becomes a two-way street (parts \rightarrow genes and genes \rightarrow parts). If not, then one can imagine that the RNAs deliver the instructions for assembling individual parts (e.g., enzymes), but mechanisms located in the cytoplasm (organelles?) decide how to put the parts together to solve problems. Given the complexity of the solutions, it seems likely that cells would apply advanced methods requiring continuous

calculations and exact timing. In turn, this would predict the presence of cell computers and sets of instructions. Microtubules, for example, appear to display properties consistent with quantum computing (Hameroff and Penrose 1996, Markus et.al., 2009) and reports of quantum effects occurring in cells already exist in the literature. But do microtubules exist in non-dividing hepatocytes? Yes.

Notice in Figure 4.2 that the developmental process advances from left to right through a series of changing patterns - displaying varying degrees of order - wherein a collection of subgroups become assembled and differentiated. A temporary solution (identified as a cell recipe) appears from days four to six, followed by the start of the next change the following day.

Recipe changes (ΔED)

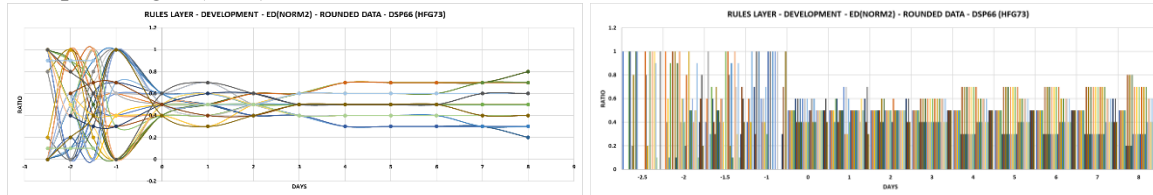


Figure 4.2 Ratios become the recipes that define the mix of parts, connections, and relationships (Original data from Dallner et al., 1966; HFG73).

Reverse engineering the partial phenotype shown in Figure 4.2 into the first subgroup (0.5:0.5) tells us that the solution for the group of paired enzymes found their solution two days after birth (Figure 4.3). When combined, all the subgroups from all the data pairs define the recipe (the solution) of the partial phenotype. In this way, hepatocytes deliberately grow into the solution by creating and fine-tuning individual subgroups. Simply put, cells appear to use rule-based ratios to become the solutions.

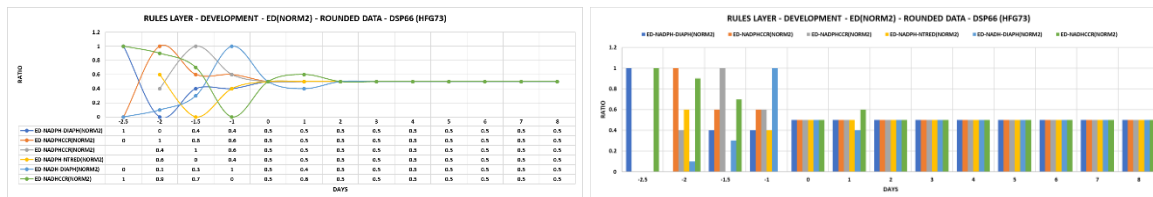


Figure 4.3 Solutions to biological problems appear as persistent ratios (three consistent data points) displayed by subgroups (e.g., 0.5:0.5) (Original data from Dallner et al., 1966 HFG73).

By entangling data pairs, we can speculate that biology could then use the properties quantum mechanics to make the calculations needed to solve the problem of optimizing the relationship of one part to the other – within and across the subgroups. For the single subgroup shown in Figure 4.3, the enzyme densities of this subgroup would have become “optimized” by postnatal day two. Furthermore, we could speculate further that the optimization would occur hierarchically, first in the hepatocytes, then in the liver, and finally systemically.

Complexity Model for Change: Biological changes operate within a theory structure based on complexity, which we are learning to copy from biology via the literature. When detecting a change within the bounds of complexity theory, the single point of failure analysis (SPOF) becomes a useful way to gauge our progress. When a change occurs, the cells follow a given protocol that we can duplicate to detect the same change. Since biology cannot afford a single point of failure, we’ll be copying a strategy designed specifically to succeed. For biology, delivering the best possible change often becomes a pass-fail test with survival providing the incentive.

For hepatocytes, the point in changing their phenotype is to solve problems by changing cell recipes that deliver advantageous outcomes. The table below identifies the data needed to detect and interpret a biological change experimentally using data from molecules, organelles, cells, and organs.

A biological change operating within the complexity model, includes six data types, one ratio for morphology, one for biochemistry, and one for defining the relationship of structure to function (Table 4.2).

Table 4.2 Parts needed to detect a biological change experimentally.

PART	DEFINITION
S_i	surface area of a membrane compartment i
g	gram of liver
$S_i/g \text{ liver}$	surface area of compartment i per gram of liver
$S_i(\text{liver})$	surface area of compartment i per liver
$W(\text{liver})$	weight of the liver
U_j	units of activity for enzyme j
$U_j/g \text{ liver}$	units of activity for enzyme j per gram of liver
$U_j(\text{liver})$	units of activity for enzyme j per liver
U_j/S_i	enzyme density (ED) – units j divided by surface area i

In living hepatocytes, for example, the items listed in Table 4.3 would produce a biological change because the data types and equations work as written. A single point of failure (SPOF) would not occur.

Table 4.3 Complexity theory: the change model applied to hepatocytes.

COMPLEXITY THEORY: CHANGE MODEL	
EXPERIMENTAL DATA	SPOF
Morphology	
$S_i/g \text{ liver}$	Passed
$S_i(\text{liver}) = (S_i/g \text{ liver}) \cdot W(\text{liver})$	Passed
$W(\text{liver})$	Passed
Biochemistry	
$U_j/g \text{ liver}$	Passed
$U_j(\text{liver}) = (U_j/g \text{ liver}) \cdot W(\text{liver})$	Passed
Morphology + Biochemistry	
$ED = U_j/S_i$	Passed

But how do our current experimental outcomes compare to those of biology? Although we can copy biology's model for change theoretically, applying it in the lab will most likely turn five of the six items into SPOFS (Table 4.4). Why? Biology's approach gives the correct results, whereas ours needs work. With the single exception of the liver weight, our experimental methods routinely corrupt the results. While all the basic sciences suffer from methodological damage, minimizing the damage becomes an essential part of any experiment.

To copy biology acceptably, we need to improve our estimates for the five failed SPOFs by updating the experimental procedures. The following two tables (4.4, 4.5) identify the problems and suggest mitigations.

Table 4.4 Errors and biases associated with laboratory data.

LABORATORY DATA	SPOF	PROBLEMS
Morphology		
S_i/g liver	Failed	Section Thickness, Volume Distortions, Undefined Number of Cells/g
$S_i(\text{liver}) = (S_i/g \text{ liver}) \times W(\text{liver})$	Failed	Section Thickness, Volume Distortions
$W(\text{liver})$	Passed	
Biochemistry		
U_j/g liver	Failed	Undefined Number of Cells/g, No Data from the Homogenate
$U_j(\text{liver}) = (U_j/g \text{ liver}) \times W(\text{liver})$	Passed	But No Data from the Homogenate and No recoveries
Morphology + Biochemistry		
$ED = U_j/S_i$	Failed	All the Above

BROKEN DATA (SPOF = 6)	REPAIRED DATA (SPOF ≈ 0)
Morphology	
S_i/g liver	S_i : corrected for section thickness biases.
	g liver: corrected for volume distortions.
	g liver: corrected for changes in the number of cells.
$S_i(\text{liver}) = (S_i/g \text{ liver}) \times W(\text{liver})$	
Biochemistry	
U_j/g liver	g liver: corrected for changes in the number of cells therein.
	g liver: corrections for microsomes (recoveries calculated).
$U_j(\text{liver}) = (U_j/g \text{ liver}) \times W(\text{liver})$	
Morphology + Biochemistry	
$ED = U_j/S_i$	

The table below identifies correction factors (CF) designed to repair experimental data and deliver a better copy of a biological change.

Table 4 5 Corrections for methodological errors and biases.

BIOLOGY	SPOF	UPDATED LAB DATA	SPOF
Morphology		Morphology	
S_i/g liver	Failed	$S_i/g \times (CF-STC)$	Passed
$S_i(\text{liver}) = (S_i/g \text{ liver}) \times W(\text{liver})$	Passed	$S_i(\text{liver}) = S_i/g \times (CF-STC) \times W(\text{liver})$	Passed
$W(\text{liver})$	Passed	$W(\text{liver})$	Passed
Biochemistry		Biochemistry	
U_j/g liver	Failed	$U_j/g \times (CF-E+N)$	Passed
$U_j(\text{liver}) = (U_j/g \text{ liver}) \times W(\text{liver})$	Passed	$U_j(\text{liver}) = (U_j/g \text{ liver}) \times W(\text{liver})$	Passed
Morphology + Biochemistry		Morphology + Biochemistry	
$ED = U_j/S_i$	Passed	Ratios	Passed

The correction factors resolve the following problems:

1. Correction for section thickness and compression (CF-STC)
2. Correction for volume distortions produced during preparation for microscopy (CF-VD) – if necessary.
3. Correction for recovery of microsomes (CF-E+N).

A Remaining Problem: In practice, enzyme activities are related to a mg of protein and then to a gram of liver. The Lowry test for protein (Lowry, et al., (1951), which uses a protein standard based on bovine serum albumin (BSA), may cause problems because it assays only two (tyrosine and tryptophan) of the twenty-two amino acids. If the newly synthesized proteins do not contain these two amino acids in the same proportion as BSA, then the data reference will not work as imagined.

Comments on Specific Details:

1. When the total number of cells in the liver remains constant, relative changes in hepatocytes (and components) remain the same when related to an average cell or to the liver.
2. The ratios in the rules layer provide a sampling test. When all the individuals used for a given data point have the same data pair ratios, the population is homogenous. A heterogeneous population includes individuals displaying different data pair ratios (different rules).
3. When trying to reproduce previously published results, first check the ratios (rules) and experimental designs for SPOFs. Use normalized data pair ratios to demonstrate and aggregate similar results.

4.3 Classical Entanglement

Definition: Classical entanglement occurs when two biological parts become connected. The test for entanglement is predictability. By knowing the value of one part, the observer automatically knows the value of the other. This situation occurs whenever the values of two connected parts form ratios (rules). Using databases populated with mathematical markers (alphanumeric strings of named ratios), one could show that populations of the same markers occurred routinely within and across cells and species (Bolender, 2019). This observation demonstrated the presence of widespread reproducibility within the biology literature.

Simple Changes: A single biological part has three options when a change occurs. It can increase, decrease, or remain the same.

Complex Changes: A complex biological change (two or more connected parts) has far more than three options. For a pair of biological parts (the basic unit of complexity), each part can (1) increase, decrease, or remain the same and (2) exist as part of a ratio with one of nine possible values. Given that increasing the number of possible outcomes fits with a basic design principle of living systems (it improves the likelihood of survival), modelling complex changes with data pairs seemed the best place to start. This thinking eventually extended to the strategies used to develop and test the phenotype models.

However, it's one thing to define a data pair ratio as an example of classical entanglement but something else to associate it with quantum entanglement. What's missing is an experimental outcome so improbable in classical biology that it points to the involvement of other theories such as quantum mechanics. Recall that the major advantage complexity theory has over reductionism comes the fact that we can use it to explain how a change occurred and eventually why. While we know that biology has found solutions to the problem of translating quantum properties into classical applications, the underlying theory for such a feat remains unknown. Without a working theory, we have little help.

Entanglement of Cell Parts: In the next section, we'll encounter hepatocytes using classical entanglement to synchronize changes in two parts having the same values (+ or -) using their version of calculus while tracing two near identical wave functions 180° out of phase (complete interference). If this observation holds up, we now seem to know when and where to look experimentally for an explanation to an inexplicable series of quantum like events.

4.4 Unexplained relationship of Classical to Quantum Mechanics

If we imagine that our world view based on classical mechanics derives from collapsed states of quantum mechanics, then explanations for the baffling events we encounter in published data might have something to do with quantum theory. When updating a paper, for example, changes appeared that defied explanation. Hepatocytes recovering from phenobarbital treatment synthesized new ER membranes, transferred half of them to the SER, and retained the rest as RER. However, they did it in such a way that that the surface area of the newly produced ER didn't change. Such an expected finding hinted that such a result might have originated quantum mechanically but somehow became apparent in the results of a classical experiment. This seemed to suggest that a strategy based on copying biology extended to exploring the way classical biology interacts with quantum mechanics. A closer look at the changes in the ER membranes uncovered several puzzling events.

Hepatocytes Recovering from Phenobarbital (PB) Treatment: When updating biological changes according to complexity theory, unusual events occurred that didn't fit the classical model (Bolender and Weibel, 1973). A pair of organelles (RER and SER) appeared to fit the narrative of quantum entanglement. A change in one part influenced the change in the other exactly and the membrane changes generated a pair of symmetrical waves displaying interference. The waves were sinusoidal, 180° out of phase, fit similar polynomials, and the RER and SER surface areas changed at the same rates but in opposite directions (except when changing direction). In effect, the RER and SER of hepatocytes (1) behaved as entangled compartments, (2) identified time points traced waves, and (3) the production and distribution of the organelles displayed variable rates of change displaying mirror-image symmetry.

The publication uncovered these curious events at a time when hepatocytes were recovering from five days of phenobarbital treatment. After updating the results (applying corrections for section thickness compression), we'll look at the rules-based approach used by the cells to time the production and distribution of the newly synthesized ER to the RER and SER.

To reestablish the pattern of the changes, we'll extend the dataset to include the missing data point at recovery day 4 (Figures 4.4, 4.5). Since cells in developmental studies typically display cyclic growth patterns (Bolender, 2019), the missing point at day 4 disrupted the expected pattern. Applying the values of day 2 to day 4 recovered the expected symmetry of the changes (Figure 4.6).

Original Data

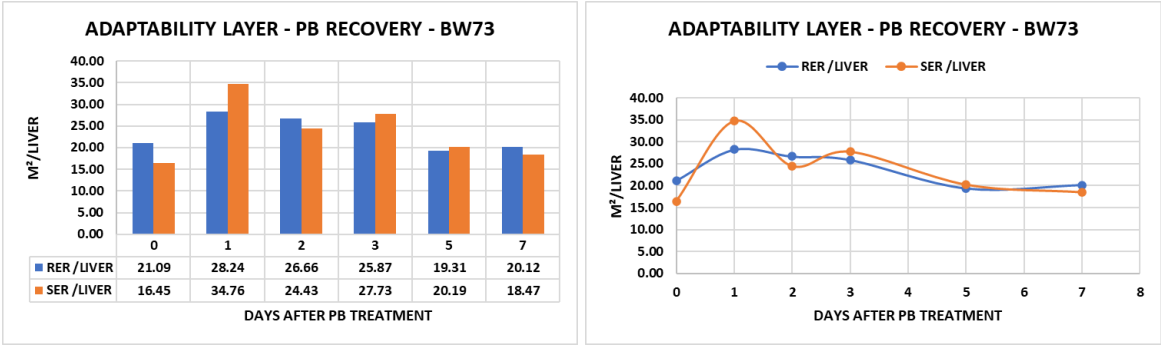


Figure 4.4 Crisscrossing curves in the adaptability layer can signal an interaction between the components (Original data from Bolender and Weibel, 1973).

Rules Layer

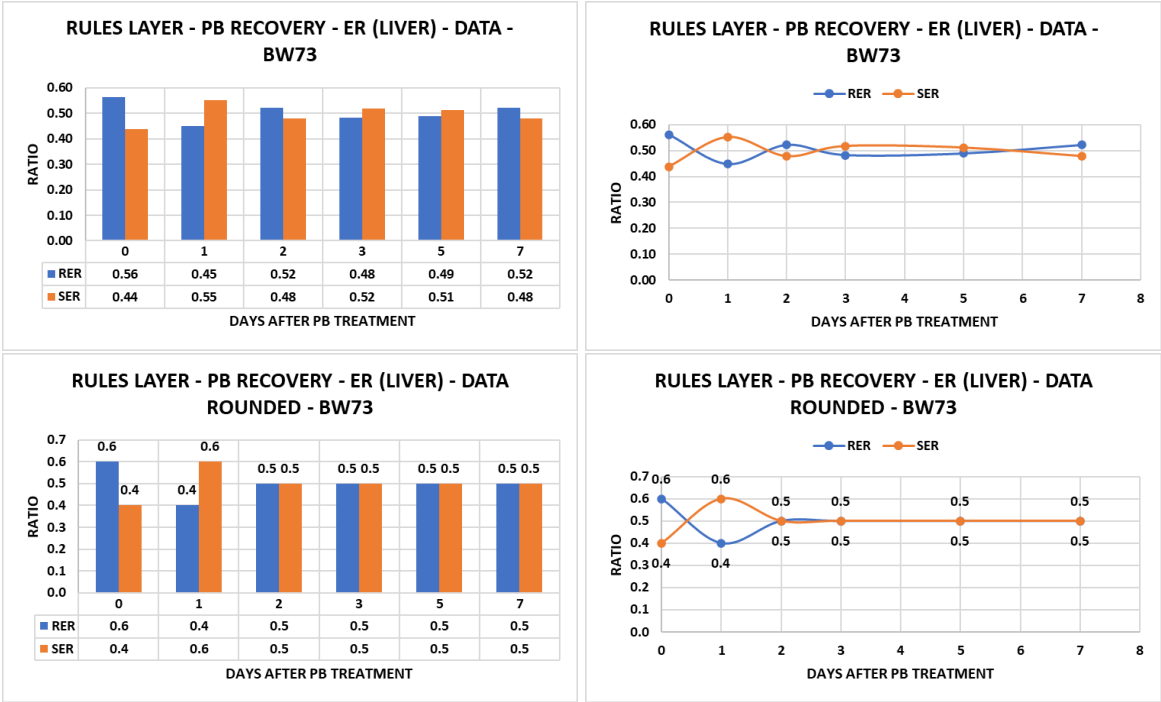
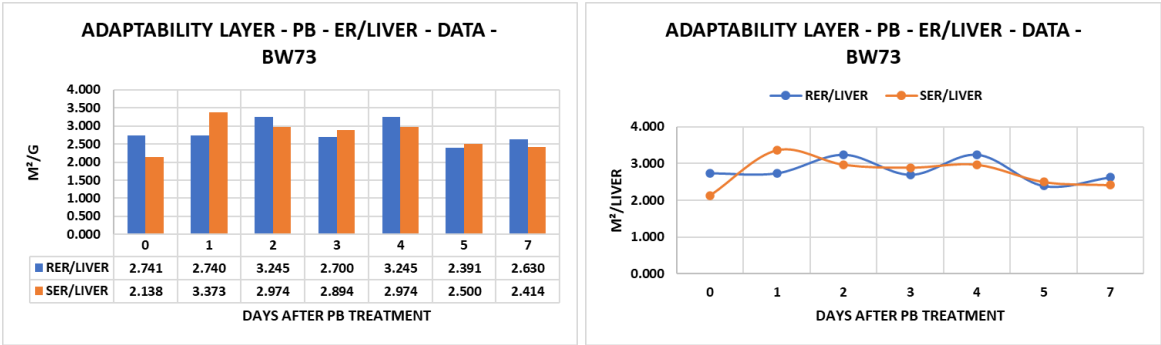
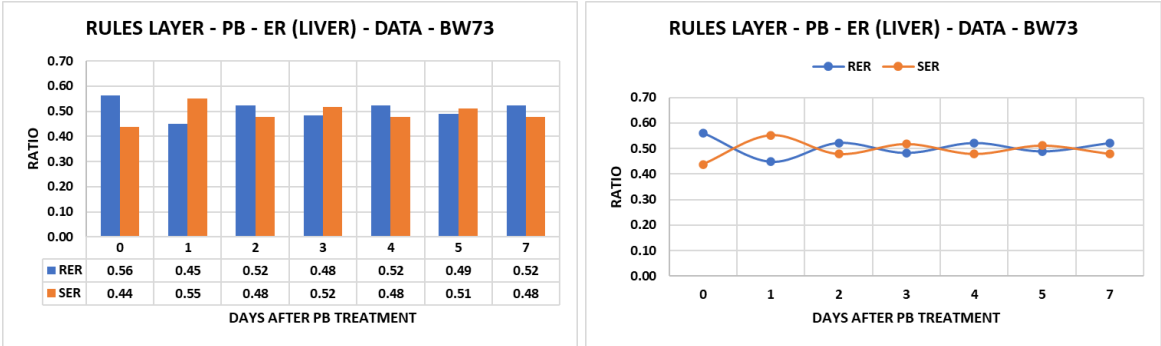


Figure 4.5 The same data expressed in the rules layer displayed a RER-SER symmetry with a solution (0.5:0.5). Note that data points were missing from days 4 and 6 (Original data from Bolender and Weibel, 1973).

Original Data Expanded (Missing Day 4 Replaced with Data Shared from Day 2)



Rules Layer (Day 4 with Data Added)



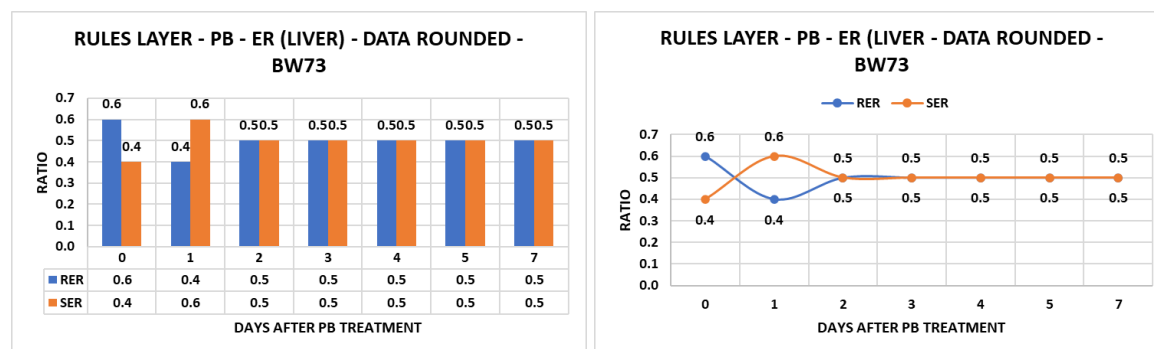


Figure 4.6 By adding back the expected value at day 4, the relationship of the RER to SER became symmetrical and the rounded data continued to show no changes in the data pair ratios (0.5:0.5) in the rules layer from days 2 to 7 (Original data from Bolender and Weibel, 1973).

A Classical-Quantum Model for a Biological Change: By expanding the dataset and selecting a segment of the RER and SER curves (1.0 to 3.5 days after PB treatment), the changes in RER exactly counterbalanced those of the SER (Figure 4.7). When added together, the two polynomial curves cancel because they share the same wave-like equation and are 180° out of phase (this explains the 0.5:0.5 ratio seen in Figure 4.6). The two curves showed that 50% of the newly produced ER membrane remained as RER and the remaining 50% went to the SER. The timing, precision, and complexity of the process seemed remarkable. Since at a given time point, one can use the polynomial equations to calculate identical plus and minus values for the RER and SER membranes, such a result could be consistent with an entanglement of the RER and SER. Moreover, entanglement of the data pair ratios also appeared to be in play with the change model used by the data pair ratios to forward and reverse engineer phenotypes. In effect, entanglement of parts may account for biology's widespread use of ratios as rules. If we know one ratio, we automatically know the other. When a cell changes one ratio, it automatically changes - and knows - the other. This is the reasoning behind quantum mechanics.

How did the hepatocytes continually vary and synchronize the rates of change across the two different membrane compartments? Although we know how to detect such changes after the fact, how did biology produce this remarkable feat in the first place? In Figure 4.7, the hepatocytes predicted the next point – instant by instant – to generate the two polynomial equations by continually varying the rate of change for the two membrane compartments simultaneously. While one can posit that the synchronization of the changes resulted from an entanglement between the RER and SER membranes, controlling the variable rates of change suggest the application of a calculus-like approach to problem solving. If true, then calculus would qualify as a first principle of a biological change. Since the calculations occurred in hepatocytes, computers must be intrinsic to cells. Published reports suggest that microtubules may have computational properties (Hameroff and Penrose 1996). Moreover, one can find microtubules in electron micrographs of hepatocytes juxtaposed to the ER.

Moreover, the on-the-fly addition of data points means that the hepatocytes can predict the changes that we see after the fact as the two crisscrossing polynomial equations shown in Figure 4.7. The likelihood that such carefully timed and precise changes occurred under the direct supervision of gene expression seems remote because of close and simultaneous timing of the changes. In any case, given the (1) widespread distribution of microtubules throughout the cytoplasm of cells and (2) their close association to cell parts, they already meet several requirements of an information processing system. If microtubules play a role in the events shown in Figure 4.7, then one could postulate that their total length and distribution would change during periods of sustained membrane growth and distribution. In other words,

changes in the total length of microtubules would also be expected to parallel total ER surface areas and induced enzyme activities as the hepatocytes increased their drug-metabolizing capacity.

Rules Layer (Δ RER VS Δ SER)

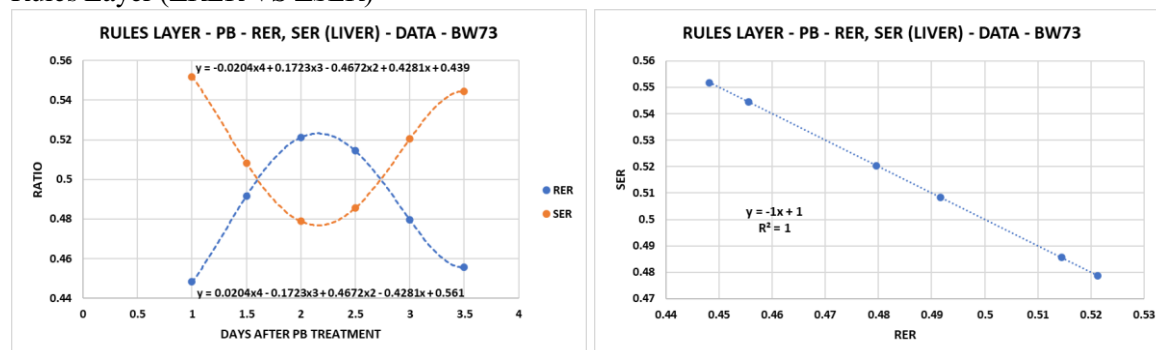


Figure 4.7 Hepatocytes appear to be solving the membrane replacement problem with two crisscrossing polynomial curves, 180° out of phase, displaying mirror image symmetry, and synchronizing changes in RER and SER. Since the cells generated the polynomial curves two points at a time simultaneously and at exact locations, they appear to be evaluating the polynomials one step at a time. This is the way we would expect a computer to do it. Notice that the ratios received no rounding, and one would expect cells to do this calculation in the rules layer (Original data from Bolender and Weibel, 1973).

By integrating the polynomials shown in Figure 4.7 between days 1.5 and 2 according to the mean value theorem of calculus, one can show that the hepatocytes would have used an average rate of change for the RER at day 1.762 and for the SER also at day 1.762. This means that the hepatocytes timed the production and distribution of ER such that the changes in RER and SER were equal in amount but opposite in direction. This suggests that hepatocytes figured out how to use the rules of quantum mechanics (or something else) to solve a change problem by applying an algorithm equivalent to one we use in integral calculus. More importantly, perhaps, it helps to explain why biology prefers ratios to single numbers because ratios define symmetries that can lead mathematically to elegant solutions.

The Classical-Quantum Model for Changing Cell Phenotypes: We can reconstruct phenotypes from published data by following relative changes in basic units of information (data pair ratios) as they work their way through a problem-solving event. Figure 4.8 shows a single subgroup of data pairs (expressed as ratios) taken from several published studies wherein hepatocytes responded to five days of exposure to phenobarbital (PB). The problem created for the cells by the experiment included the task of neutralizing the drug by replacing their current phenotype with one containing optimal amounts of drug-metabolizing and housekeeping enzymes. Notice that the input (exposure to PB) appears at the left of figure, the output at the right (solution), with the problem-solving zone located in between.

The task of replacing one phenotype with another involves resetting relationships across nested sets of increasing complexity. A change begins with two parts (a data pair ratio), continues as multiple subgroups of data pair ratios sharing the same solution, and concludes with a collection of subgroups that redefine the properties (recipes) of the new cell phenotype. This means that the classical part of model posits that a biological change is based on changes to the ratios of parts, not to changes in individual parts. Moreover, this identifies a major difference between complexity and reductionist theories.

The phenotype model postulates that pairs of parts, which include data pair ratios consisting of two enzyme densities, become entangled. In addition to keeping the parts connected, entanglement allows the relationships of the parts (one to one and one to many) to become optimized as they pass through the gauntlet of change to produce the best result within and across data pairs. The discovery of connected subgroups by reverse engineering the phenobarbital phenotype supports this view.

Figure 4.8 includes the solution (output) of one subgroup to the PB problem as a consistent ratio of 0.5:0.5. In turn, the subgroup can be reverse engineered into multiple subgroups (Figure 4.9).

Enzyme Density Subgroup (0.5:0.5)

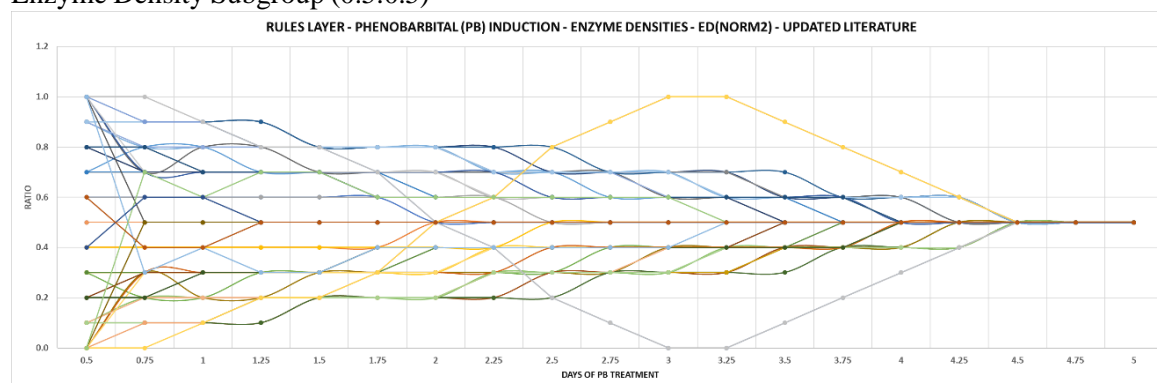


Figure 4.8 The phenobarbital phenotype combined data from several publications (see Chapter 9 for details). The 0.5:0.5 subgroup, named according to the last three ratios includes additional subgroups (two appear in Figure 4.9).

By continuing to reverse engineer the 0.5:0.5 subgroup shown in Figure 4.8, we can see that one subgroup solved the problem faster than another (Figure 4.9). Also note that the same enzyme densities coming from two different publications (ED-DEM(OE66)-NORM2, ED-DEM-(SHW69)-NORM2) posted reproducible results. In effect, global phenotypes include built-in tests for reproducibility.

Two Subgroups of the 0.5:0.5 Subgroup

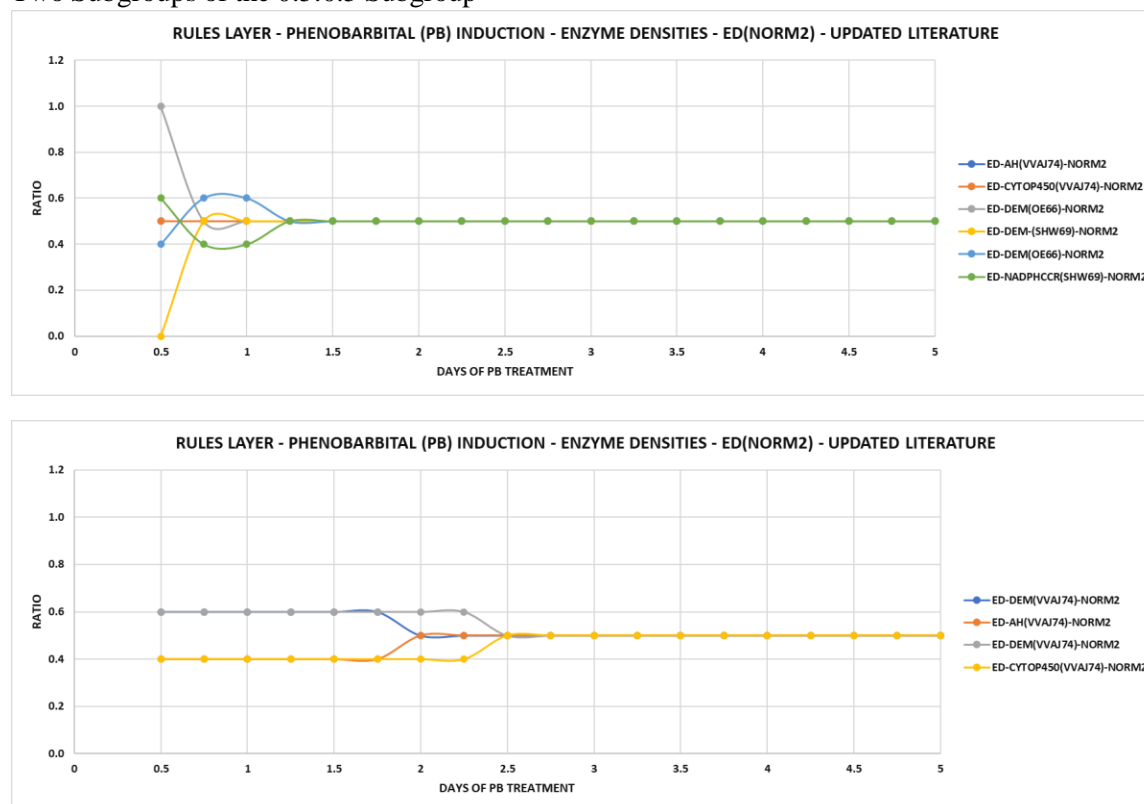


Figure 4.9 Subgroups of subgroup 0.5:0.5 reverse engineered from the phenobarbital induction phenotype show how combining data pairs within and across publications uses reproducibility to reconstruct complex changes (See chapter 9 for further details).

CHAPTER 5

DEVELOPMENTAL PHENOTYPE

SUMMARY

A science defines itself by the way it detects and interprets change. Working with biological changes becomes a challenging task because four factors come into play: (1) the changes in the part of interest, (2) the changes (biases and errors) added by the experimental methods, (3) the theory structure that provides the rules, and (4) the validity of the result. So far, only one player has managed to deal successfully with three of the four factors (1, 3, 4) and that credit goes to biology. This means that by copying the way biology changes, we reduce the task of detecting a change to minimizing the changes added by the methods. Since the patterns of change displayed by living systems begin with ratios, our model for change will do the same by starting with the simplest ratio (data pair ratio) and then allow the data pairs to grow into the complexity of a biological change (n parts taken two at a time). The interpretation of the results posits that the connected parts (data pairs) became optimized and ordered into optimal subgroups to deliver the solution (the change). In short, a biological change became the solution because the cell was the solution. The following case studies update published results in line with the model to the extent that the original data allow.

5.1 Generating Cell Phenotypes from the Literature

The Basic Strategy: Updating the biology literature operates at two levels – local and global. At the local level, we update the results of a published study and then reanalyze the data within the framework of a complexity theory copied from biology. Next, we expand, normalize, and aggregate the local results into global phenotypes. This combines several publications into datasets capable of generating global patterns. At both levels, change and reproducibility adhere to same rule-based order.

The four phenotypes chapters represent exercises wherein the beginner works through the mechanics of the literature update under a wide range of experimental settings. One of the primary lessons to take from the exercise will be the understanding of why it's so difficult to detect a biological change correctly when dealing with incomplete datasets. The message is simple. It takes the right data to get the right results.

The Questions: Since change becomes our primary window into the way biology works, updating published data focuses on expanding datasets and working out solutions to the larger problem of aggregating data across seemingly incompatible studies. If biology changes by making a transition from one phenotype to the another, how does it bring order to a system in which everything seems to be in flux? Within this seemingly chaotic setting, biology maintains order by connecting its parts according to rule-based ratios. When aggregated across publications, we'll use such ratios – expressed as data pairs, equations, and patterns – to follow the way biology defines a change within the context of a cell phenotype that grows its way into a solution.

Of special interest to us here are the patterns of change displayed by the different pairs of parts. If, for example, we know when a pair of parts changes similarly or differently, when one pair of parts mimics the changes of another, or when several pairs change together, then we can work out the complexity of a

change. Moreover, by seeing how biology replaces one phenotype with another in different settings, we gain insights into the process of forward and reverse engineering a change within the reality of a theory structure basically copied from biology.

The challenging part of the exercise involves figuring out how to compare changes occurring within and across publications when most or all parts are changing and when the experimental methods are detecting changes differently from the way they occur biologically. By managing the technical problems, we can begin to use phenotypes to identify the cytoplasmic strategies cells use to solve problems. Specifically, when a cell changes, we want to know what does what, when, where, and how? Emergent properties explain the why.

Mixed Data References: When calculating an enzyme density, we divide units of enzyme activity (U/G) by a membrane surface density (S/G): $ED = [(U/G)/(S/G)] = U/S$. We expect the gram of liver (G) to cancel out and it does. When updating the literature, however, authors often prefer to relate biochemical results to a mg of protein and those of morphology to a cm^3 of hepatocyte cytoplasm. Since such references fail to cancel out, we cannot calculate the enzymes densities unless both references become the same. This requires changing the biochemical reference from a gram of liver to a cm^3 of hepatocyte cytoplasm or vice versa. Since publications don't include basic data and calculations, we have no way to fix the mg of protein reference without the uncertainty produced by one or more assumptions.

When normalized, however, only the numbers remain, which means that we can use mixed data references when necessary to calculate enzyme densities. We can do this because changing a gram to a cm^3 has no effect on the shape of the normalized curve because multiplying a gram of liver by a constant does not change the shape of the curve (see worked examples included herein). Using the mg protein reference as a mixed reference assumes that its changes occur roughly in parallel with changes related to a gram of liver or a cm^3 of hepatocyte cytoplasm. Such an assumption becomes risky because updates of pre and postnatal development showed both parallel and nonparallel changes when compared to other references.

The Checklist: When updating a publication or interpreting the results of an ongoing experiment, we can simplify the process by reducing it to a checklist. It keeps one on track, minimizes mistakes, and discourages questionable assumptions.

CHECKLIST FOR UPDATING A PUBLICATION (LIVER)

1. ☐ Collect published data from figures, tables, and text.
2. ☐ If possible, relate the data to a gram of liver and to the liver.
3. ☐ Enter the raw data and subsequent calculations into Excel worksheets.
4. ☐ For stereological data (S and S/V), apply corrections for section thickness and compression.
5. ☐ Adaptability Layer: Include a copy of the original raw data.
6. ☐ Adaptability Layer: Fit the original data to polynomial curves with regression analysis.
7. ☐ Adaptability Layer: Expand the original data in order by evaluating the polynomial equations.
8. ☐ Adaptability Layer: Plot the expanded data.
9. ☐ Adaptability Layer: Normalize (NORM1) and plot the data.
10. ☐ Rules layer: Calculate ratios (original and normalized) - data (0.##) and rounded data (0.#).
11. ☐ Rules layer: Generate patterns from the original and normalized data; identify duplicates.
12. ☐ Adaptability Layer: Calculate enzymes densities – from original and normalized data.
13. ☐ Rules layer: Calculate ED ratios (original and normalized): data (0.##) and rounded data (0.#).
14. ☐ Rules layer: Generate ED patterns from original and normalized data; identify duplicates.
15. ☐ Adaptability Layer: Report solutions as recipes.
16. ☐ Adaptability Layer: Assemble EDs into phenotypes (forward engineer).
17. ☐ Adaptability Layer: Reverse engineer phenotypes to identify subcompartments and solutions.

The Changes: Updating publications focuses on the complexities of change - biological and methodological. Focus on finding answers to the following questions.

What can hepatocytes do to change? They can:

- Change the amounts of and relationships between enzymes.
- Change the amounts of and relationships between membranes.
- Change the relationships of enzymes to membranes (enzyme densities).
- Change membrane recipes (the amounts and proportions of membrane bound marker enzymes).
- Change the rates at which enzymes and membranes change.
- Change the biochemical and morphological capacities of the liver.

Specifically, how do hepatocytes change as data pair ratios? They can:

- Change the amounts of enzymes (A) and the amounts of membranes (B).
- Change the enzyme and membrane recipes (C).
- Change the enzyme densities of a membrane organelle (D).
- Change the rates at which enzymes and membranes increase or decrease in amount (E).

When changing, what are the options? Hepatocytes can:

- Use some or all five variables (A, B, C, D, E) in any combination to solve a problem.
- Solve the same problem using a different combination of the variables (A, B, C, D, E).

In effect, a biological change defines a variable rich environment. Reporting a change as an event occurring to one or a few variables ignores the fundamental nature of a biological change. Instead, detecting a change requires copying the complex sequence of events orchestrated by cells to solve a specific problem. The details tell the story of what the cells changed and explains how.

The Models: Starting with morphological and biochemical data, we can apply models to explore the effectiveness of different strategies for detecting and aggregating biological changes. To this end, patterns play a key role in identifying and interpreting the rule-based relationships of parts to parts within and across publications. These relationships include the following data pairs (expressed as ratios):

- 1) Units of Enzyme Activity (U_A) to Units of Enzyme Activity (U_B),
- 2) Membranes Surface Area (S_A) to Membranes Surface Area (S_B),
- 3) Enzyme Density A (U_A/S_A) to Enzyme Density B (U_B/S_B).

The change models range from interpreting results from a single experiment to integrating published data across many publications. For example, each model includes basic components.

- 1) Publication Model – Single Study Model (Publishing new results)
 - a) Enzyme Model [Original Data] - (Identify changes in the adaptability and rules layers)
 - b) Membrane Model [Original Data] - (Identify changes in the adaptability and rules layers)
 - c) Enzyme Density Model [Original Data]- (Identify changes in the adaptability and rules layers)
- 2) Global Model – Multiple Study Model - (Updating published data)
 - a) Enzyme Model [Normalized Data (NORM1)] - (Identify rule-based patterns of change)
 - b) Membrane Model [Normalized Data (NORM1)] - (Identify rule-based patterns of change)
 - c) Enzyme Density Model [Normalized Data (NORM2)]- (Identify rule-based patterns of change)

Summary: Although copying biology’s approach to change is inherently straightforward and easy to understand, once we try to detect a change experimentally, we add experimental changes that turn practically everything we measure or use as a reference into an altered variable. In effect, our experimental methods unleash a flood of uncertainty. When most of our data points (controls and experimentals) and data references become altered variables, detecting and reproducing experimental results correctly becomes nearly impossible. With practice, however, we can avoid most pitfalls by knowing where the problems occur, learning how to fix them, or figuring out how to avoid them. The case studies include examples.

5.2 Developmental Phenotype

Chapter 5 focuses on two developmental phenotypes (hepatocyte and liver) operating at two levels (local and global).

Phenotypes: A local phenotype records the data of a given experiment, whereas the global option combines the normalized data of several local phenotypes. Figure 5.1 summarizes the developmental phenotype.

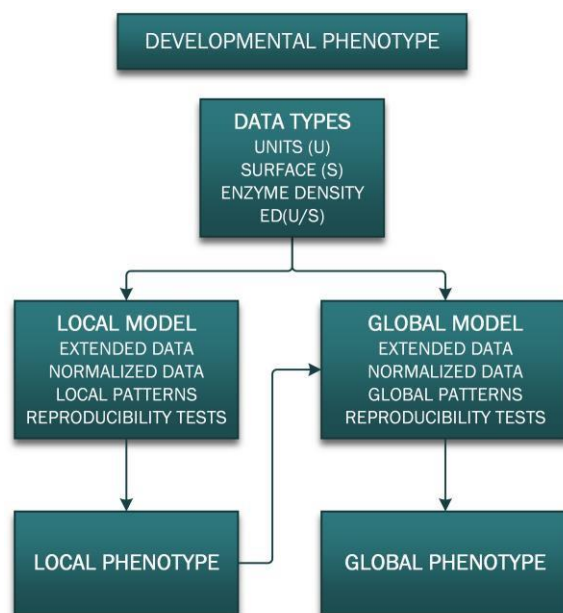


Figure 5.1 The developmental phenotype includes a local model for publishing original data and a global model for comparing current results to those previously published. Both models extend data, use normalized results, rely extensively on patterns, and test for reproducibility.

5.3 Case Studies

A case study revisits a publication and when possible, updates the original data into local and global phenotypes. In turn, we’ll use the reworked version of the data to track the same biological changes as a

standalone paper (local phenotype) and as a dataset capable of combining its results with the data of other papers (global phenotype).

Such a strategy, which includes integrating data within and across publications, works reasonably well when encountering incompatible data types, sharing data, generating phenotypes, and analyzing results. Since a biological change represents the culmination of many individual changes occurring over time, the phenotype becomes the model for summarizing biology's approaches to problem solving. To view and interpret changes, we'll draw upon data coming from both the adaptability and rules layers to generate the patterns needed to reconstruct phenotypes.

Specifically, these exercises create opportunities for the beginner to explore biology as a complexity and to experience the benefits of treating the biology literature as a renewable resource. The overarching strategy becomes one of exploring new approaches to organizing published data with the goal of optimizing the immediate and long-term effectiveness of published data.

5.3.1 Case Study 1: Development (Enzyme Differentiation) – GHK72 (HFG73)

Source: Update applied to the original data from Greengard O., Herzfeld A., Knox E. (1972) Cytomorphometry of developing rat liver and its application to enzymic differentiation. J Cell Biol 52: 261-272.

Topic: Morphological and biochemical development of the liver including responses of cells to hormones.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, calculate enzyme densities [$ED = [(U/G)/(S/G)]$; $ED = [(U/G)/(S/G)](NORM1)$; $ED(NORM2)$], analyze patterns, and report biological solutions (recipes).

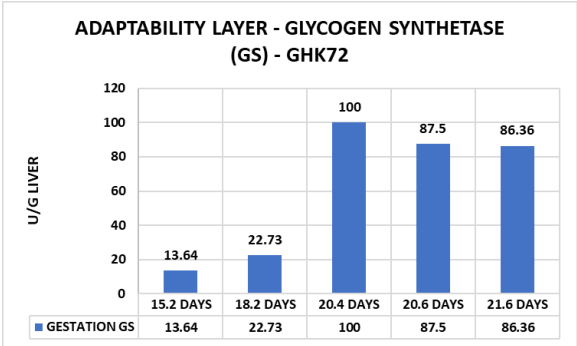
Dataset: Using local and global models, we'll follow the updated changes in five enzymes [(glycogen synthetase (GS), tyrosine aminotransferase (TAA), glucokinase (GK), aspartate transcarbamylase (ATCA), phosphoserine phosphatase (PP), and one membrane (ER) during early development.] The normalized enzyme densities (NORM2) used mixed references (U/G of liver, S/CM³ of hepatocyte cytoplasm) - see 5.3.10.

Greengard et al., (1972) reported changes in enzyme activities during development, which included glycogen synthetase (GS), tyrosine aminotransferase (TAA), glucokinase (GK), aspartate transcarbamylase (ATCA), and phosphoserine phosphatase (PP). ER surface areas came from a study by Hertzfeld et al., (1973; HFG73). Note that the titles of the figures often carry two reference codes based on the initials of the authors last names and the publication date – e.g., GHK72 (HFG73). The first code identifies the updated paper - GHK72, whereas the second one in brackets (HFG73) credits the source of shared data (in this case ER surface area).

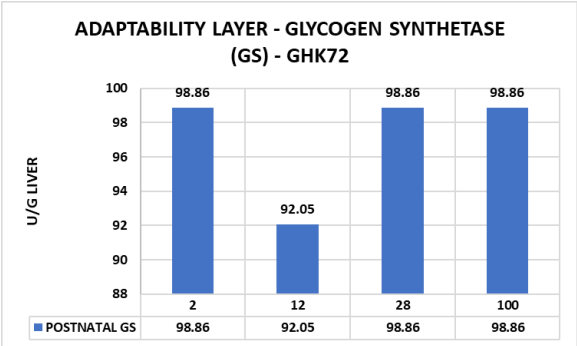
Local Model (Enzymes) – Adaptability Layer

Figure 5.2 shows that changes in the activities of the enzymes displayed a wide range of values, ranging from < 1 to > 1000 U/G. Since these estimates came from a gram (G) of liver, we would expect that the number of hepatocytes filling a gram of liver changed during the development as the cells developed. By relating results to the liver, we can remove the cell packing error and can see how the gram of liver reference influenced the results (Figure 5.3).

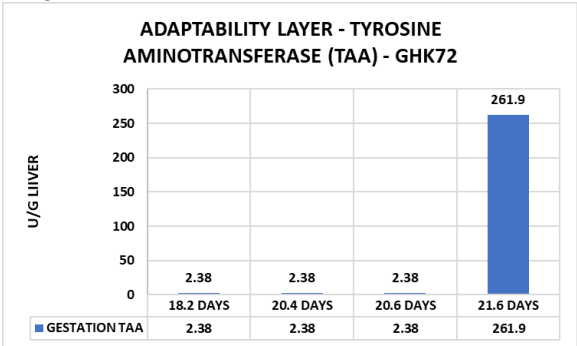
Original Data (Gestational)



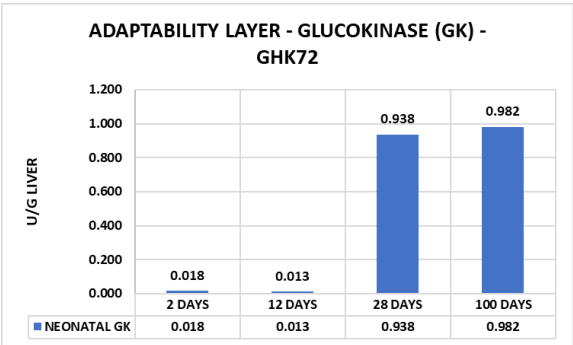
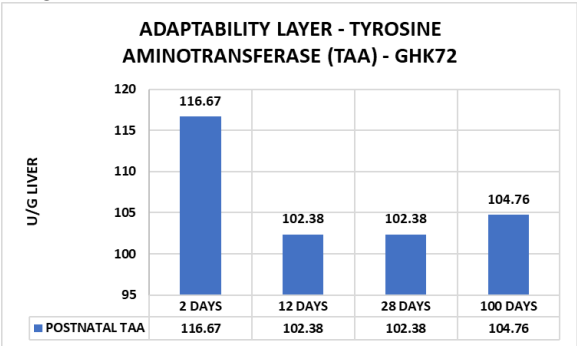
Original Data (Postnatal)



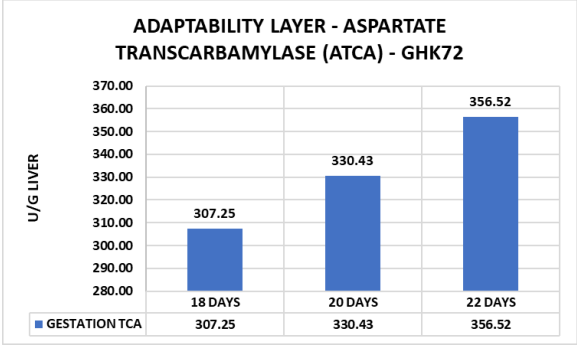
Original Data (Gestational)



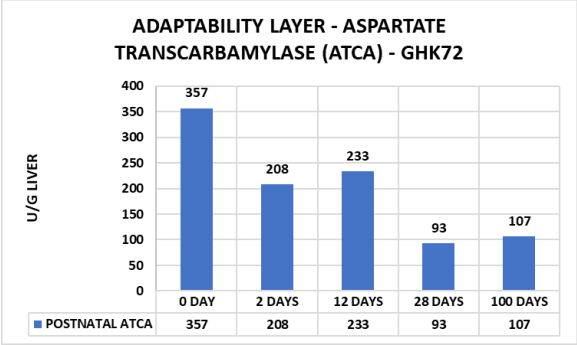
Original Data (Postnatal)



Original Data (Gestational)



Original Data (Postnatal)



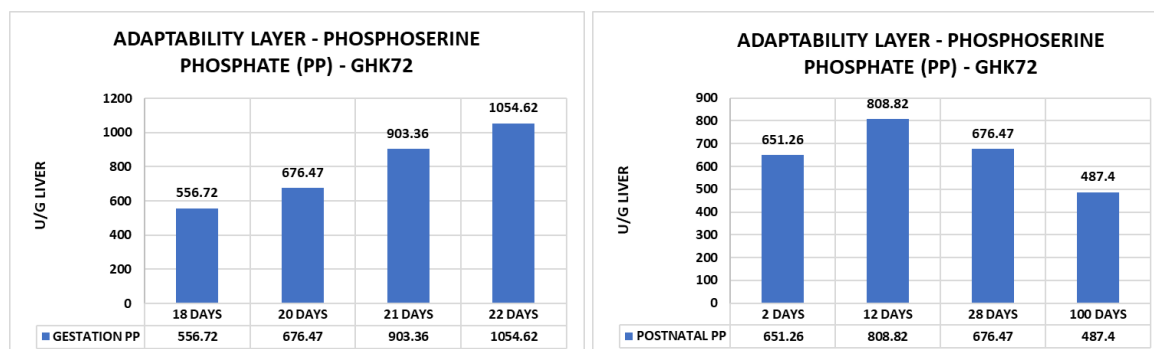


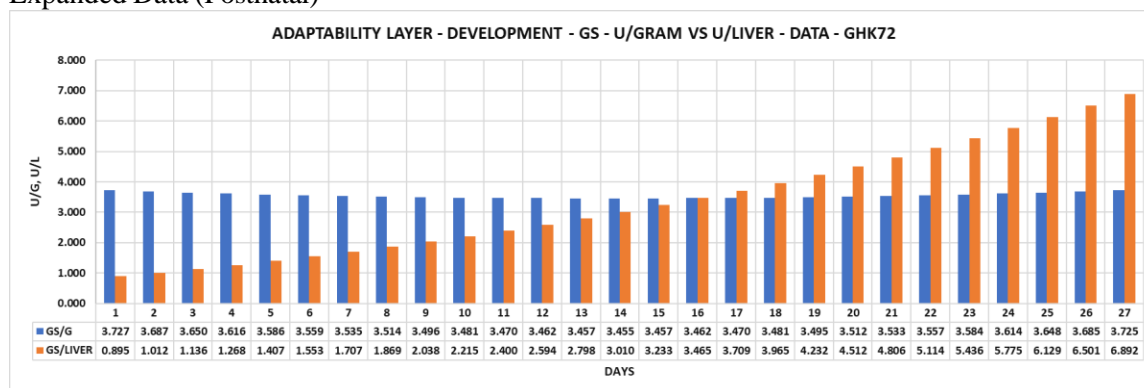
Figure 5.2 Original data related to a gram of liver (gestational and postnatal) displayed in the adaptability layer.

By expanding the dataset, Figure 5.3 shows that a gram of liver and the liver detected the same changes differently in the adaptability layer. As the number of hepatocytes filling a gram of liver changed, the enzyme activity of each subsequent data point came from a different number of cells. In effect, the gram of liver reference produced incorrect results because the developing cells changed their volumes. Notice in Figure 5.3 the severity of the problem. The per gram data decreased (incorrect result) while the per liver data increased (correct result). However, ratios calculated with data related to a gram of liver or to the liver always gave the same result. Why? Because they differ only by the weight of the liver. Multiplying two values by the same number does not change the ratio.

Multiplying data related to a gram of liver by the weight of the liver removes the cell packing error. The point? Estimates coming from cells related to a mg of protein or to gram of liver routinely become incorrect when the cell volume or protein content of the cell changes. Unfortunately, most investigators don't know (1) that such a problem exists and (2) how easy it is to fix. Since progress in the biomedical sciences depends importantly on connecting – mathematically - changes in the phenotype to those in the genome (because we want to predict one from the other), becoming adept at detecting biological changes *correctly* becomes a basic skill.

By attaching data tables to the plots, the reader can recalculate results, make future updates quickly, and have ready access to published data.

Expanded Data (Postnatal)



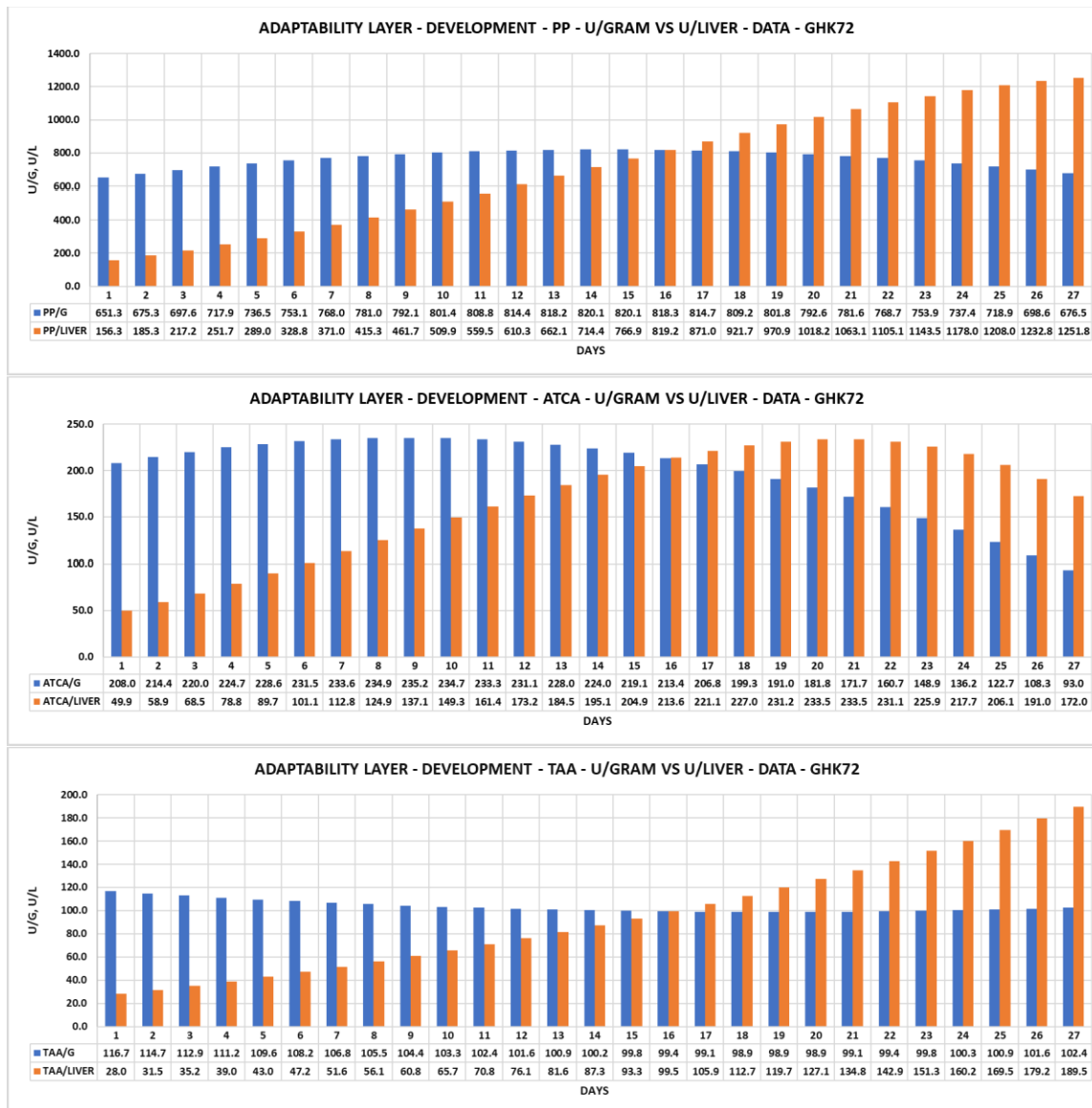


Figure 5.3 Postnatal data displayed in the adaptability layer. A gram of liver reference gives one result, the liver clearly another. What these biochemical plots can't tell us is that when cells change, they can alter their parts and recipes (phenotypes) as well as their shapes, sizes, and volumes. Treating change as a simple, isolated event defies reality. Try to answer the following question. Using data from the figure, if one reference (gram or liver) detected a significant increase and the other a significant decrease (p value + effect size) would both results still be publishable as standalone papers?

During liver development, hepatocytes run the developmental program by changing relationships of structure to function, which for the purposes of the literature update will focus largely on enzymes attached to or associated with membranes. Enzyme activities related to a unit of membrane surface area and to the activities of other enzymes define the enzyme-membrane recipes that characterize the ongoing solutions of the developmental process. Together, the recipes and total liver values can supply correct answers to many of the questions posed by the experimental objectives. Recall that the enzyme density (ED) defines the membrane-enzyme recipe quantitatively as units of enzyme activity related to one square meter of membrane surface area ($ED = U/S$). For the update of this study, we'll follow the changes in enzyme densities calculated from both the original and normalized data.

Using the expanded data in Figure 5.4, which includes enzyme activities and ER surface areas, we can calculate the local enzyme densities. However, the calculation assumes that a cm^3 of fixed tissue is roughly equivalent in amount and composition to a gram of fresh liver, which, of course, may not be the case. By switching to normalized data, we'll mitigate this suspected incompatibility of the data references by removing the data units and using instead the shape of the curve to represent the change.

Expanded Data

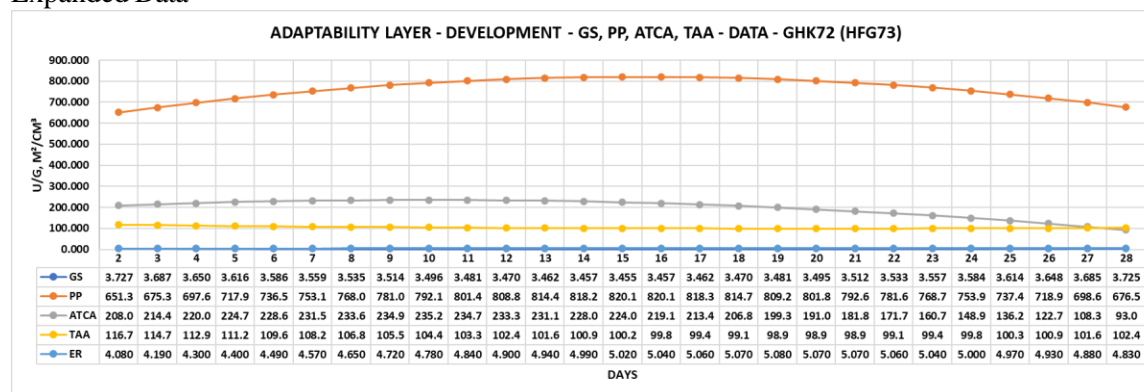


Figure 5.4 Expanded enzyme and membrane data displaying changes during early postnatal development. [Note that the ER surface areas per CM^3 of hepatocyte cytoplasm came from a paper published by same research group (HFG73).]

Local Model (Enzyme Densities) – Adaptability Layer

The first calculation for the enzyme densities (ED), which tracks the changes in the amount of enzyme activity associated with one square meter of ER surface area, uses the expanded data (Figure 5.5). The local model for enzyme density requires the same data reference (e.g., per gram liver) for both the morphology and biochemistry. It uses the following equation: $ED = [(U/G)/S/G] = U/S$, where U refers to units of enzyme activity, G to gram of liver, and S to membrane surface area. However, we have a problem with the surface area of the ER because it's related to a cm^3 of hepatocyte cytoplasm and not to a gram of liver.

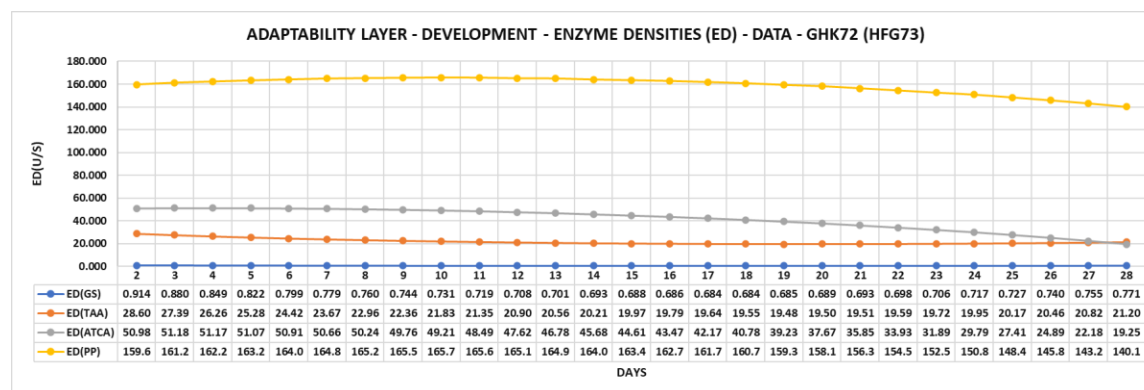


Figure 5.5 Enzyme densities calculated incorrectly with data from the adaptability layer (Figure 5.4). The ED data in the table are incorrect because the calculation does not work mathematically; $ED \neq U/S \neq [(U/G)/(M^2/CM^3)]$. Since many authors report their stereological estimates related to a CM^3 of hepatocyte cytoplasm, how do we estimate enzyme densities using mixed data references? One solution removes both the units of numerator and the denominator by normalizing the data. Instead of breaking the mathematical rules by making assumptions, we can mitigate the problem by using just the normalized curves to make the calculations. Of course, we'll check the method when the opportunity occurs.

First, let's see how the enzyme density works. It allows us to switch from a variable reference (gram of liver) to one that remains constant (1 m^2) by calculating enzyme densities from enzyme activities (U) and the ER membrane surface area (S) – both related to a gram of liver. The constant reference - one meter squared – exists because of the calculation.

The enzyme density tells us how the enzyme activities change when related to a square meter of ER membrane. As a complex data type, it contains three pieces of information wherein any two parts can predict the third ($ED = U/S$, $U = ED \cdot S$, and $S = U/ED$). Since hepatocytes solve problems by changing the absolute and relative amounts of these three variables, one needs the enzyme density to explain how the cell solved the problem. The point? Detecting and explaining a biological change requires both biochemical and morphological data.

Background: Since the design of most published experiments focus on detecting simple “changes” as significant differences and not on the complexities of a biological change, a central challenge of the updating process became the incompatibilities of data types and references. In biology, complexity derives from the relationships of many parts connected mathematically by rule. Consequently, putting biology back together using a literature defined by many different and often incompatible data types questioned the reality of a goal aimed at reorganizing the literature with a model based on complex changes.

Does biology suffer the same shortcoming? No, because it's a rule-based system that knows perfectly well how to change as a complexity. Since biology can change and we can copy biology, our job reduces to shifting the focus of the updates from the methodological tangles to just the biological changes. Recall that the ratio data in the rules layer work as well as they do because they report relative changes and amounts as dimensionless numbers (no units). Normalizing data produces the same result. By generating changes within a range of 0 to 1, normalization removes all the data units and thereby most of the methodological obstacles. More importantly, following a strategy based on normalization simplifies the task of making the transition from standalone local data to the more realistic massive datasets populating the information processing systems used by biology.

Global Model (Enzymes-NORM1 and Membranes-NORM1) – Adaptability Layer

Generating normalized enzyme densities (NORM2) involves a three-step process. First, we normalize the individual parts (enzyme activities and membrane surface areas), then use the normalized parts to calculate the enzyme density [$ED = U(\text{NORM1})/S(\text{NORM1})$], and finally normalize the result ($ED\text{-NORM2}$) to recover the 0 to 1 range of the values lost by the previous step. Figures 5.6 and 5.7 illustrate the process.

Expanded Data (NORM1)

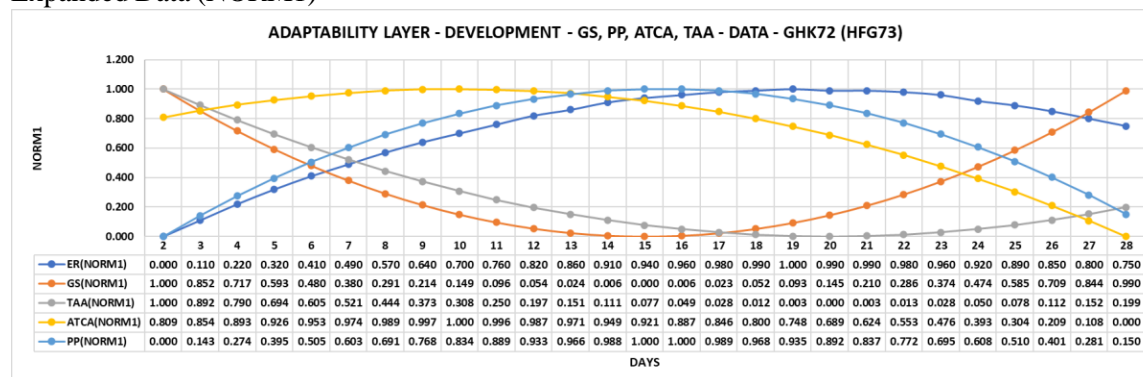


Figure 5.6 Normalizing the data adjusts all the changes within a range of zero to one, wherein zero represents the smallest number and one the largest. Normalization removes everything (data units and references) leaving just the changes, shown as curves defined by polynomial equations.

Global Model (Enzyme Densities – NORM2) – Adaptability Layer

$$\text{ED-NORM2} = [(\text{U})\text{-NORM1})/(\text{S})\text{-NORM1}] \rightarrow \text{NORM2}$$

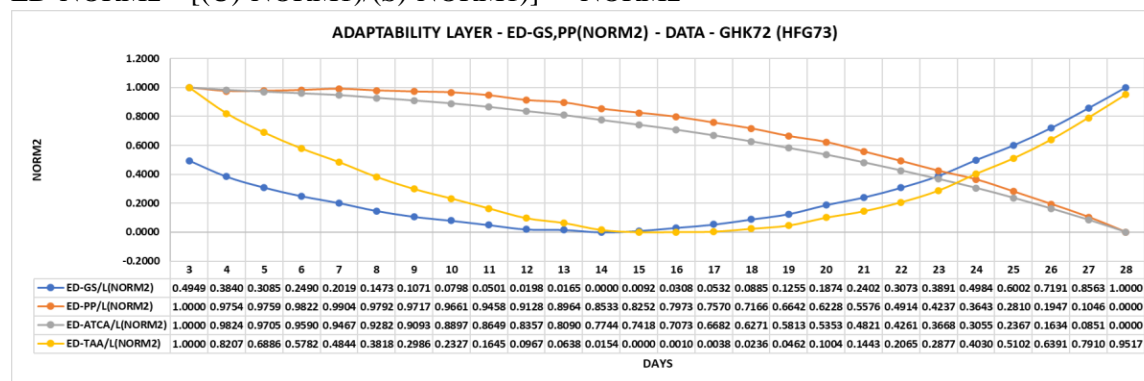


Figure 5.7 A normalized enzyme density (ED-NORM2) equals the normalized amount of enzyme activity divided by the normalized amount of ER membrane surface area. As with the original calculation (wherein both enzymes and membranes are related to a gram of liver), the division relates the enzymes to one standard unit of membrane. To translate a CM^3 of cytoplasm into a gram of liver, we would multiply it first by its volume density in the liver (roughly 70%) to get M^2/CM^3 of liver and then by the density of the liver ($1.07 \text{ G}/\text{CM}^3$) to get M^2/GRAM . Since the process involves introducing two constants into the calculation with minor changes in cytoplasmic volume densities produced by changes in the liver volume, we can expect both references (M^2/CM^3 and M^2/GRAM) to produce parallel curves. While such an approach is cumbersome, it nonetheless works well enough to allow the updating process to continue. In short, by normalizing the enzyme and membrane data before calculating enzyme densities the problems related to data units and calculations largely disappear. Note that the data sciences routinely use normalization to deal with such data related problems. Nonetheless, we'll use several case studies to test the unfamiliar practice of mixing data references when calculating normalized enzyme densities.

Global Model (Enzyme Densities – NORM2) – Rules Layer

In the next step, we move the NORM2 enzyme densities to the rules layer and use them to find duplicate patterns of change. This shows us how pairs of enzyme densities change relative to each other as they progress toward a solution. Overlapping patterns, for example, tell us that the enzyme densities of different enzymes change according to the same rules. In turn, we'll use these aggregated sets of duplicated patterns to forward and reverse-engineer phenotypes.

If we assume that cells optimize outcomes, when, where, and how do the optimizations occur?
 Does an enzyme density optimize the relationship of biochemistry to morphology? Does an
 entangled pair of enzyme densities optimize the relationship of one enzyme density to another?
 If the answers are yes, then how do we ask the cells for the algorithms?

Taking four enzyme densities (NORM2) two at a time results in a combination of six data pairs (without concern for the order). Note that data in the adaptability layer become ratios in the rules layer, wherein the ratios display two digits (labeled as DATA) or contain only one (labeled as DATA ROUNDED). After plotting the results separately (Figure 5.8) and together (Figure 5.9), we can combine the duplicates (Figure 5.10).

ED-NORM2- Rules Layer – Data Rounded

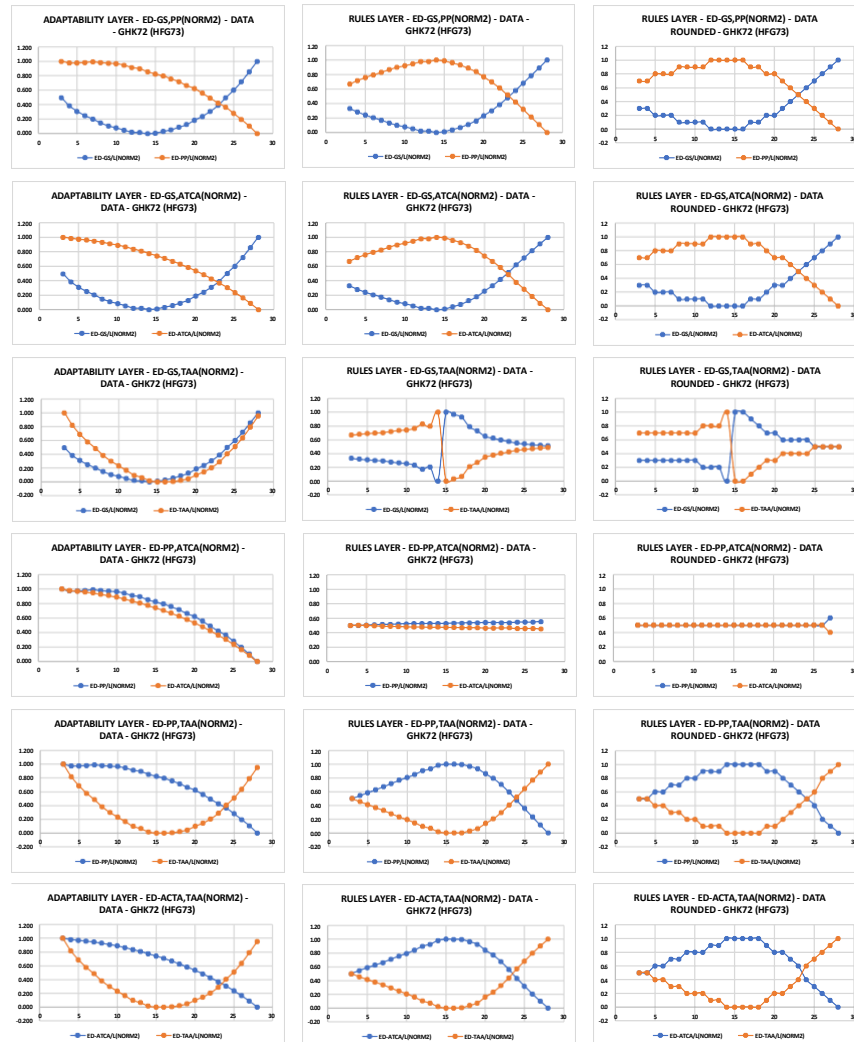


Figure 5.8 The rules layer detects relative changes in the ratios of the data pairs. The y axis identifies ratios and the x axis days.

Figure 5.9 displays a pattern of data pair changes that begin with a simple ED recipe (postnatal day 3), followed by enzyme densities (recipes) alternating between periods of less activity (Days 3, 15, 26) to more activity (the days between).

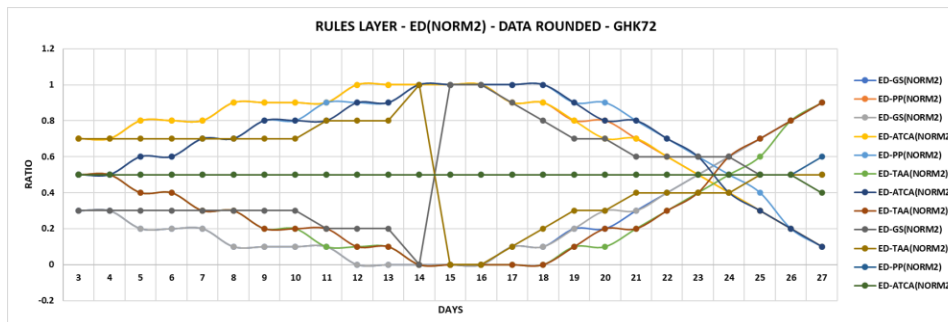


Figure 5.9 When combined, the patterns formed by the data pairs in Figure 5.8 produce a small segment of the larger developmental phenotype. At day twenty-six, for example, the data pairs (EDs) displayed two fleeting solutions (0.5:0.5, 0.2:0.8), which then transitioned to the next set of changes.

Figure 5.10 shows the overlapping data pair ratios. In response to the developmental program, the patterns of the enzyme pairs overlapped to varying degrees. When two data pairs share the same ratio (e.g., 0.5:0.5), the changes in EDs become synchronized. For example, the enzymes of the ED-PP: ED-ATCA data pair predict that the genes expressed equal amounts of the two enzymes between days 3 and 26.

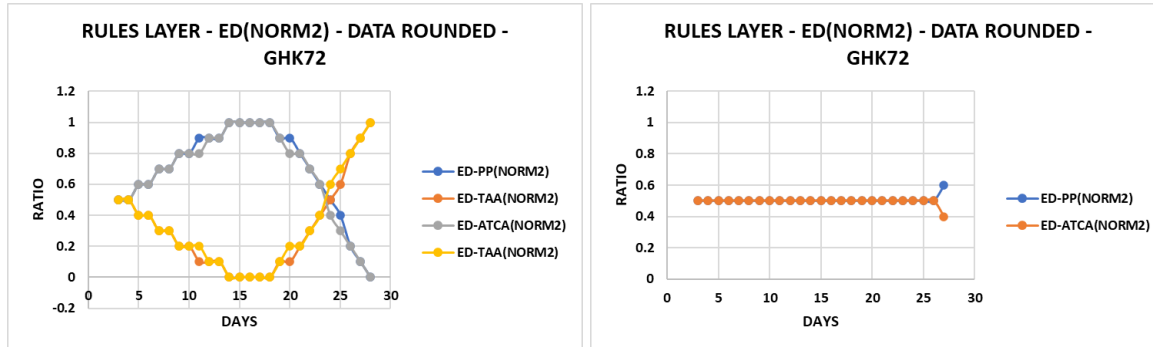


Figure 5.10 If the two data pairs (left) superimpose and share the same enzyme (TAA), then the unshared enzymes (PP, ATCA) predict the result shown in the figure at the right. While the plot at the left predicts continuous changes in the relative amounts of gene expression for both data pairs, the plot at the right predicts no variation in expression for the single data pair until day twenty-seven. Such information may become helpful when attempting to reconstruct large scale patterns of gene expression from phenotypes.

Methodological Problem Solving

Fortunately, we can use the data of this updated publication to explore a methodological problem created by preparing tissue samples for electron microscopy. In response to fixing, dehydrating, and embedding, the volume of a fresh piece of liver can change. This means that an equal volume of fresh and fixed liver would not contain the same number of cells if the fixed tissue underwent shrinkage or swelling.

Fortunately, estimating surface densities and expressing them as ratios eliminates the units and thereby any errors and/or biases associated with a volume distortion. This is not the case, however, when using the hierarchy equations of stereology to estimate the total surface area of a membrane compartment in the liver. Two worked examples introduce the problem and approximate the extent of the volume related distortions.

Volume Distortions Produced by Tissue Fixation (Example 1): Since preparation for electron microscopy can produce changes in tissue volumes (shrinkage and/or swelling), stereological estimates for membrane surface areas can become biased (Bertram et al., 1986, West 2013). The source of the problem seems to involve tissue compartments that swell (connective tissues) and shrink (cells), wherein one cancels the other - to some unknown extent. By normalizing the data, we'll use the change models to estimate the effects of the shrinkage and swelling on the shapes and relative positions of the curves.

The first model consists of making changes to the original data (glycogen synthetase - GS), normalizing the changes in the enzymes and membranes, and observing how tissue shrinkage and swelling affects the results. Starting with the original ER data (m^2/g), we can decrease the values by 10% to simulate swelling and increase them by 10% for the shrinking. The differences appear in Figure 5.11.

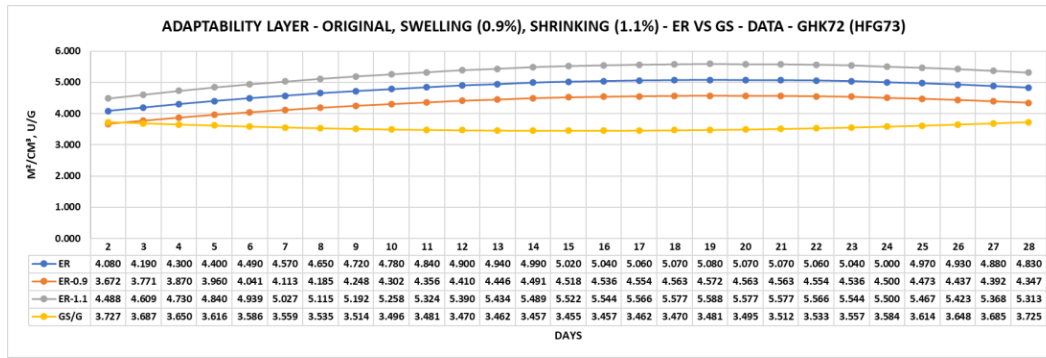


Figure 5.11 ER (original data); ER-0.9 (ER diminished by 10%, ER-1.1 (ER increased by 10%); GS/G (enzyme activity per gram of liver).

After normalizing the four rows of data (ER and enzyme data) shown in Figure 5.11 and plotting the results, the original and distorted datasets produced similar results (Figure 5.12). Why? Because normalizing the data puts the different changes in ER surface areas into the same range (0 to 1).

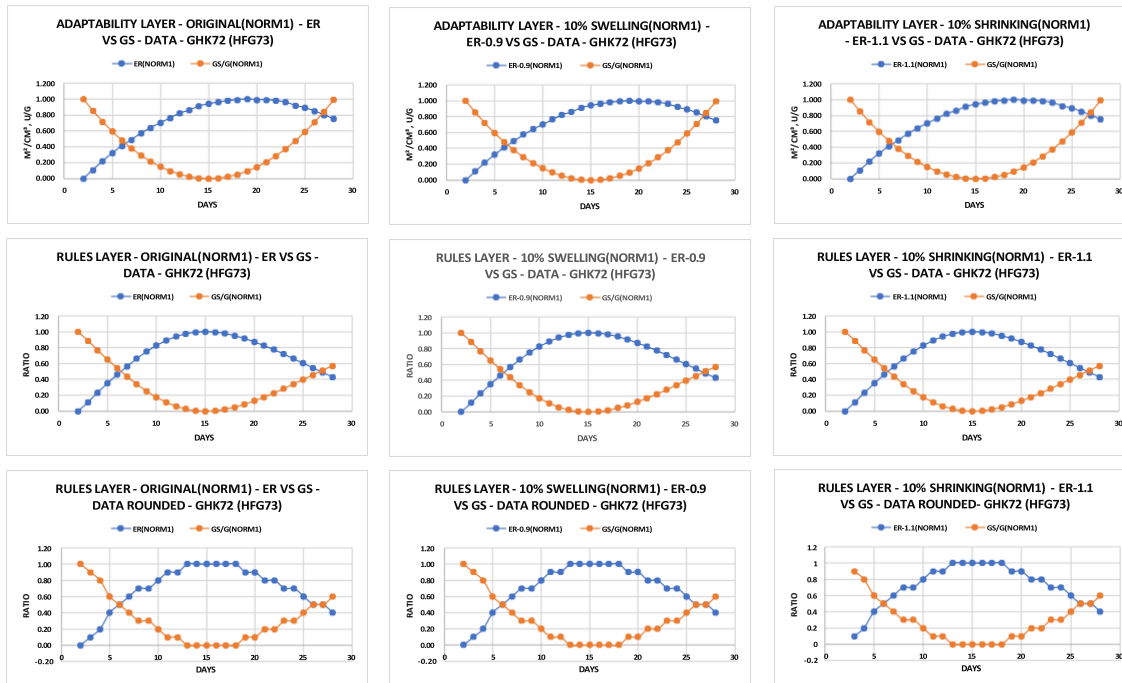


Figure 5.12 The results of the shrinkage and swelling experiment showed that the normalized data detected no important differences between the original and altered datasets.

When we make the same calculations with the original data (not normalized) the patterns changed (Figure 5.13). Without normalization, we detect the effects of the volume distortions in both the adaptability and rules layers.

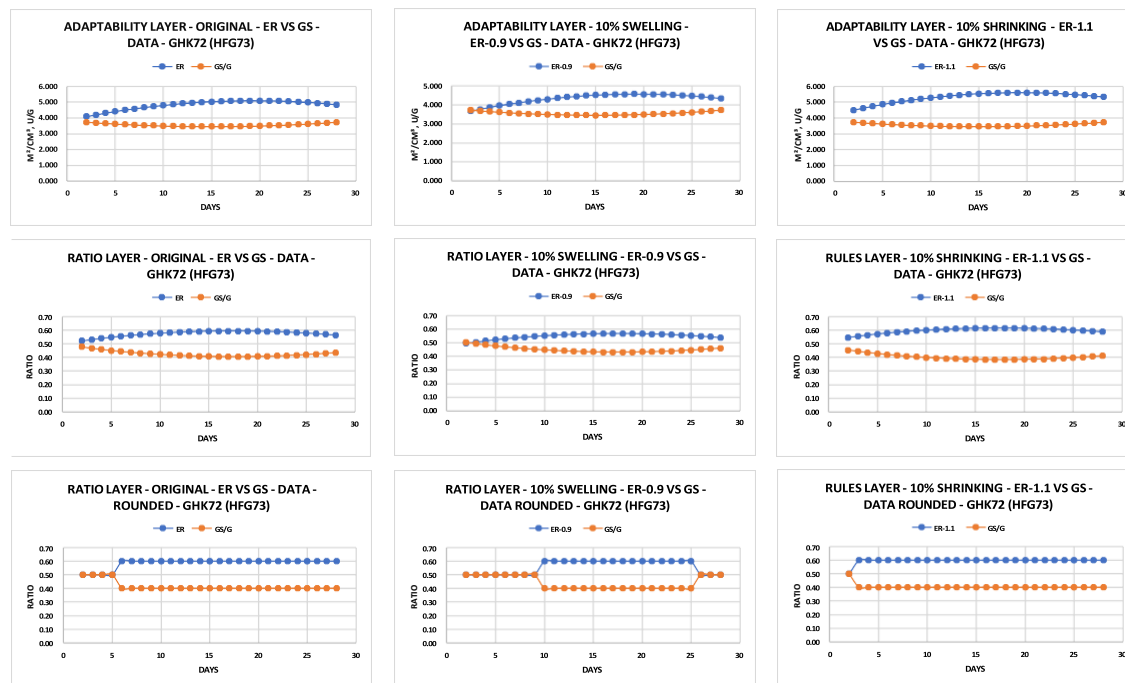


Figure 5.13 As expected, differences appeared in the original data because changes in the relationships of activities to the ER surface areas changed.

Volume Distortions Produced by Tissue Fixation (Example 2): One of the important lessons to come from the first primer (Bolender, 2019) was the importance of using data from living material (e.g., biochemistry and MRI) when dealing with problems arising (1) from changes in volume related to data references and (2) from volume distortions produced by preparing specimens for electron microscopy. Managing two volume related changes occurring at the same time identifies the challenge. While we can deal with the problem of changing numbers of cells packed into a gram of tissue by relating the data to the liver (i.e., multiply units/gram or m^2/gram by the weight of the liver), identifying corrections for volume distortions related to preparing tissues for microscopy remains a work in progress.

First, let's look at the changes in the original data. By relating the same data to different references as shown in Figure 5.14, we can see how changes in the average cell volumes and the tissue fixation influenced the results. Since many published studies report ER surface areas relative to a cm^3 of hepatocyte cytoplasm, we'll compare it to a gram of liver. Notice in the figure below that data related to the liver showed a steady increase, whereas data referenced to a cm^3 increased and decreased or to a gram of liver gram decreased and increased.

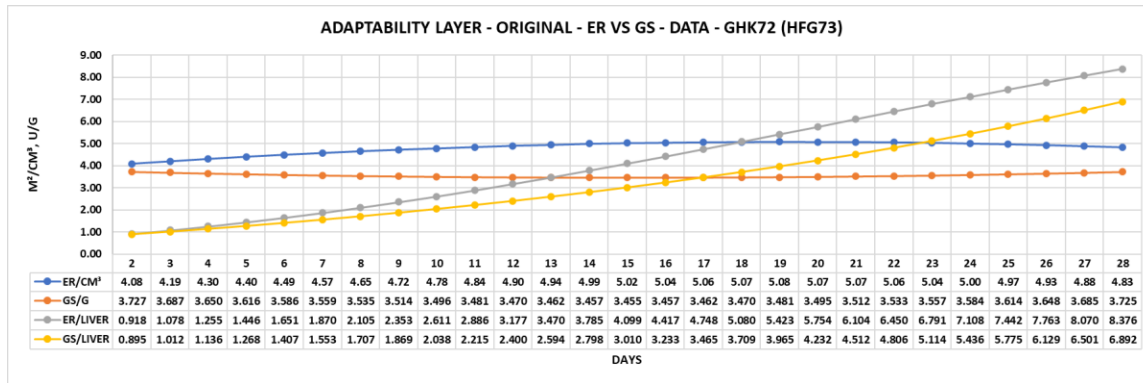


Figure 5.14 The figure indicates that data related to the liver showed a steady increase, whereas cm³ and gram of liver references showed variable responses.

If, however, we express the results in Figure 5.14 as percentages (Figure 5.15), then both the biochemical and morphological data displayed similar results. This suggests that the volume distortion associated with tissue preparation for microscopy may represent a minor error. In any case, the results indicated that the volume distortions represented largely a constant bias and not a variable error.

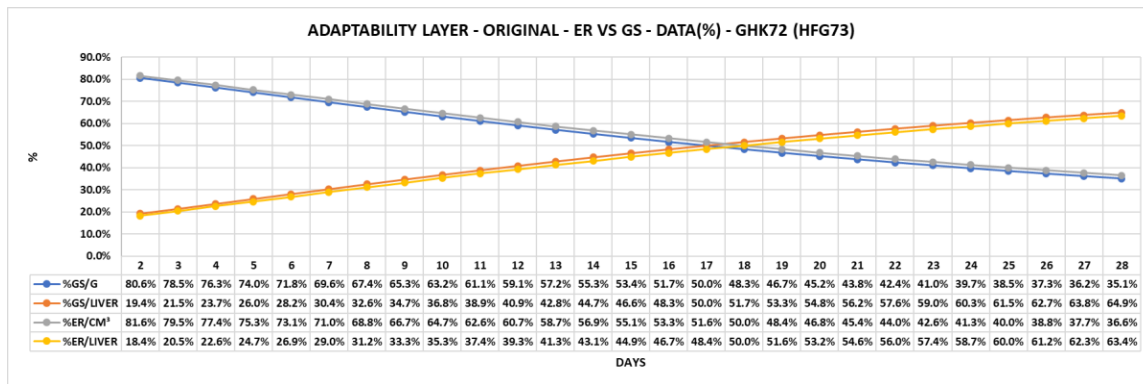


Figure 5.15 When expressed as percentages, both the biochemical and morphological data displayed similar results for the two sets of references.

Using data from figure 5.15, we can see that the extent of the tissue distortions due to swelling (Figure 5.16) and shrinkage (Figure 5.17) amounts to less than 2%.

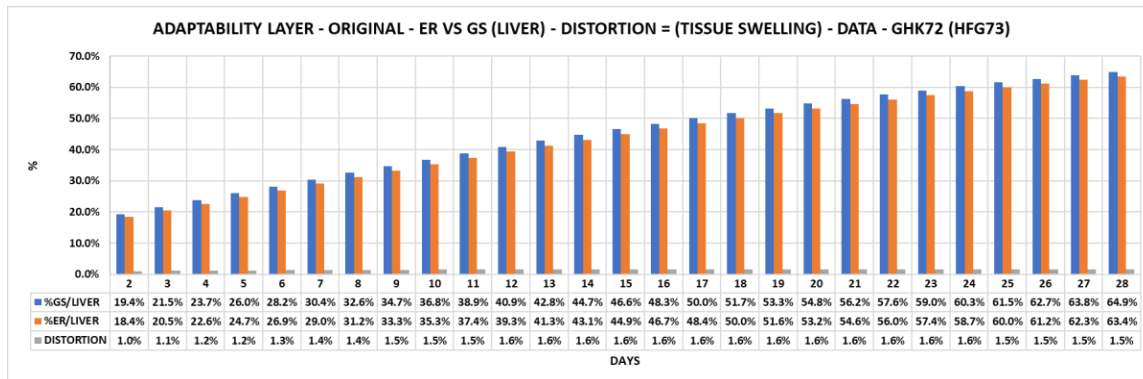


Figure 5.16 When viewed as a histogram, results related to the liver suggest that the tissue swelling error of the ER amounted to less than 2% (1.0 % to 1.6 %).

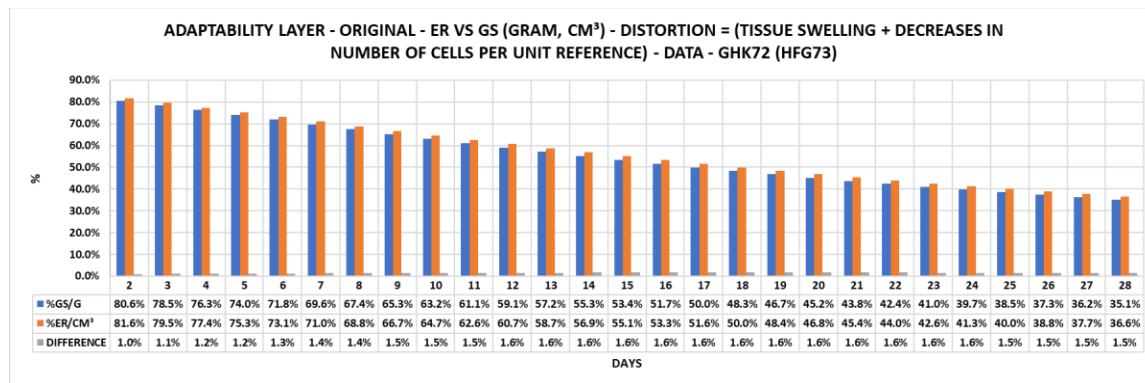


Figure 5.17 When we look at the changes in the enzyme activities and membrane surface areas related to a unit reference (gram, cm³), we get the opposite result – the decreases suggest tissue shrinkage. Once again, the error remains small at less than 2%. [Note that the liver density (ρ) equals the liver weight (W) divided by liver volume (V), which means that the liver volume is slightly larger numerically than its weight ($VL = WL/\rho$) because the liver density (ρ) equals about 1.07 grams/cm³. A gram of liver would have a volume of about 0.93 cm³.]

Summary: These results suggest that the volume distortions related to tissue preparation appear to represent a minor error. Moreover, the histograms suggest that substituting ER/cm³ for ER/gram can lead to comparable results, especially when normalized.

5.3.2 Case Study 2: Regeneration (Hepatocytic Membranes) – PZGM75

Source: Update applied to original data from Pieri C., Zs-Nagy I., Giulì C., Muzzufferi G. (1975) The aging of rat liver as revealed by electron microscopic morphometry II. Parameters of regenerated old liver. *Exp Gerontol* 10:341-9.

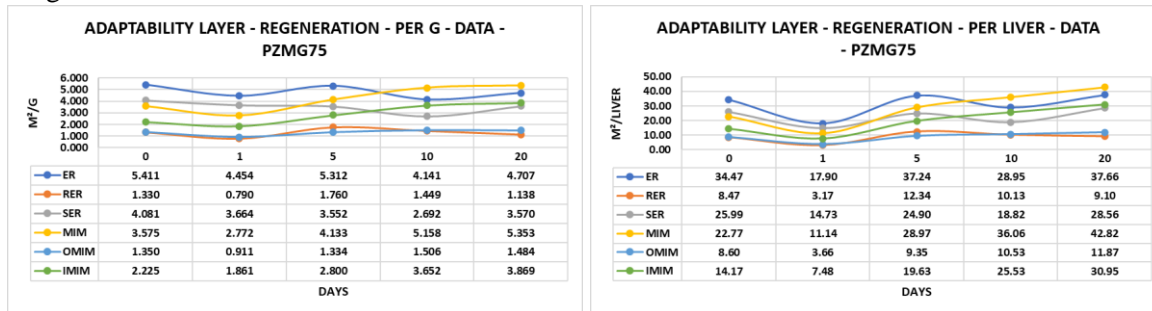
Topic: Morphological evaluation of aging in the rat liver.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, and generate patterns.

Dataset: For twenty days following partial hepatectomy, the update follows the regeneration of ER, RER, SER, MIM, OMIM, and IMIM using both the gram of liver and the liver as references.

In this study of liver regeneration (Pieri et al., 1975; PZGM75), which resembles development in that it involves the rapid production of new hepatocytes, we can follow changes in the surface areas of membrane organelles as the liver recovers from a partial hepatectomy (Figures 5.18 to 2.20). Results include data first related to a gram of liver and then to the liver.

Original Data



Expanded Data

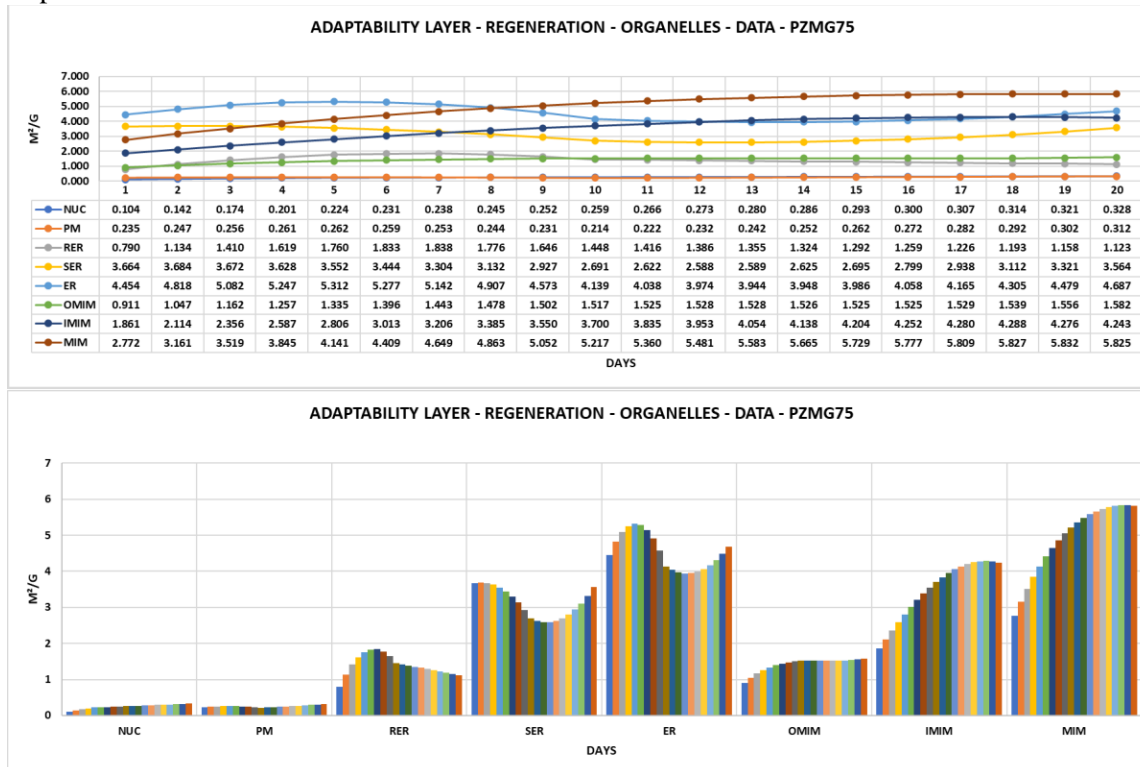


Figure 5.18 Original and expanded data (S/G) showing changes in hepatocytic organelles during liver regeneration.

Expanded Data Normalized (NORM1)

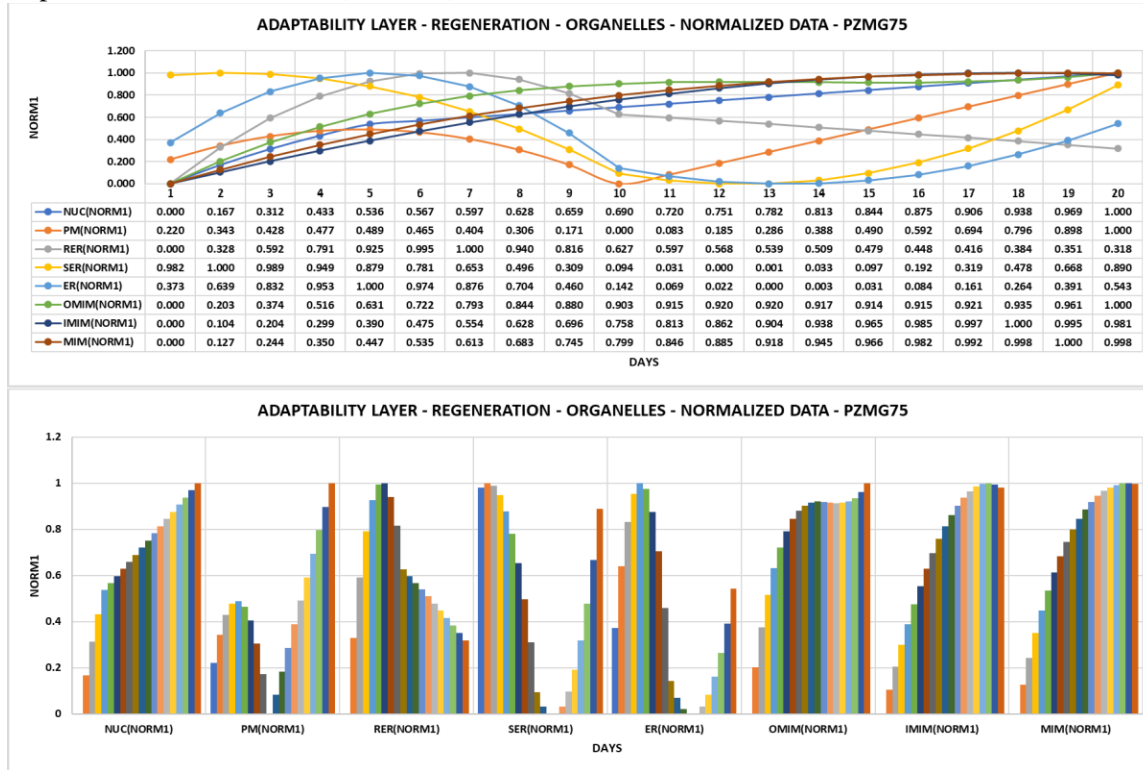


Figure 5.19 Normalized changes in regenerating organelles (from S/G data). Since the normalized data share the same range (0→1), all the histograms have the same height. Now the membranes with varying amounts of membranes just show their changes within the range of 0→1. Using the normalized data, we can identify membranes that change similarly by inspection (with histograms) or by plotting data pair ratios that compare changes in one membrane to those of another (Figure 5.20).

Finding Duplicate Patterns of Change in the Rules Layer: To look for changes in data pair ratios during regeneration, we can link two normalized organelles (Figure 5.19) and follow their changes expressed as ratios in the rules layer (Figure 20). The exercise consists of finding duplicate patterns to identify the relationships of organelles during rapid growth.

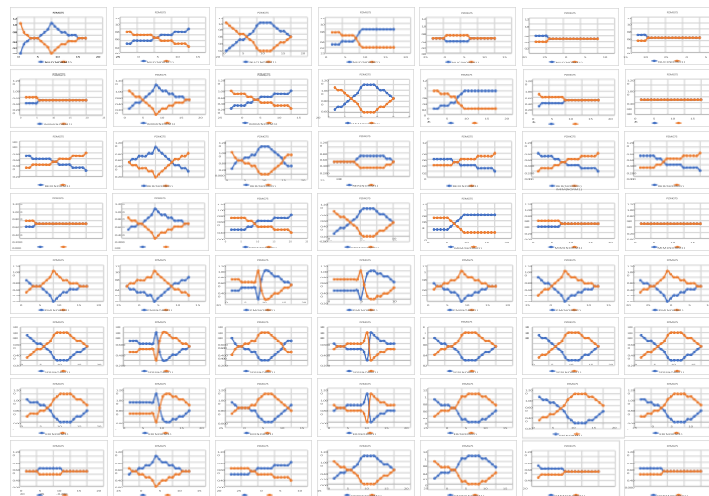
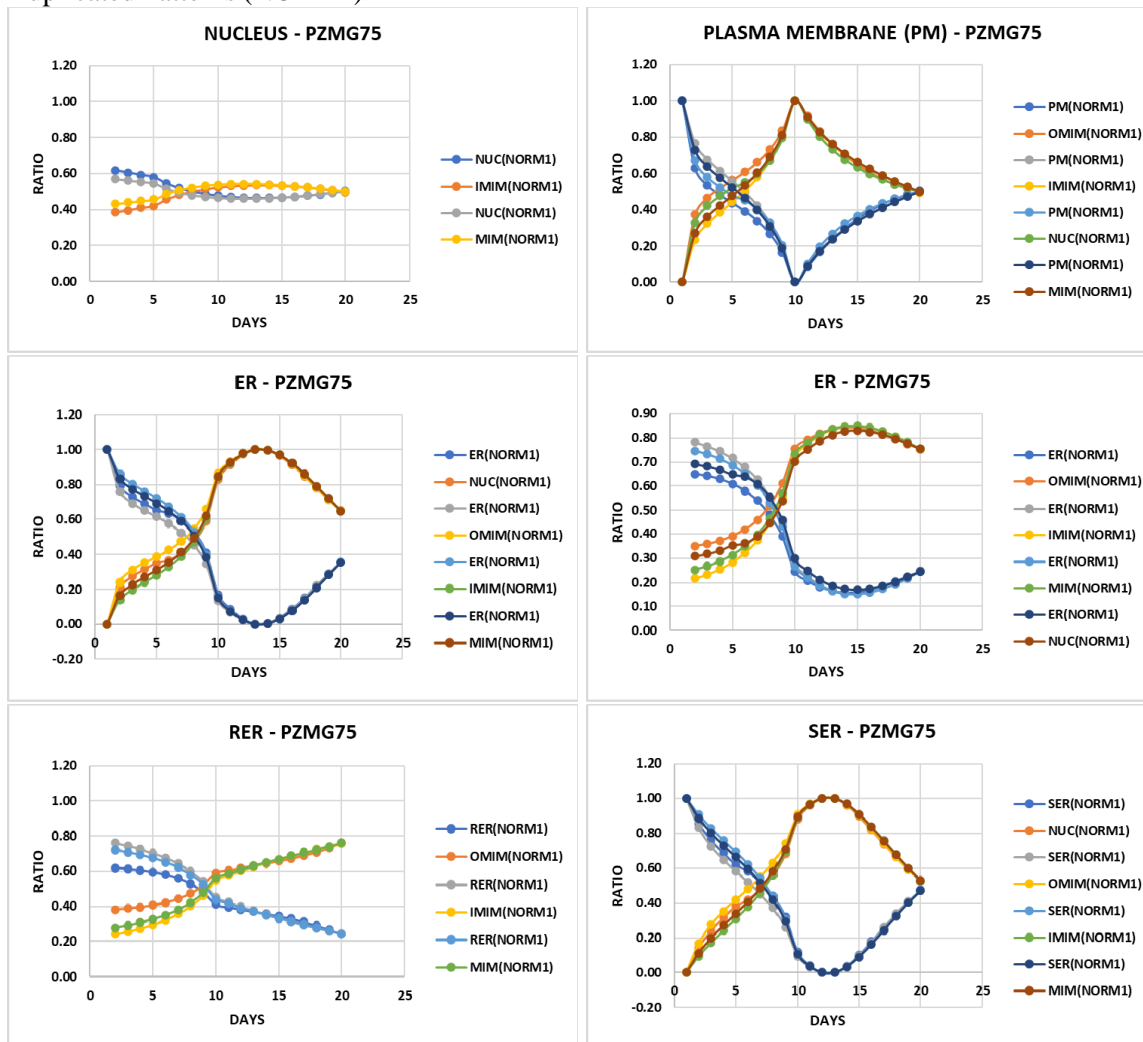


Figure 5.20 The patterns above include data pairs plotted in the rules layer (data rounded only). Duplicate plots identify data pairs changing similarly. Figure 5.21 summarizes the duplicated data pairs.

Using the set of unrounded data pair ratios (two decimal places showing), we can stack the duplicated patterns one on top of the other to show that many membrane pairs changed similarly during the regenerative process. This pattern of applying similar changes to different pairs of parts identifies a general principle of a biological change. In this study, for example, all the updated membrane organelles displayed this property. Notice that the closeness of the duplications improved over time as the regenerating cells worked their way toward solving the problem triggered by the hepatectomy. Figure 5.21 shows the extent of the duplications for various data pairs. For example, changes in the plasma membrane (PM) eventually paralleled those of the outer mitochondrial membrane (OMIM), inner mitochondrial membrane (IMIM), and the nuclear membrane (NUC) as the hepatocytes replaced the cells lost to the hepatectomy.

Duplicated Patterns (NORM1)



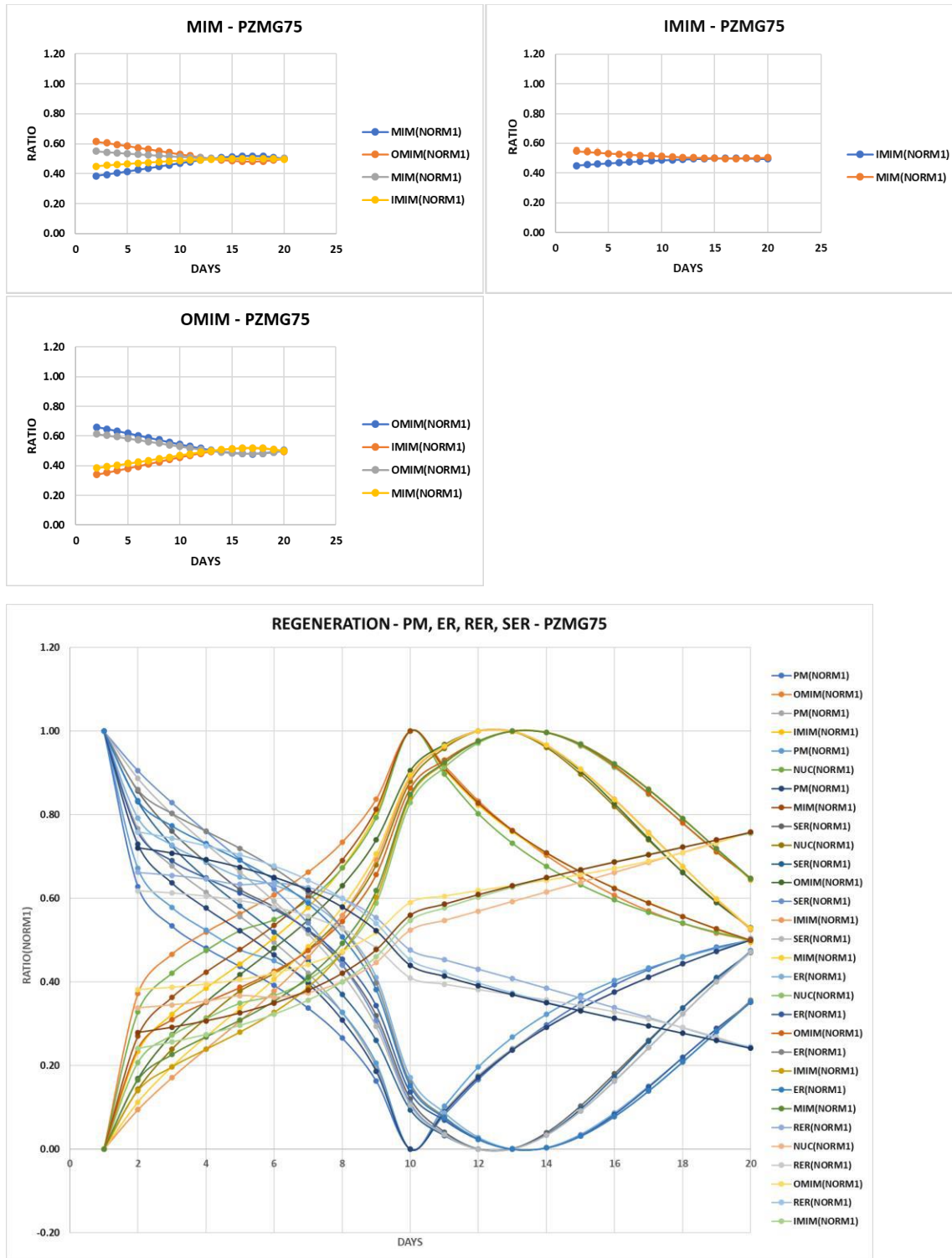
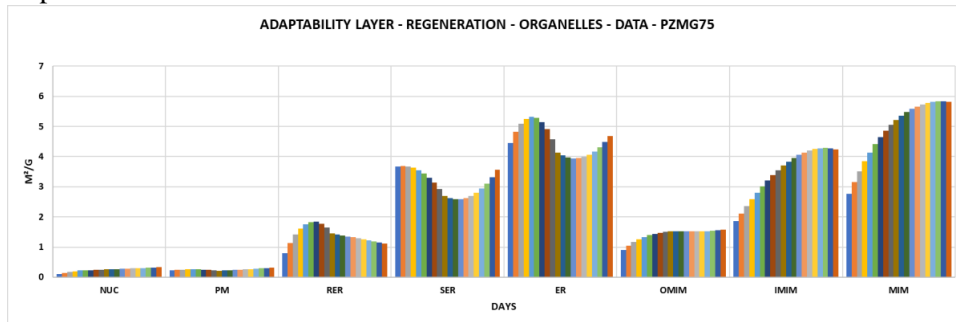


Figure 5.21 Replacing lost cells with new ones, involves restoring relationships between and among membrane compartments. When changing, membrane data pairs assembled from the PM, ER, and SER displayed extensive synchronization with other membranes. These results suggest that biology uses a design principle based on changing multiple data pairs with similar rules. In effect, the data pairs described the changes that occurred during the differentiation of hepatocytic organelles. The summary plot of the PM and ER membranes shows how the hepatocytes differentiate these organelles during cell regeneration (data pair ratio model for change). The morphological patterns predict the extreme complexity of working out the sequences of gene expression during regeneration. Alternatively, an enzyme density plot would need just a name change: enzyme \rightarrow gene.

Comments on Working with Normalized Data: Normalization allows us (1) to aggregate published data by first removing data units and references and then (2) to summarize the events of a change by forward and reverse engineering the cell phenotype. Starting with one or more columns of data points wherein some columns contain small numbers and others large, min-max normalization (see Wikipedia) resets all the values – small and large – to the same range – zero to one. The smallest value becomes zero and the largest one. Since we'll use normalization frequently, becoming familiar with some of its properties will prove useful.

Figure 5.22 displays the original data (m^2/g) before and after normalization. Notice that the NORM1 data display values ranging from 0 to 1.

Expanded Data



Expanded Data Normalized (NORM1)

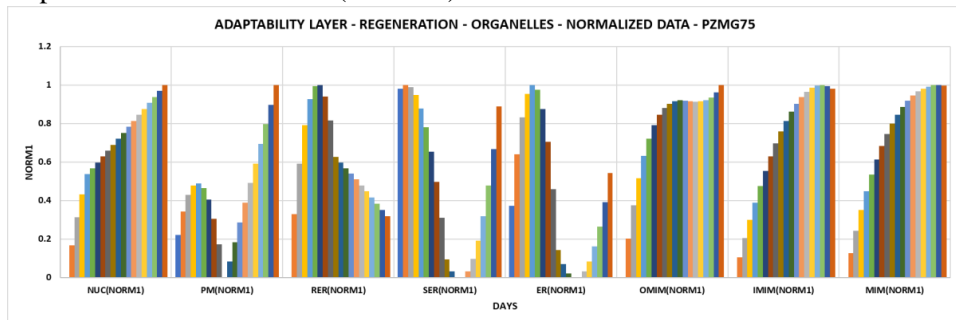
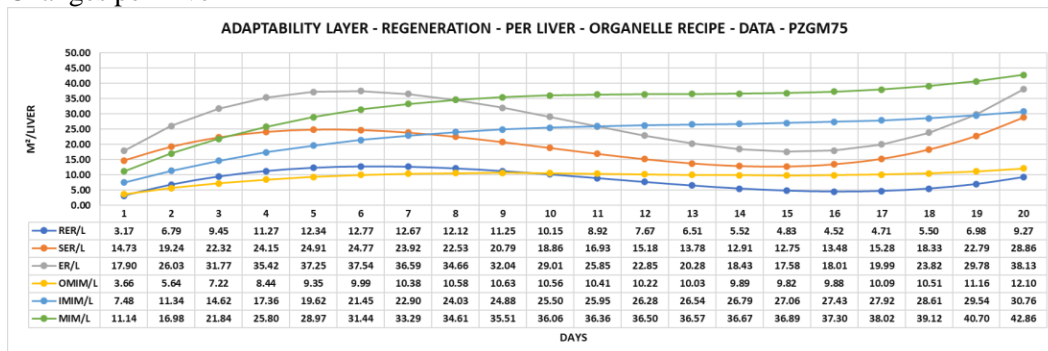
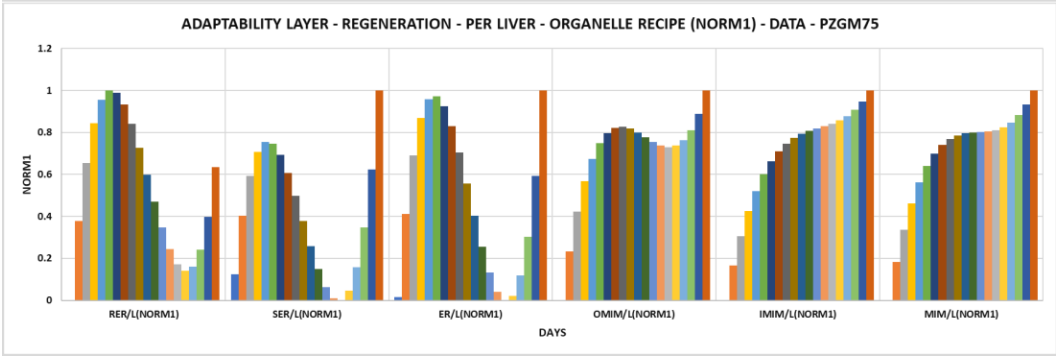
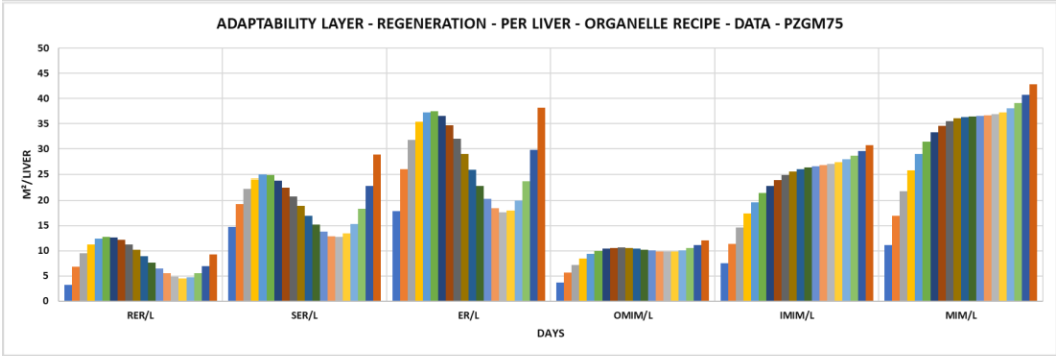
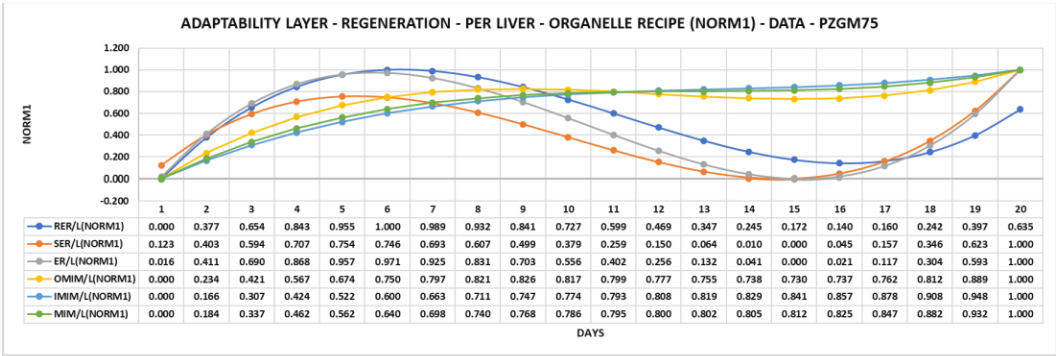


Figure 5.22 This histogram and the one above show how min-max normalization works. The original data display the changes sitting on “pedestals” that normalization removes by resetting changes from zero (smallest value) to one (largest value). When fitted to a regression line, the normalized data can characterize a change with an equation (usually a polynomial).

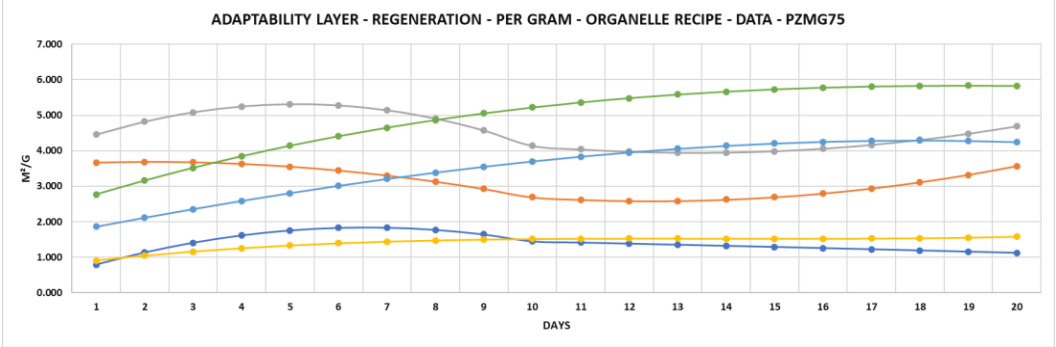
Changes Per Liver vs Changes Per Gram of Liver: Since data related to a gram of liver (Figures 5.18, 5.19) have one set of numbers and the same data related to the liver have another, normalization produces two different sets of curves (Figure 5.23).

Changes per Liver





Changes per Gram of Liver



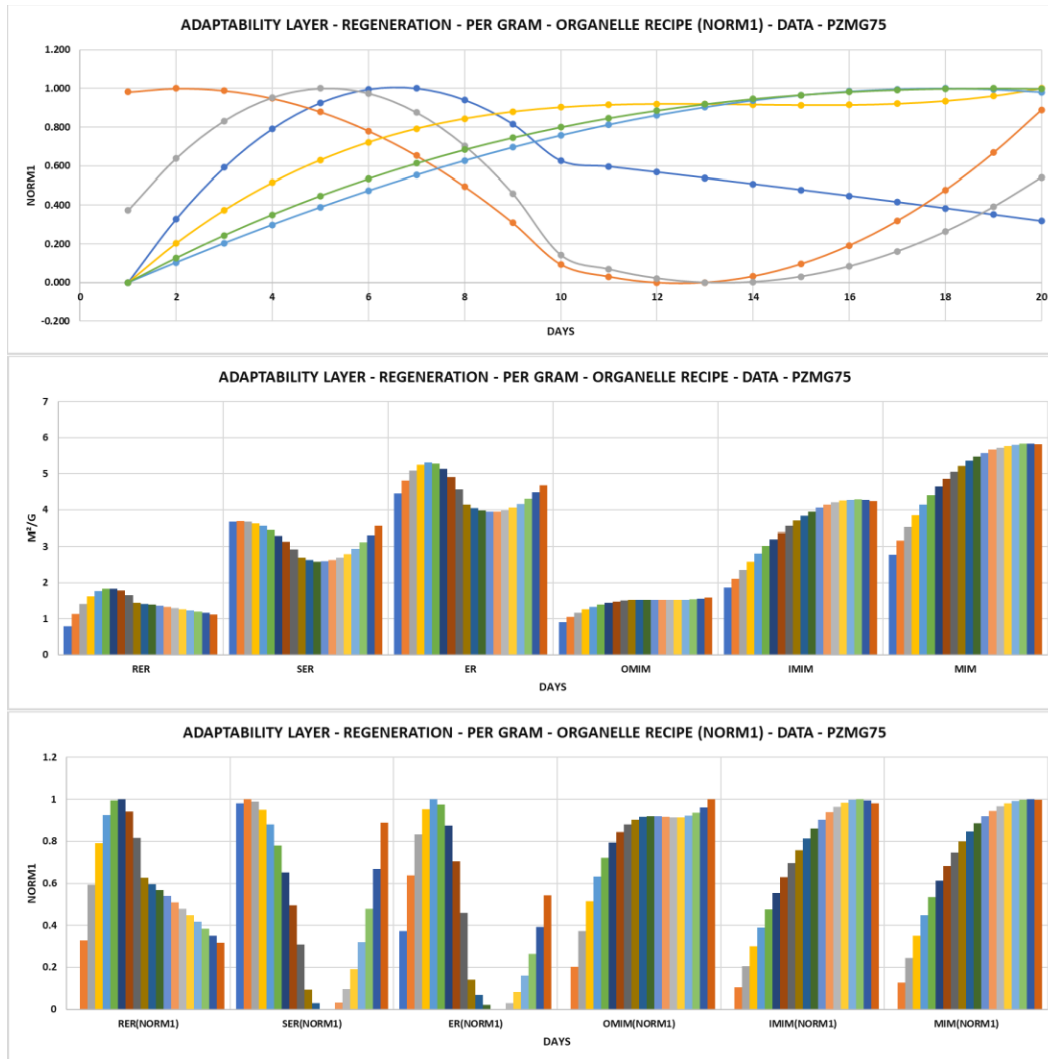


Figure 5.23 Comparing changes per liver to changes per gram of liver. By comparing the two normalized histograms shown above to the ones per liver, we can see the effect of the cell packing bias.

Analyzing Results with Just Morphological Data: How did the hepatocytes respond to a two-thirds hepatectomy? Since we're missing two-thirds of the data needed answer the question, the update can describe what happened morphologically but not address the larger question. As shown in Figure 5.21, the patterns of the duplicated ratios indicated that membranes changed surface areas similarly, but without the biochemical data we don't know what's happened to the recipes of the marker enzymes populating the membranes.

We can, however, generate data pair ratios from the membrane surface areas and use them to gauge the hepatocytes progress toward the solution. As a growth process, regeneration focused on manufacturing new parts (ER: membranes and enzymes) and increasing the energy supply (MIM: mitochondrial membranes and enzymes). They both go hand in hand.

First question. In Figure 5.24, did the ER to MIM ratio at recovery day 20 match the control at day 0? No, which suggests that the solution to the problem – being solved by the change – was still in progress.

A disruptive event, such as a partial hepatectomy, changed the control phenotype into a regenerative phenotype. For the hepatocytes and the liver, fixing the partial hepatectomy problem involved two major changes: control phenotype \rightarrow regenerative phenotype \rightarrow control phenotype. We can follow the progress of the hepatocytes by checking on the data pair ratios in the rules layer.

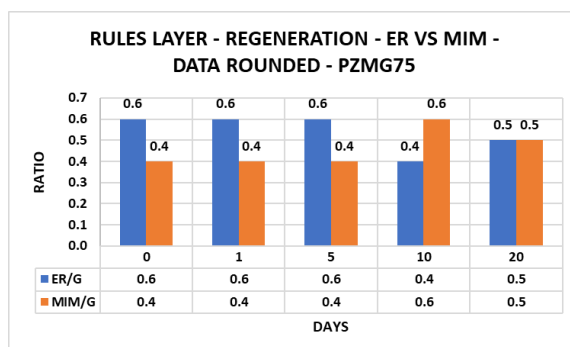


Figure 5.24 In response to the hepatectomy, the hepatocytes assembled the “machinery” for replacing the lost cells (increased mitosis, production of parts, and energy supply). Twenty days after the intervention (partial hepatectomy) the cells continued the liver recovery (ER:MIM - 0.5:0.5). When completed, the organelles will return to the control ratio (ER:MIM – 6:4).

Next question. When we look at the RER to SER ratio (Figure 5.25), day twenty resembles day zero. Does this mean that a solution existed for the production side of the problem? Maybe. Since we don’t have the enzyme densities (ED) for the ER membranes, we don’t know if days 0 and 20 share the same membrane recipes (i.e., ED) nor do we know the total enzyme capacities of the livers.

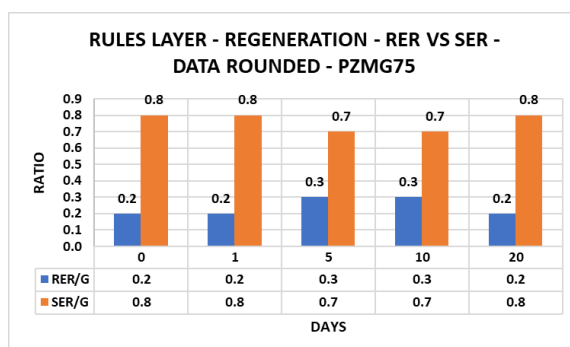


Figure 5.25 The relationship between the RER and SER appears restored (surface areas yes, enzyme densities?). Day 0 (control) and day twenty share the same ratio (rule).

In contrast, the mitochondrial membranes did not return to control values at recovery day 20 (Figure 5.26). Notice that the 0.3:0.7 ratio (OMIM:IMIM) typical of the recovery stage remained consistent at day twenty.

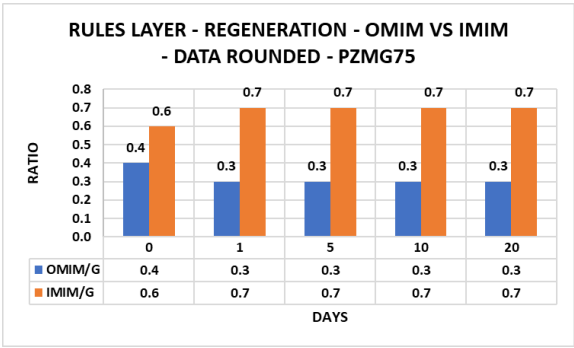


Figure 5.26 The mitochondrial membranes, which increased the relative amount of the IMIM (inner mitochondrial membrane) from 6:4 (day 0) to 7:3 (day 20) suggests that the synthetic rate remained at an elevated state on day twenty. Absent the marker enzymes of the IMIM and the total liver energy producing capacity, the state of the solution on day twenty remains an open question.

Comment: Were we intent on knowing what the hepatocytes had accomplished by day twenty, what are our options? We could go to the literature and look for biochemical papers dealing with liver regeneration under similar experimental conditions. If published marker enzyme data existed for the ER and mitochondrial membranes, we could expand the biochemical data, normalize everything, and then calculate enzyme densities and total liver capacities. In turn, this could tell us how the hepatocytes were solving the experimental problem. While such a mashup could supply provisional answers, a complete set of original data remains the preferred solution.

5.3.3 Case Study 3: Development (Aging of the ER in the Hepatic Lobule) – SMJ78

Update applied to original data from Schmucker D. L., Mooney J. S., Jones A. L. (1978) *Stereological analysis of hepatic fine structure in the Fischer 344 rat - influence of sublobular location and animal age*. *J Cell Biol* 78: 23-41.

Topic: Morphological changes during development and aging in central and portal hepatocytes.

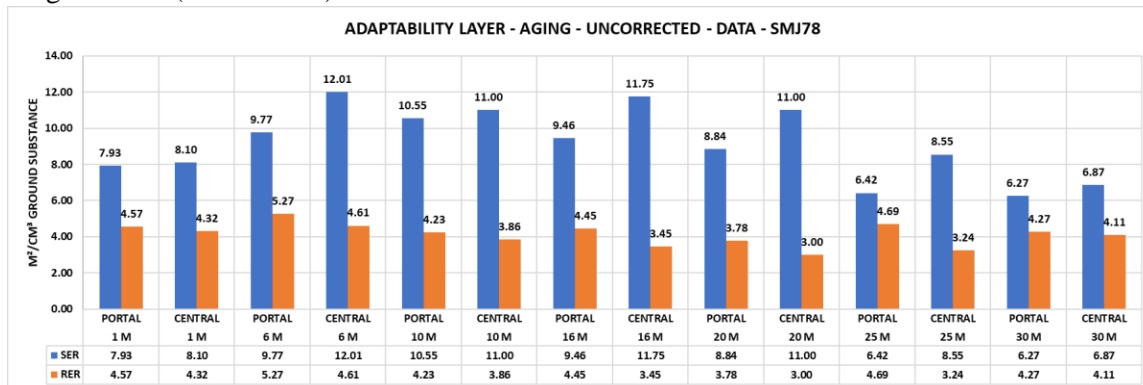
Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, generate patterns, and analyze results.

Dataset: The study follows changes in the two ER compartments – RER and SER.

In the liver, hepatocytic organelles age differently within the portal and central locations of the liver lobule (Schmucker et al., 1978; SMJ78). By updating this publication, we can use data pair ratios from the rules layer to follow the aging of organelles within and between portal and central hepatocytes. This will show us how aging changes the rules.

The liver consists of functional units called lobules, which include portal and central zones populated with hepatocytes named according to their lobular location. By correcting the published data for section thickness and compression and interpreting the data in the rules layer, we can follow the effects of aging on the relationships of the SER and RER in both portal and central hepatocytes (Figure 5.27).

Original Data (Uncorrected)



Original Data (Corrected)

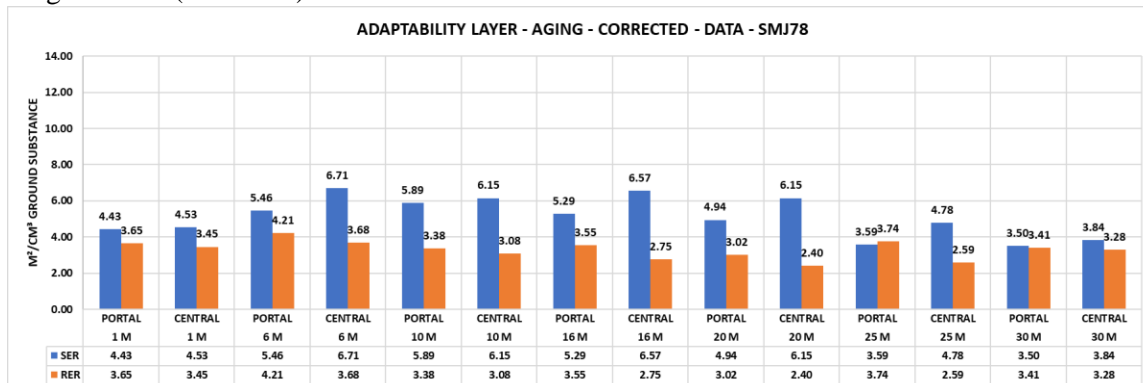


Figure 5.27 Note the remarkable difference between the uncorrected and corrected data. In some cases, substantial differences between the SER and RER surface areas disappeared.

Once corrected for section thickness and compression, we can expand the ER data of the portal and central hepatocytes by fitting the original data to polynomial equations and then use the equations to generate equally spaced time points (Figure 5.28). In turn, we can normalize the data (Figure 5.29).

Expanded Data (Portal and Central)

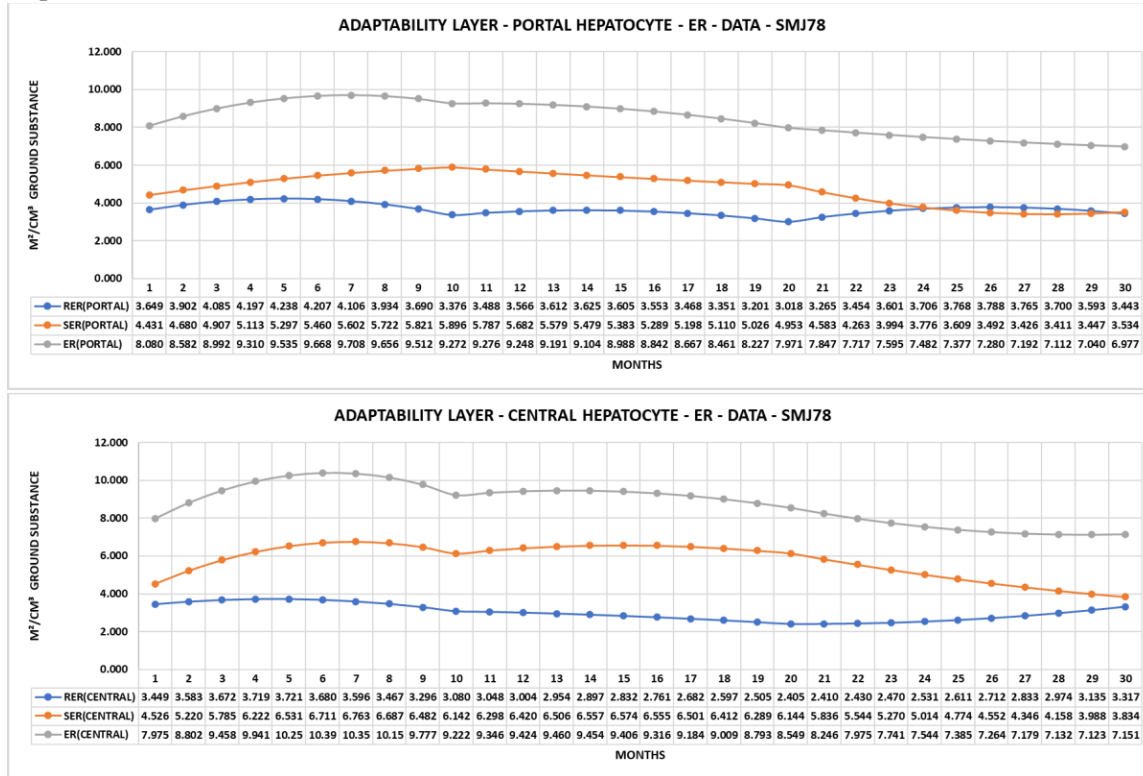
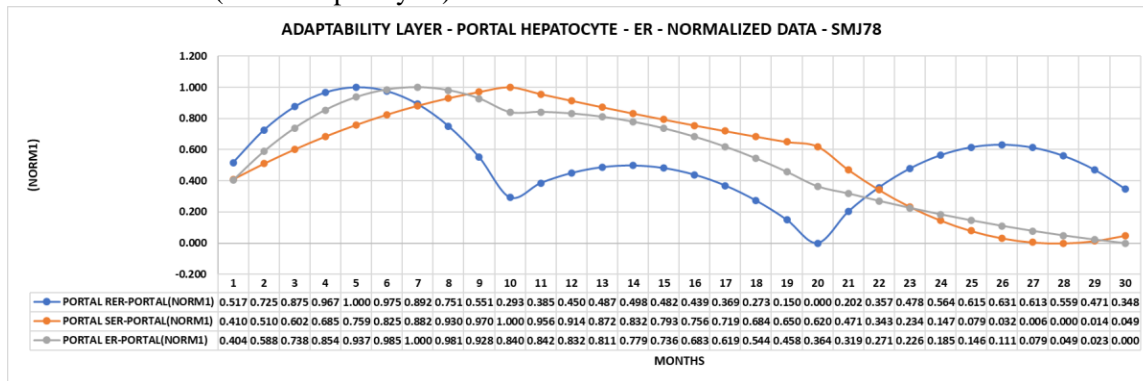


Figure 5.28 The portal and central plots display differences.

Normalized Data (Portal Hepatocytes)



Normalized Data (Central Hepatocytes)

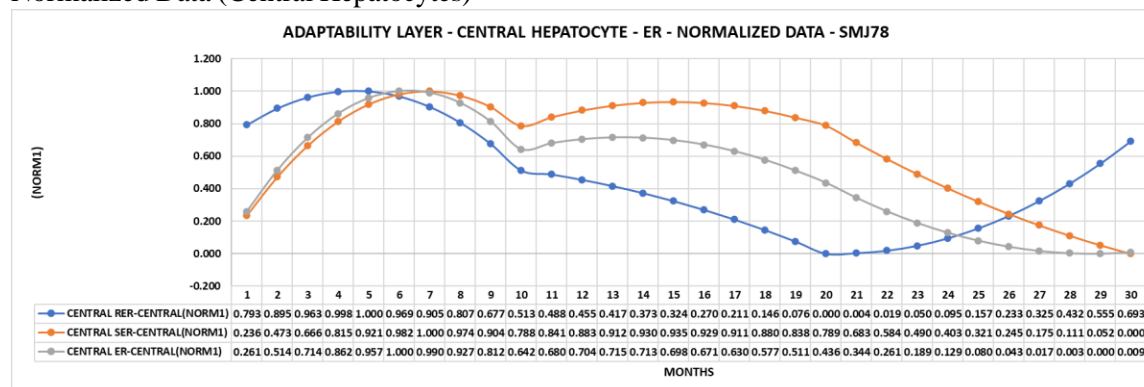


Figure 5.29 When normalized, however, the changes based on the same range ($0 \rightarrow 1$) become more pronounced. Note that fitting the data to the portal and central hepatocytes (days 1 to 28) required three polynomial regressions to obtain the $R^2 = 1$ outcomes.

Portal vs Central: Next, we can use the normalized data to generate data pairs ratios in the rules layer. In the first row of Figure 5.30, the ER ratios (data rounded) showed largely equal changes in the portal and central ER until month twenty-three at which time the portal ER increased while the central ER decreased. In rows two and three of the figure, the ratios (data rounded) showed that the RER of the portal hepatocytes became the dominant player (larger ratio value), whereas the SER of the central hepatocytes had the larger ratio value.

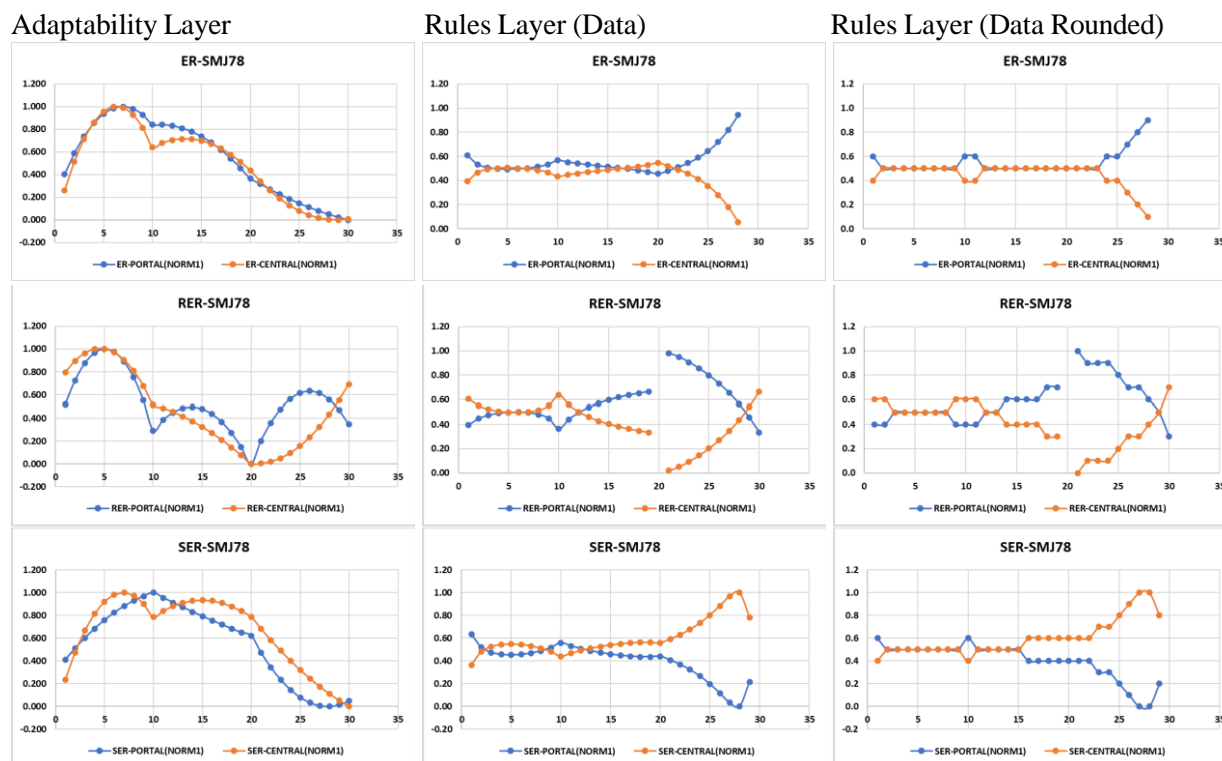


Figure 5.30 The patterns show that similarities and differences exist between the organelles in portal and central hepatocytes. It depends where and when we look. The 0.5:0.5 ratio indicates equal rates of change for the organelle membranes of the portal and central cells. Curiously, the ER remained largely the same in both portal and central hepatocytes until something unknown happened at month twenty-three. If marker enzymes distribute similarly on the ER membranes of portal and central hepatocytes,

then solving sets of simultaneous equations becomes a workable strategy for detecting different enzyme densities of RER and SER in portal and central cells. The ratio data plots suggest when and where to look.

Combining the normalized data for the RER and SER of portal and central hepatocytes associates aging with an uncoupling of the order seen for the earlier days (Figure 5.31).

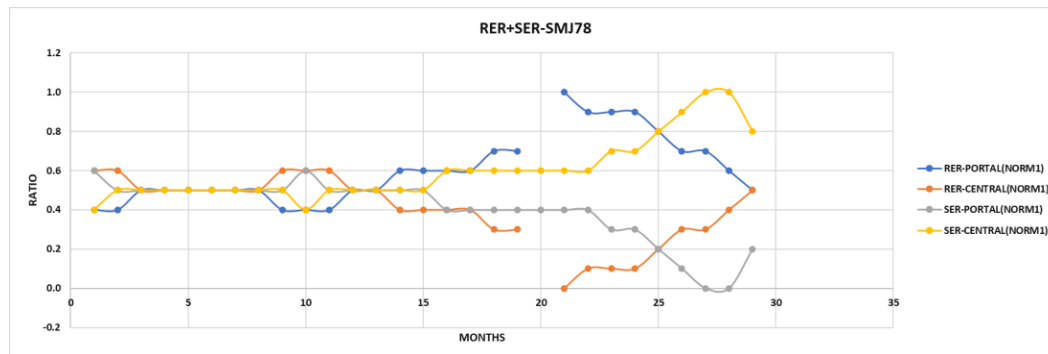


Figure 5.31 Superimposing the portal-central plots identifies the areas of similarity (0.5:0.5, 0.4:0.6, 0.2:0.8). Basically, the RER and SER at the two lobular locations included both differences and similarities in the data pair ratios tracking the changes.

Portal vs Portal; Central vs Central: Notice that the differences in the RER and SER were greater within cells (Figure 5.32) than between cells (Figure 5.31).



Figure 5.32 A different pattern appeared when comparing the RER to the SER in just portal or central hepatocytes. Now the ratios (data rounded) of RER to SER in both portal and central cells detected few overlapping points. Either each part of the

data pair had the same ED or the EDs combine to produce the same ED for the ER. [Note that EDs were not determined]. The point? Detecting a change is one thing, explaining it requires morphology, biochemistry and a basic understanding of how cells change.

Questions: (1) Can changes in organelles be similar and different when located in portal and central hepatocytes? Yes, but it depends on when and where we look. (2) Is it possible to interpret changes in portal and central hepatocytes empirically using just morphology or biochemistry? No. Why not. Cells define their organelles by the way they change morphologically, biochemically, and in the relationships of biochemistry to morphology (enzyme densities). Moreover, hepatocytes focus on two key outcomes: (1) membrane recipes and (2) total liver capacities. Although we can detect changes in one or a few parts, such changes exist out of context and consequently suffer the loss of interpretation.

5.3.4 Case Study 4: Development (Ornithine Aminotransferase) – HG69

Source: Update applied to original data from Herzfeld A., Greengard O. (1969) Endocrine modification of the developmental formation of ornithine aminotransferase in rat tissues. *J Biol Chem* 244: 4894-4898. ER membranes: Herzfeld et al., (1973);HFG73.

Topic: Morphological and biochemical development of ornithine aminotransferase in the rat liver.

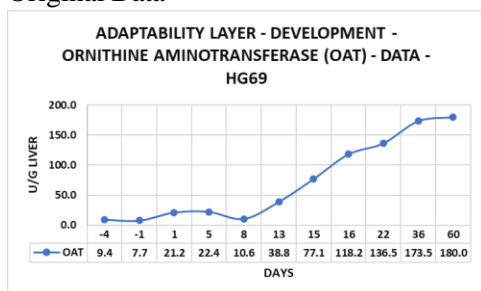
Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, and calculate enzyme densities [ED(NORM2)]. The difference between NORM1 and NORM2 explained.

Dataset: Ornithine aminotransferase and ER surface area (shared) related to a gram of liver. The normalized enzyme densities (NORM2) used mixed data references (U/G of liver, S/CM³ of hepatocyte cytoplasm) - see 5.3.10.

Hertzfeld and Greengard (1969; HG69) studied the pre and postnatal developmental of ornithine aminotransferase (OAT). The update calculated normalized enzyme densities before (ED-NORM1) and after normalization (ED-NORM2); the ER surface area data came from a 1973 study (HFG73).

Since the mixed references of the biochemical and morphological data prevented the calculation of an enzyme density from the original data, we'll use normalized data instead. However, converting the gram of liver reference to a cm³ of hepatocyte cytoplasm (multiplying the gram by 0.7) would have allowed the calculation. Figure 5.33 summarizes the original and expanded data.

Original Data



Expanded Data - ED = [(U/G)/(S/CM³)] → (Caution – mixed references for illustration only)

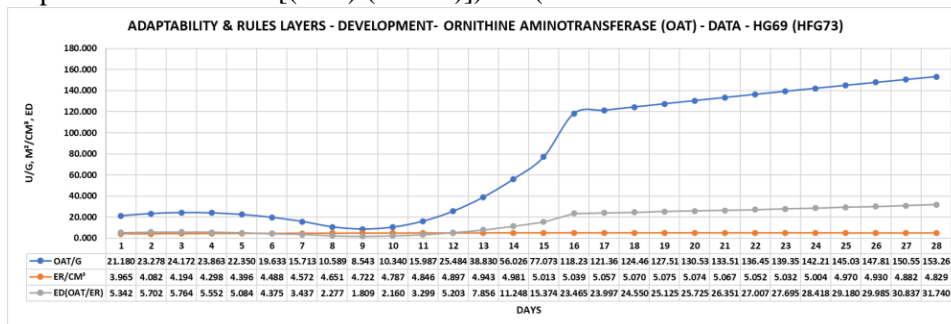


Figure 5.33 The dataset creates a problem because the biochemistry has one reference (OAT/G) and the membrane surface area another (ER/CM³). By converting the per gram of liver reference used by the biochemistry to a CM³ of hepatocyte cytoplasm, we could have calculated the enzyme density (ED). Instead, we'll solve the problem of the mixed references by normalizing the data before calculating the ED-NORM2).

Recall that when calculating an enzyme density from normalized data (ED-NORM1), the normalized data no longer fall within the range of 0 to 1. A second normalization (NORM2) restores the appropriate range (Figure 5.34).

Normalized Data, where $ED-NORM1 = [(U-NORM1)/(S-NORM1)]$

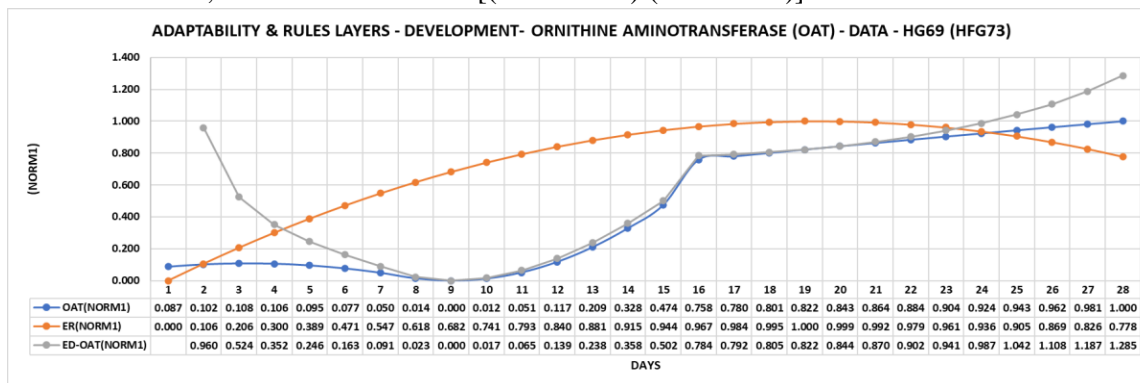


Figure 5.34 This intermediate calculation $[(U-NORM1)/(S-NORM1)]$ loses the normalization (data range goes beyond 1.0 to 1.285).

A second normalization (ED-OAT(NORM2)) mitigates the problem (Figure 5.35). Notice that the second normalization successfully retained the shape of the curve, as shown by the parallel ratios in Figure 5.36.

ED-NORM2

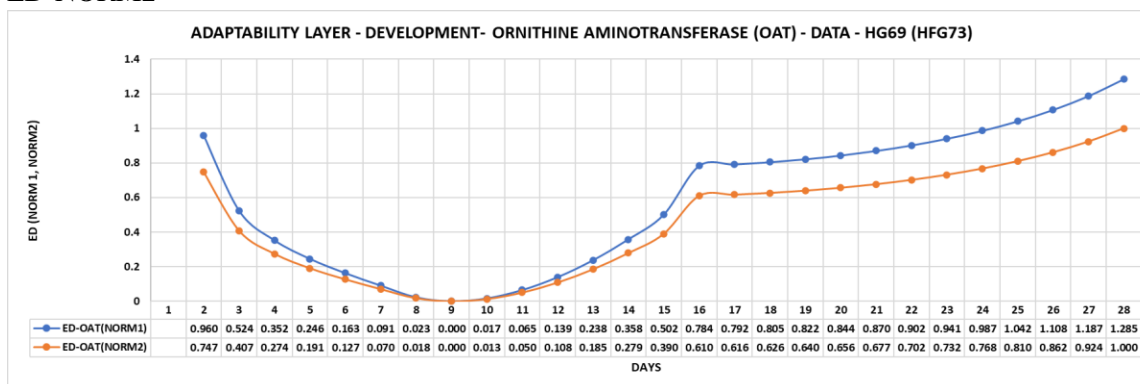


Figure 5.35 Enzyme densities before (NORM1) and after the second normalization (NORM2).

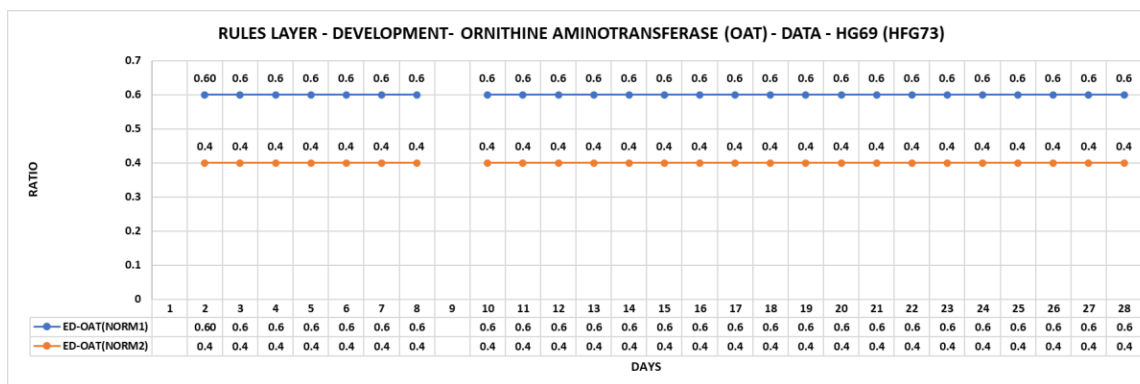


Figure 5.36 As expected, the NORM1 and NORM2 curves plotted as ratios run parallel. [Note: The missing point at DAY 9 resulted from dividing a value by 0 (lowest value). When this creates a problem, one can separate the data into two parts (e.g., day 1-9 and 9-28), expand each part, and extend the data a second time.

Comment: Generating enzyme densities (ED-NORM2) from many publications produces the basic dataset used to assemble and test cell phenotypes as a model for detecting and exploring cell changes. By exploring a strategy copied from biology, we're trying to take a step beyond our current limitation of trying to detect complex biological changes statistically by looking for significant differences between two data points.

By unfolding biological complexity, we're encountering algorithms operating presumably within the larger framework of the cells information processing systems. The hepatocyte, for example, has the capacity to generate algorithms fully capable of morphing recipes into an almost unlimited number of solutions. As we work our way through the case studies, hepatocytes always seem to know - inherently - how to fix problems or to draw from a vast library of hard coded solutions (if they exist). Either way, they're remarkably good at their job.

5.3.5 Case Study 5: Development (Cytoplasmic Membranes - Prenatal) – DDZM82

Source: Update applied to original data from Daimon T., David H., Zglinicki T. V., Marx I. (1982) Correlated ultrastructural and morphometric studies on the liver during prenatal development of rats. *Exp Path* 21: 237-260. DDZM82

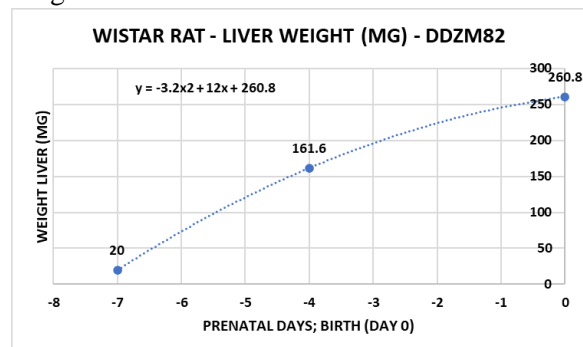
Topic: Development of hepatocytic membranes during prenatal development.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, and generate patterns using data from the rules layer.

Dataset: ER, RER, SER, MIM, OMIM, IMIM.

Daimon et al., (1982; DDZM82) described changes in the morphology of hepatocytic organelles during prenatal development. We'll use the updated data to look for duplicate patterns within and across the membrane compartments. The results include the expanded liver weights (Figure 5.37), original data (Figure 5.38), expanded data (Figure 5.39), normalized data (Figure 5.40), and duplicated ratios (Figure 5.41).

Original Data



Expanded Data

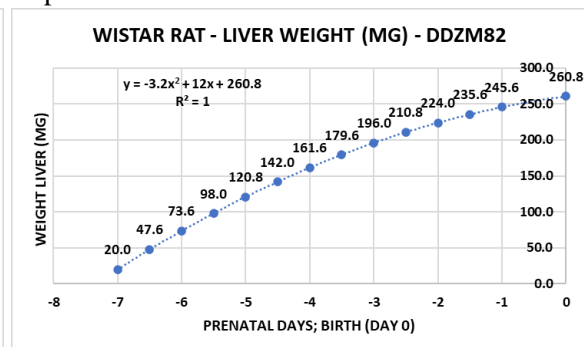


Figure 5.37 Expanding the dataset using a polynomial regression (in MS EXCEL).

Original Data

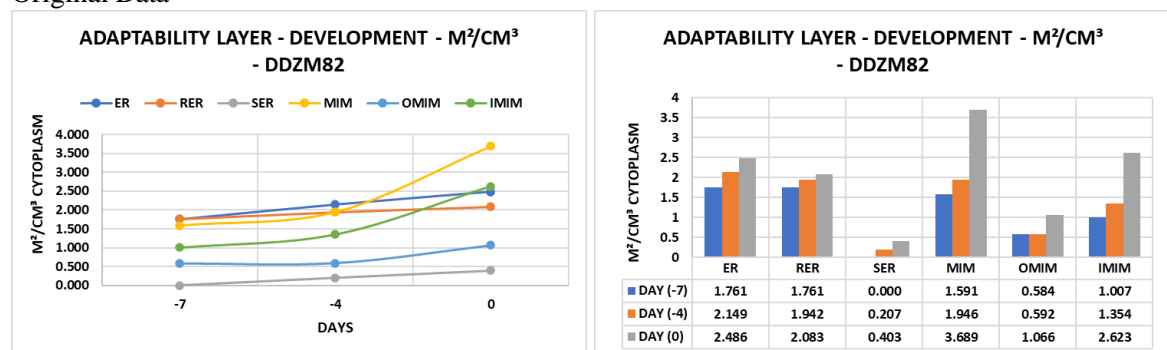


Figure 5.38 The original data corrected for section related errors. At birth (day 0), the mitochondrial membranes showed the greatest increases.

Expanded Data

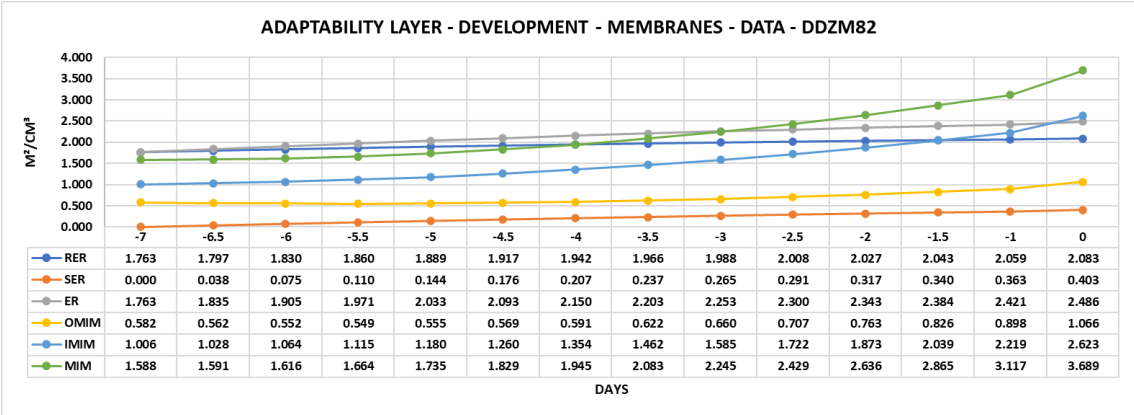


Figure 5.39 The expanded dataset suggested increases for all the membrane organelles.

Normalized Data (NORM1)

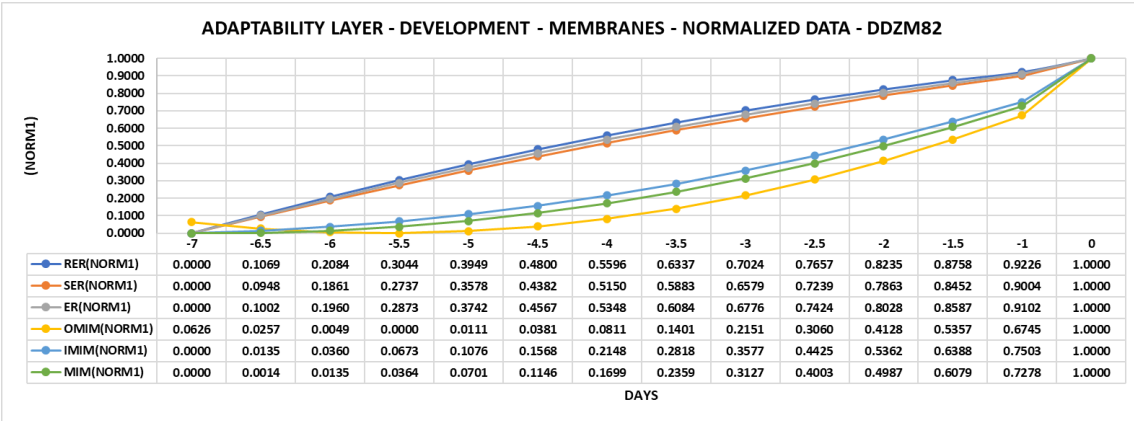
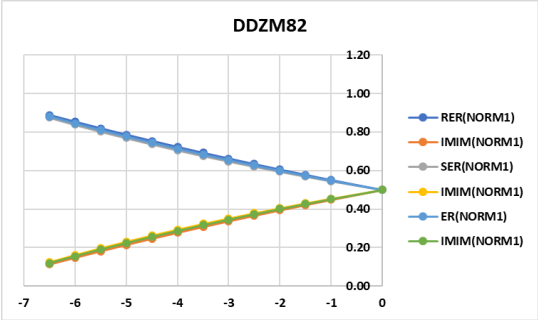


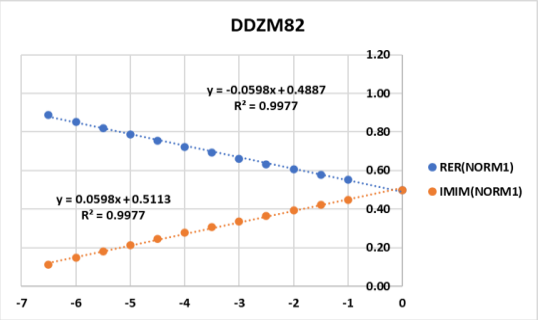
Figure 5.40 When normalized, we can see that the membranes formed two distinct groups – ER and MIM – with priority given to the ER.

By generating data pairs and forming ratios, superimposed plots show that different membrane organelles experienced nearly identical changes during prenatal development (Figure 5.41). Moreover, the organelle ratios converged to 0.5:0.5 in preparation for birth.

Duplicate Ratios



Equations



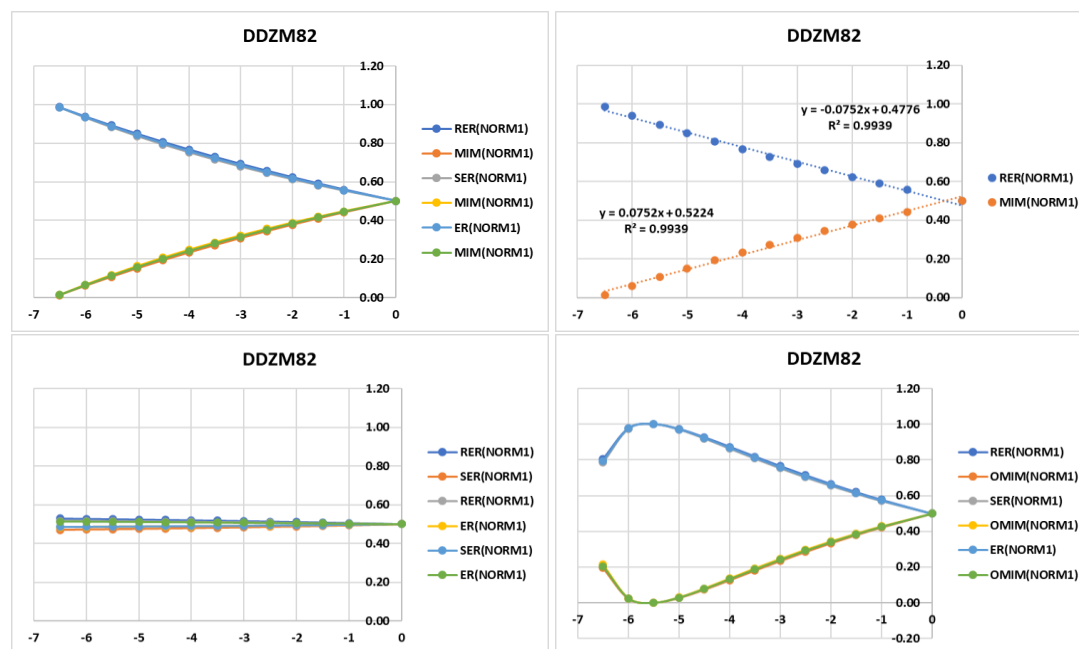


Figure 5.41 Different membrane data pairs observed the same rules (ratios) during prenatal development. The tight alignment of the duplications suggests the workings of a well-tuned system driven perhaps by an underlying need to establish the best starting point to face the unknowns of independent growth and development. Recall from Figure 5.38 that the original dataset included three data points - the regression equations predicted the rest. The R^2 s serve to highlight the linearity of the curves.

Comment: Connectivity and complexity are key to understanding biological changes. The update of this study showed that hepatocytes can connect multiple parts (membrane organelles) by rule and control their changes precisely. During the prenatal period, what was the relationship between the changes in membrane surface areas and their constituent marker enzymes? How were the changes in membrane surface areas part of a larger plan focused on producing membrane-enzyme recipes? Did the enzyme densities conform to the external surrounding prenatally? Since the biology literature most likely contains the missing enzyme data, one could use a data mashup to generate preliminary answers to such questions.

5.3.6 Case Study 6: Development (Electron Transport Enzymes, Phosphatases) - DSP66

Source: Update applied to original data from Dallner G., Siekevitz P., Palade G. E. (1966) Biogenesis of endoplasmic reticulum membranes II. Synthesis of constitutive microsomal enzymes in developing rat hepatocyte. J Cell Biol 30: 97-117; DSP66 ER membranes: Herzfeld et al., (1973); HFG73.

Topic: Development of microsomal (ER) enzymes and membranes (pre and postnatal).

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, calculate EDs (NORM1 + NORM2), and generate patterns using data from the rules layer.

Dataset: Enzymes including NADPH-DIAPH, NADPHCCR, NADPH-NTRED, NADH-DIAPH, NADHCCR, NADH-NTRED, DEM, and membrane surface areas (ER). The normalized enzyme densities (NORM2) used mixed references (U/G of liver, S/CM³ of hepatocyte cytoplasm) - see 5.3.10 and additional checks provided in the chapter.

Dallner et al., (1966) described the biochemical development of the endoplasmic reticulum (ER) using microsomal enzymes. To calculate enzyme densities, the update adds the surface area of the ER from Herzfeld et al., (1973; HFG73).

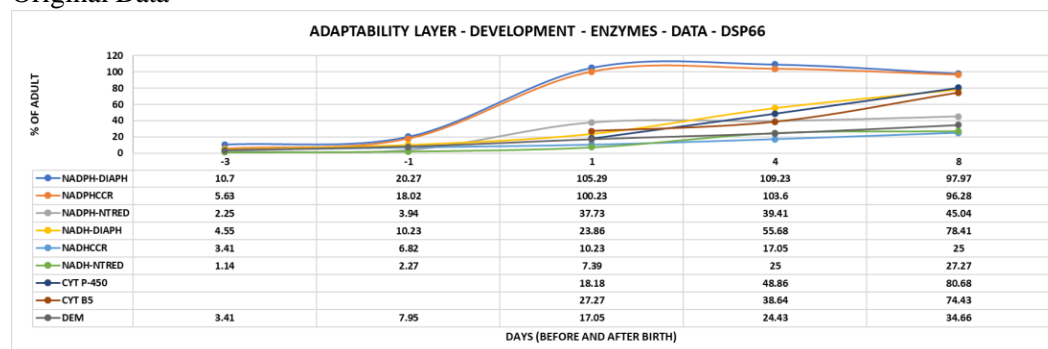
Most studies on the liver relate biochemistry to one set of references (e.g., mg protein, gram of liver, or the liver) and morphology to a similar or different set (e.g., a fixed gram of liver, liver, or cm³ of either cytoplasm or hepatocytes). Typically, the supporting data needed to translate units of one data type into another seldom appear in publications. Some authors prefer to publish results only as percentages.

Normalization alleviates several problems stemming from incompatible data units and references. It accomplishes this by cancelling out data units and restricting all the changes to the same range of 0 to 1. As noted earlier in the chapter on references, two routinely used references (mg protein and gram of liver) can detect the same changes (and rules) similarly or differently. The problem with these and other mixed pairs of references is that we often cannot determine when the references change similarly, differently, or by how much. Since we have both biochemical references (mg protein and gram of liver) in Part B of the update (phosphatases), we can observe the effect of normalization on both data references. But first, Part A updates the perinatal changes associated with the electron transport enzymes.

Part A (Electron Transport Enzymes)

The Enzymes: Notice in Figures 5.42 and 5.43 that the changes in enzyme activities fell into three roughly similar groups during development. Moreover, the groups persisted during the pre and postnatal periods.

Original Data



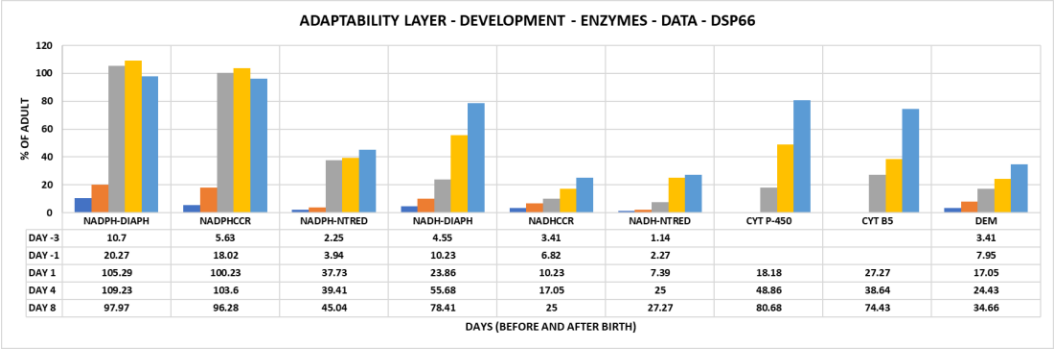


Figure 5.42 The original data summarizing relative changes in enzyme activities.

Next, Figure 5.43 summarizes the changes in the enzyme activities related to a mg of protein.

Expanded Data

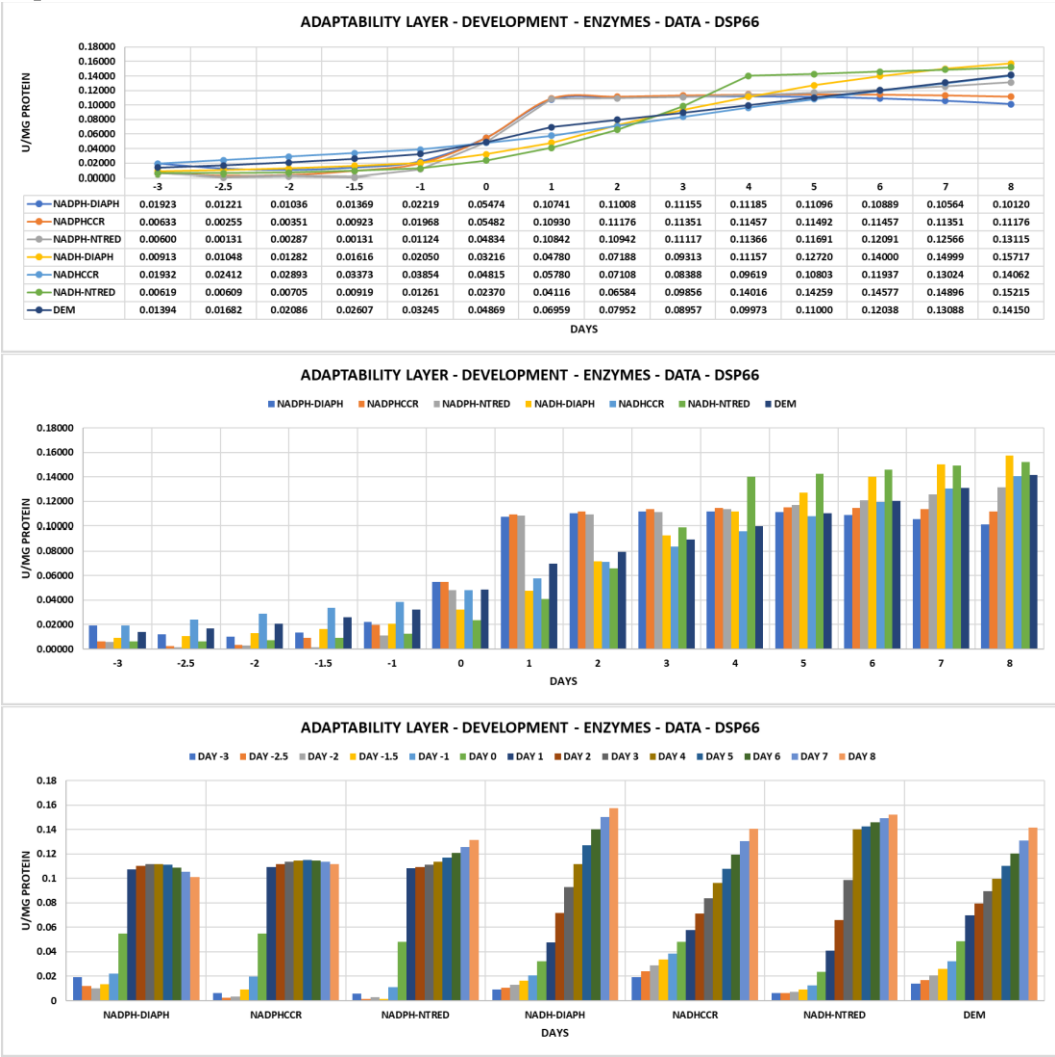


Figure 5.43 Note that some enzymes related to a mg of protein displayed similar patterns of change.

Normalized data, however, presented a somewhat different story (Figure 5.44). Notice that the similarities increased as development continued.

Normalize Data (NORM1)

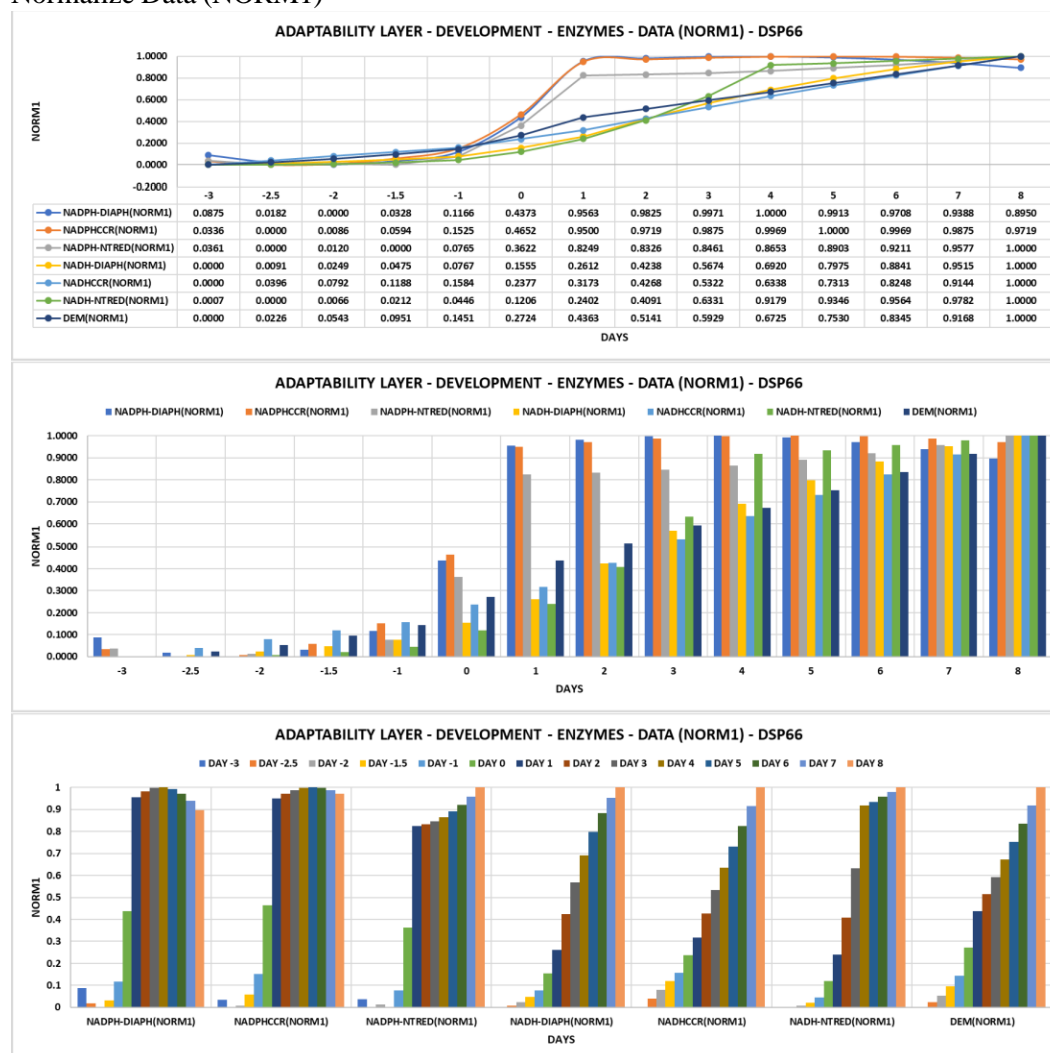


Figure 5.44 When normalized, similarities between enzymes increased particularly as development progressed.

The Enzyme Densities: The enzyme densities added a key piece of information – the amount of the enzyme activity associated with a unit of ER surface area (Figure 5.44). During the postnatal period (days 0 to 8), the hepatocytes adjusted the enzyme densities of their ER until they produced a mix consistent with its ongoing developmental program (a recipe). If we identify a persistent recipe as one that lasts for three consecutive days (maintaining three similar values), then by day eight NADPHCCR and DEM fit the definition.

[**Caution:** The ER values were originally related to a cm^3 of hepatocyte cytoplasm and the enzyme activities to a mg of protein. Since normalizing the original data removed the units and references, the ED-NORM2 calculation worked, but the interpretation required the assumption that the changes related to a mg of protein roughly paralleled those related to a gram of liver (or to a cm^3 of hepatocyte cytoplasm). Results from other updates suggest that this can happen either throughout or toward the end of a study.

For purposes of illustration, we're assuming - with a stated caution - that the normalization produced changes comparable to those coming from data when related to the same reference.]

Enzyme Densities (NORM2)

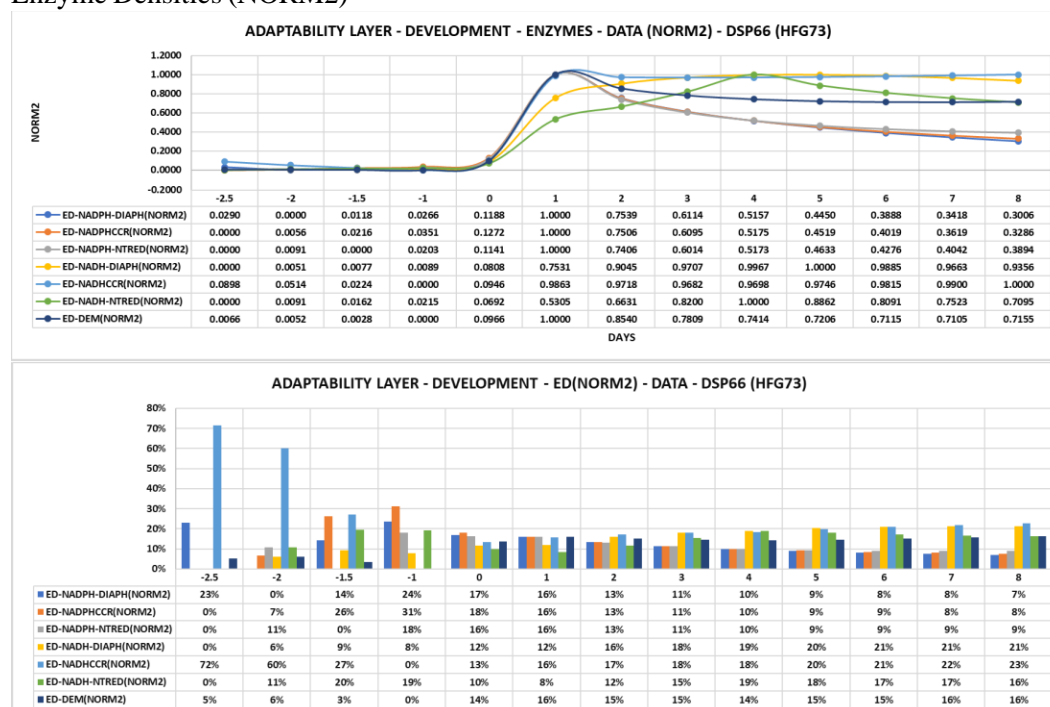


Figure 5.45 Moving left to right, the proportions of the enzymes in the recipes change less and less as the cells approach an early developmental solution.

For each day, the group of seven enzymes densities combined to form the enzyme-membrane recipe, which consisted of the relative amounts of the enzymes associated with the ER. Over time, the recipe stabilized as it approached the developmental goal. The day after birth (day 0), the hepatocytes began postnatal development by first ramping up enzyme production and then transitioning to the best mix of the enzyme activities by about day six. The daily recipe – the column of data expressed as percentages - records the proportions of the seven enzyme activities. The update illustrates the level of phenotypic detail that one can extract from the literature when, for example, wishing to predict gene expression from phenotypic changes.

By plotting the changes by enzyme, we can follow the developmental process as it unfolds and begin to identify duplicate patterns (Figure 5.46).

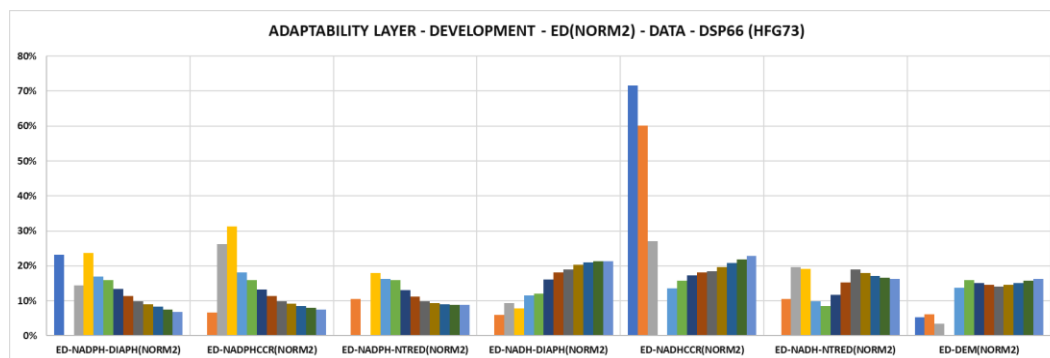
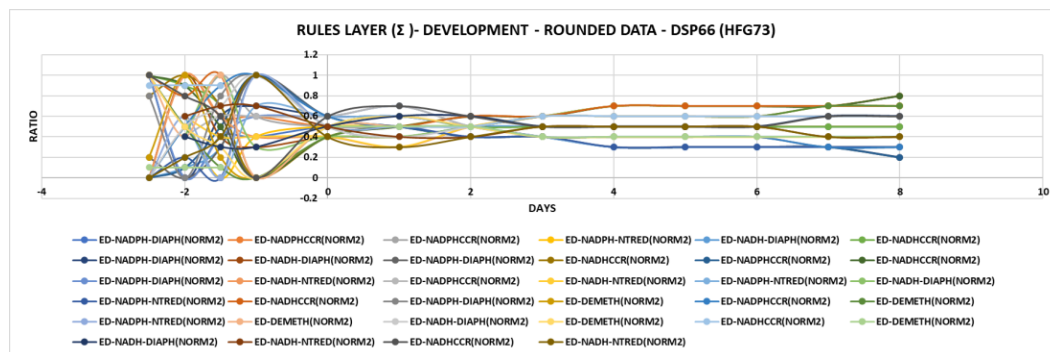


Figure 5.46 Notice the nearly synchronized changes in three enzymes (NADPH-DIAPH, NADPHCCR, NADH-NTRED) postpartum.

During the developmental process, three distinct events occur. Individual structural and functional parts change in magnitude and direction as do the relationships of one part to another. If development involves an optimizing routine that has as its output a recipe defining the functional relationships of enzymes, then pattern analysis provides ready access to such information. By taking two enzyme densities at a time, plotting the patterns they create, and collecting duplicates (as described previously), we can follow changes in the relationships within and across data pairs.

The point? During a change, there appears to be at least four subevents that become candidates for optimization. If, for example, AI can capture the algorithm(s) for these events, then we would have a mathematical model for predicting a given phenotype. Were such change algorithms to generalize, it would suggest a first principle. Moreover, one could use the developmental phenotype to predict the flow of information between phenotypes and genes, wherein reverse engineering would predict gene expression from the downstream synthesis of parts. If such an approach established quantitative connections between genes, enzymes, and organelles, then one could begin to imagine large-scale simulations.

Reverse Engineering with Duplicated Patterns: Recall that a change occurs as a transition from one type of phenotype to another or, in the case of development, through a redefinition of starting points (days 0, 2, 3). The prenatal period (days -2.5 to 0) defined a time of wide-ranging changes to the data pair ratios, but shortly after birth the patterns became regular with only minor changes (Figure 5.47).



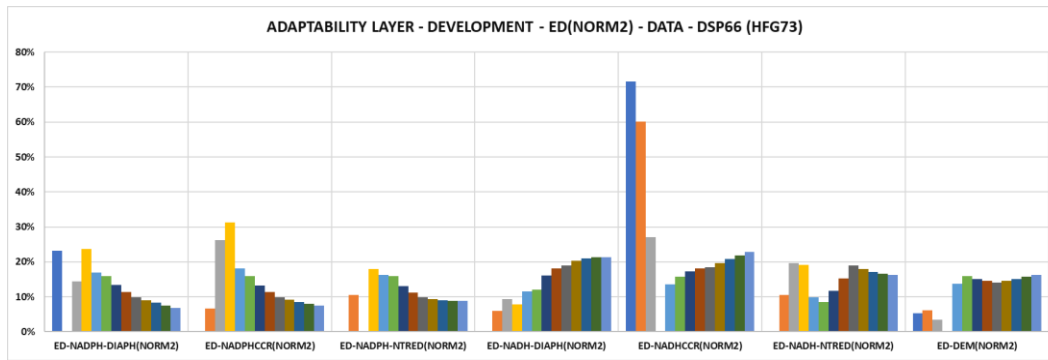


Figure 5.47 By combining all the changes of all the enzyme pairs into a single plot, we get a partial view of a developmental phenotype based on the enzyme densities in play. The three identical solutions seen at postnatal days 4, 5, and 6 suggested a temporary solution that transitioned to the next solution beginning at day seven. To reverse engineer this plot, we can sort on the outcomes (data pair ratios) to unfold the phenotype into a collection of subgroups that changed at different times and in different directions. In turn, each subgroup unfolded stepwise all the way back to the original data pairs. The point? Using a data pair ratio model, we can see that a cell change results from a series of changes punctuated by intermittent solutions appearing at clearly defined times according to well-defined rules (ratios).

Reverse engineering a change involves taking a phenotype apart down to the individual data pairs using the persistent solutions to name the individual subgroups. Since the operating definition of an outcome includes the same results for three consecutive days, the solution shown in Figure 5.47 occurred at postnatal days 4, 5, and 6.

Beginning with the first subgroup, Figure 5.48 shows the 0.5:0.5 data pair ratios unfolded from the rest. This occurred at postnatal day two when the ratio of the data pairs (two enzyme densities) became one to one (0.5:0.5). Other subgroups of enzyme densities with similar data pair ratios displayed a similar pattern of change but with different ratios. The point? Change as a solution becomes explained in terms of multiple solutions defined by subgroups consisting of individual data pairs. If true, and if hepatocytes orchestrate development exclusively from gene expression, then the changes detected with the data pair ratios of phenotypes should coincide with those of molecular biology (e.g., microarrays). By simply replacing the names of the enzymes with those of their parent genes, reverse engineering the phenotype might allow one to predict gene expression one subgroup at a time. This sets up a situation wherein one can genetically change an enzyme in one subgroup and then see how the modification influences changes within and between subgroups. This means that the phenotype model becomes thoroughly testable using the power and persuasion of reproducibility.

When using the literature as a data source, reverse engineering a change begins by forming data pairs from the enzyme densities(NORM2) and then combining all the duplicates into a partially reconstructed phenotype that presumably existed during early development. By combining the data pairs into a combined set, one obtains a reconstructed phenotype, as shown in Figure 5.48.

Duplicated Patterns - ED(NORM2): The reverse engineering begins by collecting all the data pairs that found the same solution (i.e., the same ratio).

Subgroup 1 (05:0.5) – A solution appeared at postnatal day 2 (Figure 5.48).

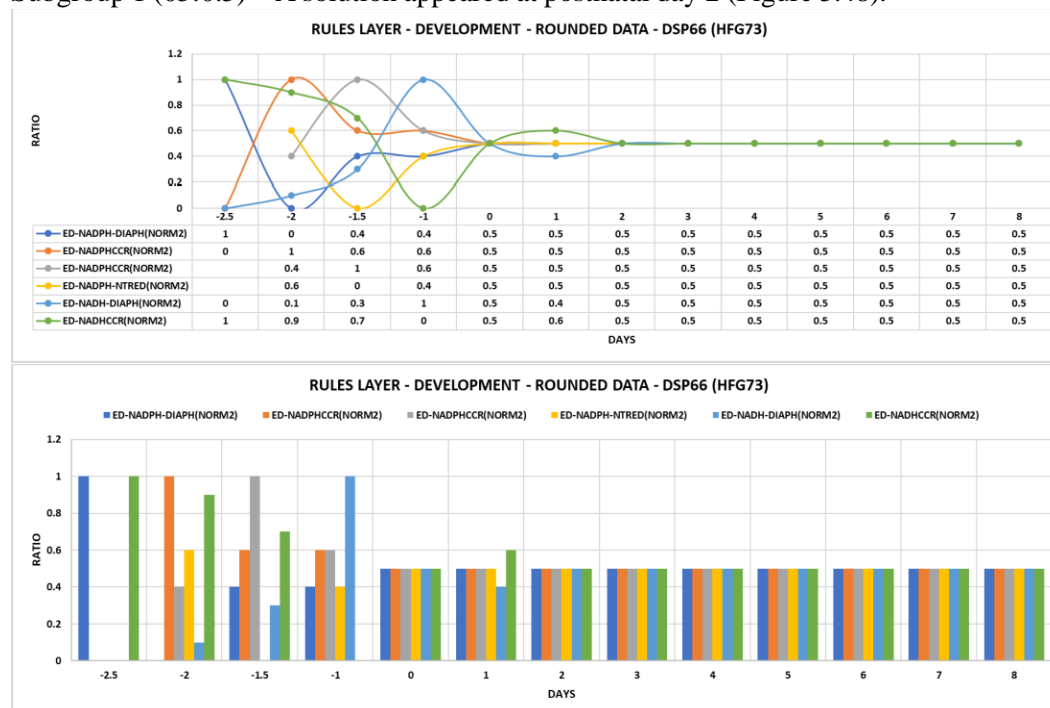
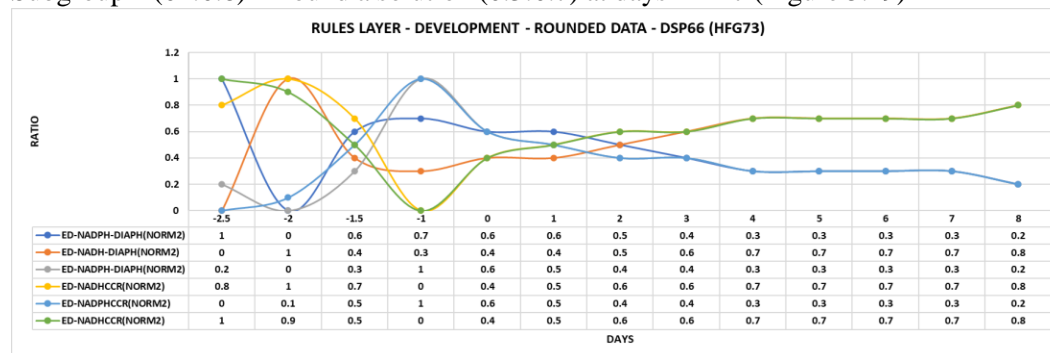


Figure 5.48 The data pair ratios associated with the electron transport enzymes, which supply energy to the cells, changed relatively to one another until birth and then abruptly stopped at postnatal day two. In effect, these enzymes were first to find a solution based on equal proportions of both enzymes in the data pair (0.5:0.5), which defined the first subgroup. This outcome implies that the developmental phenotype assigns the highest priority to establishing the energy resource.

The following ten figures unfold the phenotype into the remaining subgroups that remain interconnected (a given enzyme can make connections within and between subgroups). The plots show that a change gradually tuned data pairs, individual subgroups, and sets of subgroups simultaneously to produce a final package – the best phenotypic recipe for this group of enzymes. The question, of course, is whether this intricate collection of events needed to form a phenotype will map back to the timings of gene expression. If not, then other mechanisms may be at play in the cytoplasm. Moreover, if the multiple changes in enzyme densities represent an optimizing process, then something somewhere in the cells must be responsible for all the algorithms, heavy calculations, and enabling infrastructure.

Subgroup 2 (02:0.8) – Found a solution (0.3:0.7) at days 4 → 7 (Figure 5.49)



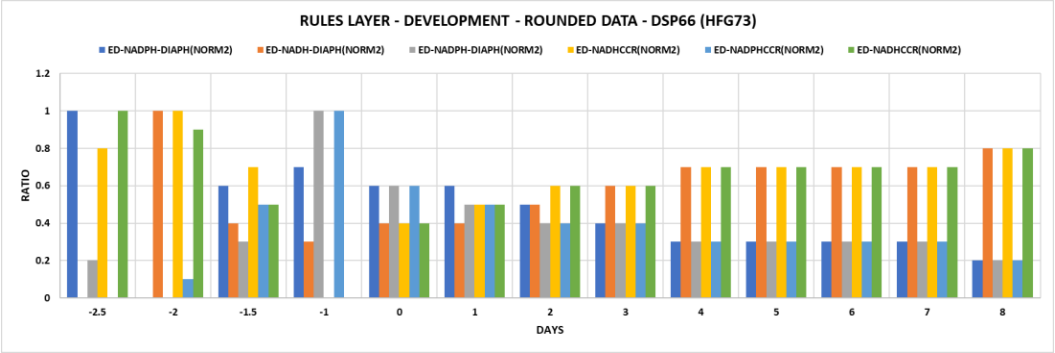


Figure 5.49 the second subgroup found its solution (days 4 to 7) and then moved on to the next stage of development.

Subgroup 3 (03:0.7) – Found its Solution at days 4 → 8 (Figure 5.50)

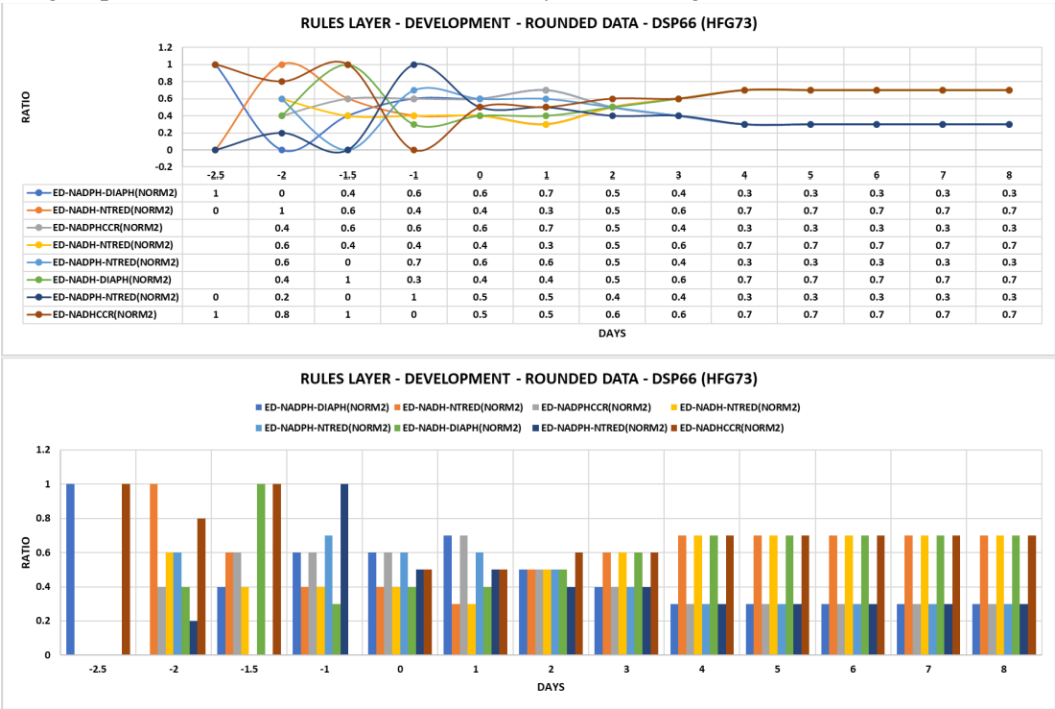


Figure 5.50 The solution (0.3:0.7) occurred on postnatal day four.

Subgroup 4 (04:0.6) – A solution at days 3 → 8 (Figure 5.51)

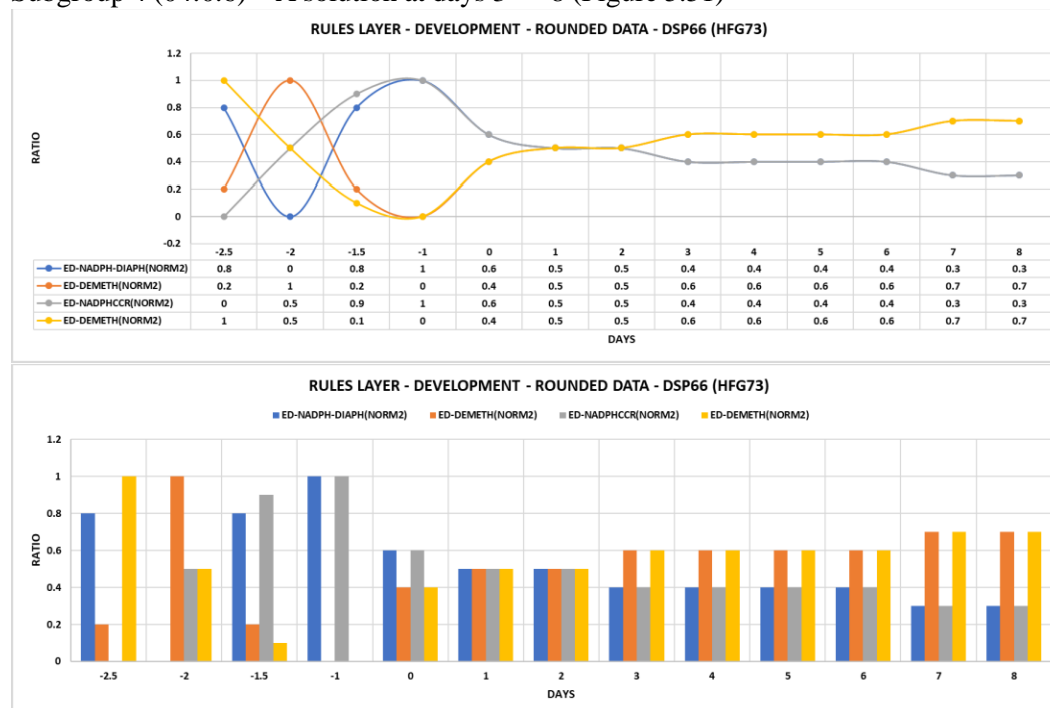


Figure 5.51 Notice how these three enzyme densities changed in parallel starting with day one. Apparently, development involves linking and synchronizing patterns of enzymes in highly specific ways.

Subgroup 5 (04:0.6) – Found a Solution at days 3 → 8 (Figure 5.52)

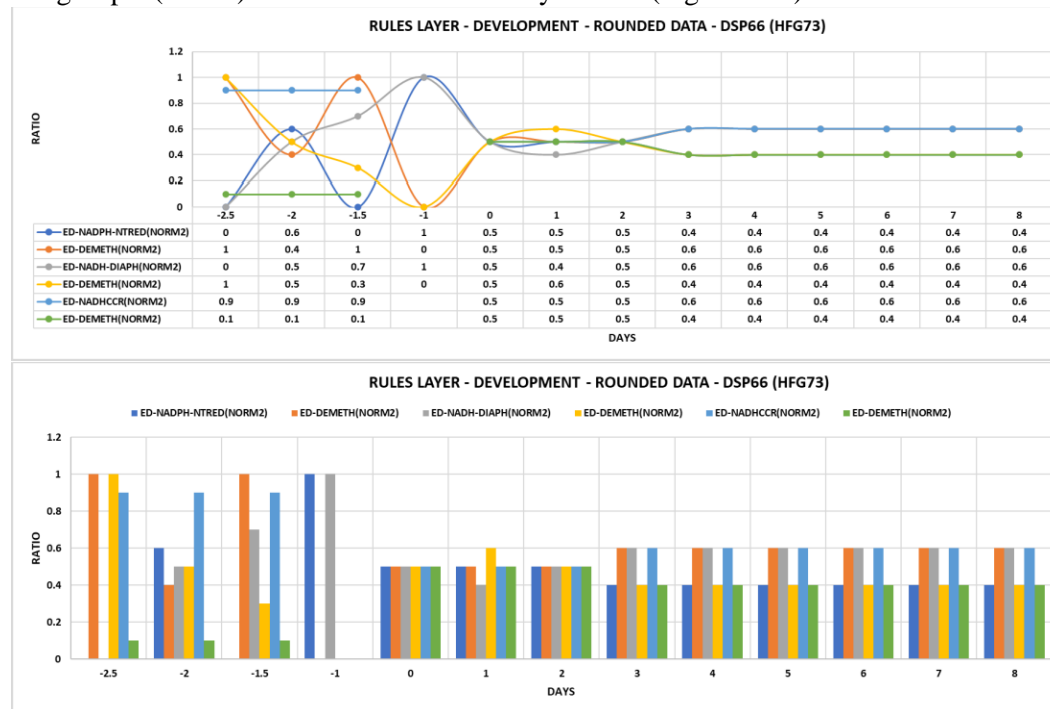


Figure 5.52 Notice the reoccurring pattern of starting out with equal amounts of activity for each enzyme in the data pair (0.5:0.5).

Subgroup 6 – Found a solution at days 3 → 6 (Figure 5.53)

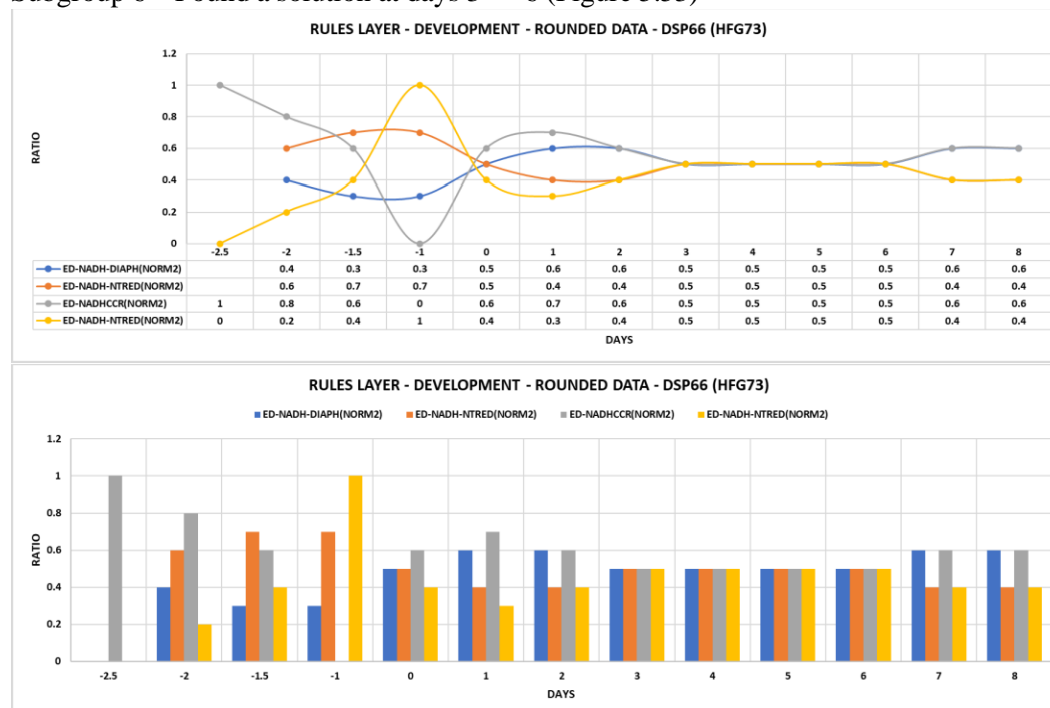


Figure 5.53 The first solution (0.5:0.5) – days 3 to 6 - changed to the next one at day seven. If 0.5:0.5 serves as an initial condition for a change, then this may represent a holding pattern waiting for something else to occur next.

Comments: When speculating on a quantum-based solution, we can imagine that the data pairs (enzyme densities) became entangled and together moved toward an optimized state, which in Figure 5.47 defined a ratio of 0.5:0.5. But consider the complexity of such a solution. While the relative changes might remain the same in the rules layer, the absolute amounts could vary considerably (in parallel) in the adaptability layer. Although a fine point, it becomes a key insight because genetic expression data appear in what we define herein as the adaptability layer but not necessarily in the rules layer. In practice, reconnecting a phenotype to its upstream genotype will require information from both players and from both layers. Also, we don't know how well genetic data survived after running their methods gauntlet.

PART B: (Phosphatases): Next, we apply a similar analysis to a group of phosphatases using data taken from Figure 2 of the original publication (Dallner et al., (1966; DSP66) However, we'll also use these data to explain two ways to calculate enzyme densities: original data for publications (ED = U/S), and ED(NORM2)]. The reason for including the two estimates is to compare the preferred method (original data with the same reference) to the workaround (original data with mixed references).

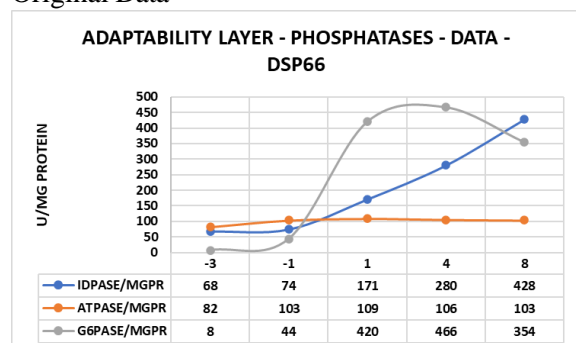
While normalized data allow the calculation of enzyme densities from mixed references, it assumes that changes in the substituted reference resemble those of the expected reference (m^2/g). Exchanging a m^2/cm^3 of cytoplasm for the m^2/g is not likely to change the shape of the curve importantly because a gram of liver and a cm^3 of hepatocyte cytoplasm differ essentially by a constant value. Multiplying m^2/cm^3 by the liver density ($1.07 \text{ g}/\text{cm}^3$) changes the reference from cm^3 to a gram and multiplying this result by 0.7 accounts for the contribution of the hepatocytic cytoplasm to the liver. However, substituting mg protein for a gram of liver when calculating EDs from normalized data comes with a substantially higher risk because a simple correction appears not to exist. We already know from results previously discussed that

the references - mg protein vs the gram of liver - can detect the same changes sometimes similarly and other times differently. For our purposes here, we will assume that normalized data based on a mg protein reference carry an unknown risk.

Figure 5.54 includes the original and expanded data with enzyme activities related to a mg of protein and membrane surface areas to a cm^3 of hepatocyte cytoplasm. The mg protein presumably relates to the protein content of the cells (plus extracellular parts) and the cm^3 of cytoplasmic volume identifies the amount of membrane coming from a changing number of hepatocytes. Moreover, the enzyme data came from microsomes. In short, we've setup an experiment using incompatible and potentially unstable parts to detect a change.

The point? To see what happens in a worst-case scenario. Fortunately, we'll discover that the experiment worked because we overlooked a key piece of information that effectively compensated for the faulty experimental design.

Original Data



Original Data Expanded

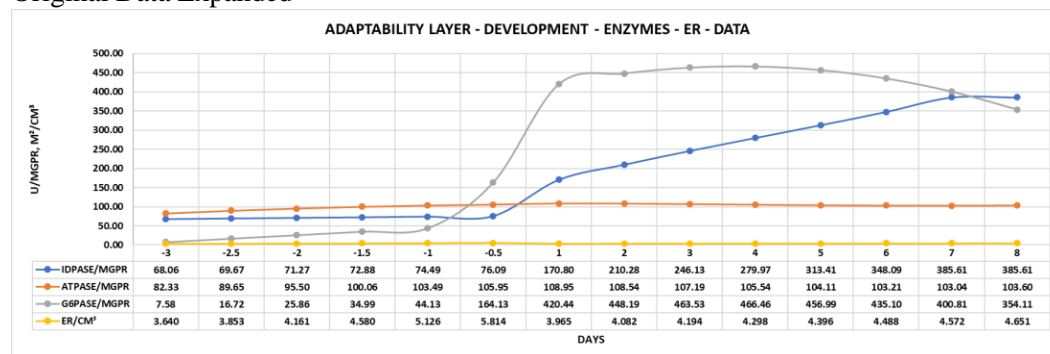


Figure 5.54 Original and expanded data.

Next, we calculate the enzyme densities from the expanded data (Figure 5.55).

Enzyme Density [ED] = $[(U/MGPR)/(M^2/CM^3)] \rightarrow$ Nothing (Mixed references – for illustration only)

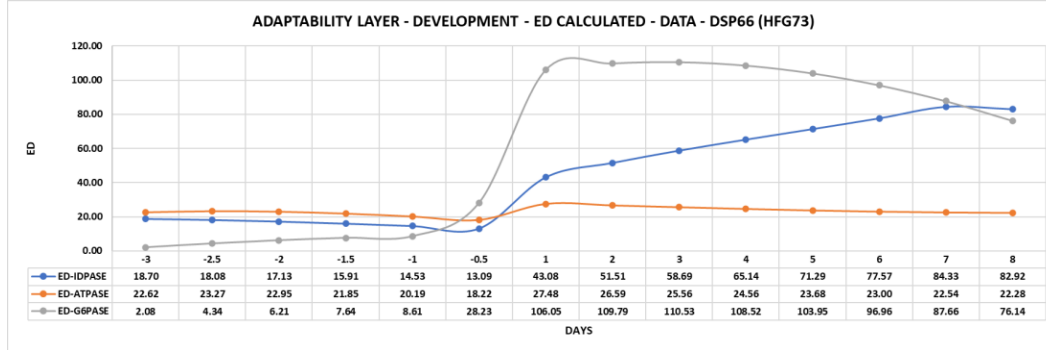


Figure 5.55 Since the references for the morphology (M^2/CM^3) and biochemistry ($U/MG PROTEIN$) didn't match, the enzyme density calculation was incorrect because the references failed to cancel out.

The work around? By normalizing the morphological and biochemical data before calculating the enzyme densities the ED calculation became possible because the resulting curves represented just the changes without the incompatible baggage (units and references). The results appear in Figure 5.56.

ED-NORM2 = $(U-NORM1)/(S-NORM1) \rightarrow$ NORMALIZED

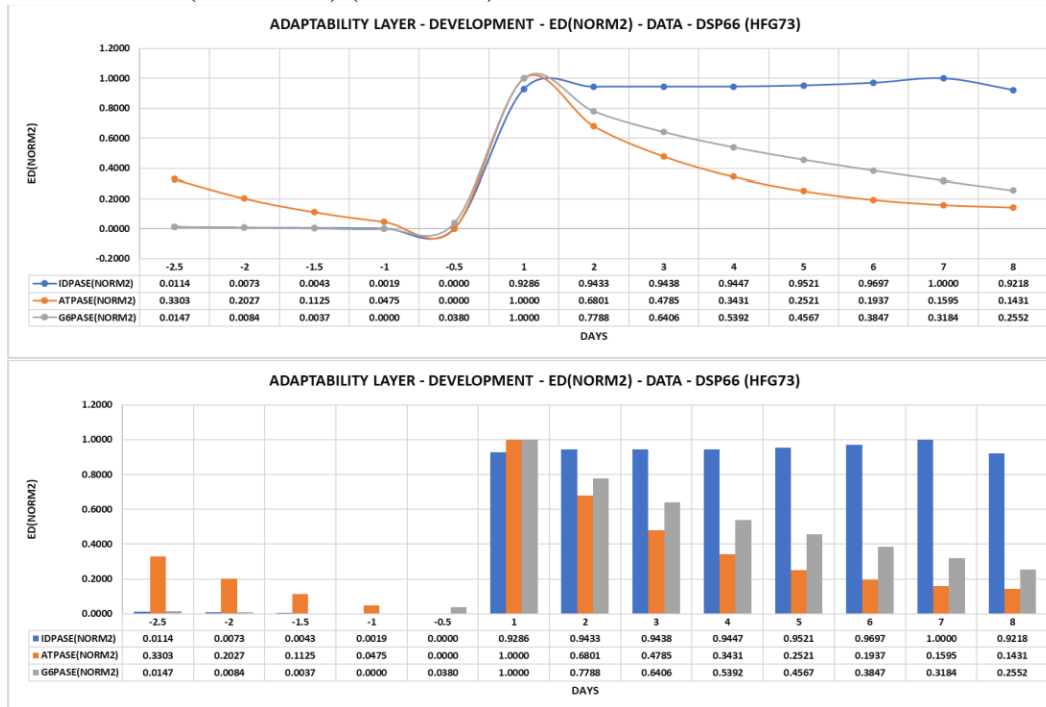


Figure 5.56 Normalized enzyme densities (NORM2) derived from mixed references ($U/MG PROTEIN$, M^2/CM^3). This required the assumption that the mixed and matched data references produced similar normalized curves. However, we can check the assumption.

When expressed as data pair ratios in the ratio layer (Figure 5.57), the ED-NORM2 data displayed two outcomes. While the changes in the phosphatase enzyme densities also synchronized around the birth day

(0), postnatally they showed a diverging pattern unlike the parallel one seen for the electron transport enzymes in Part A.

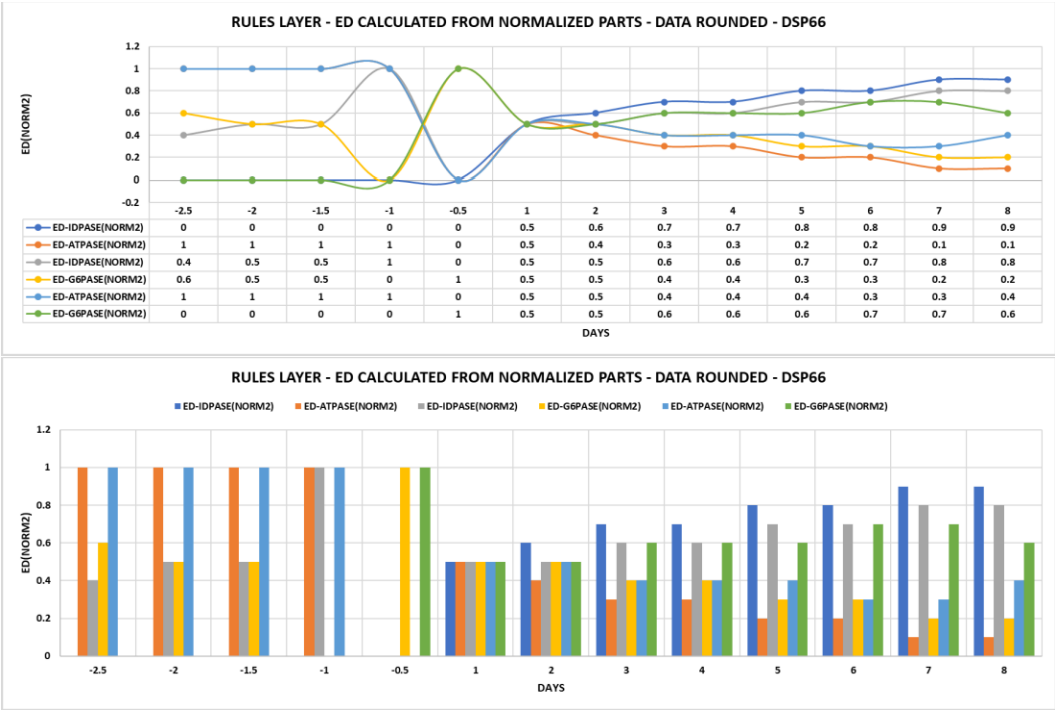
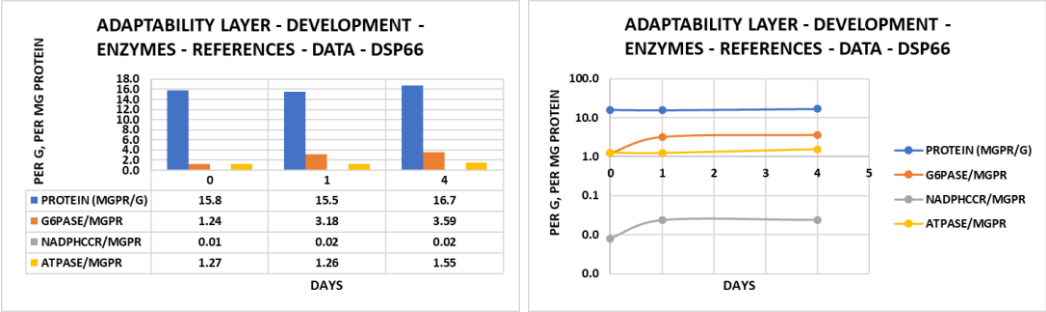


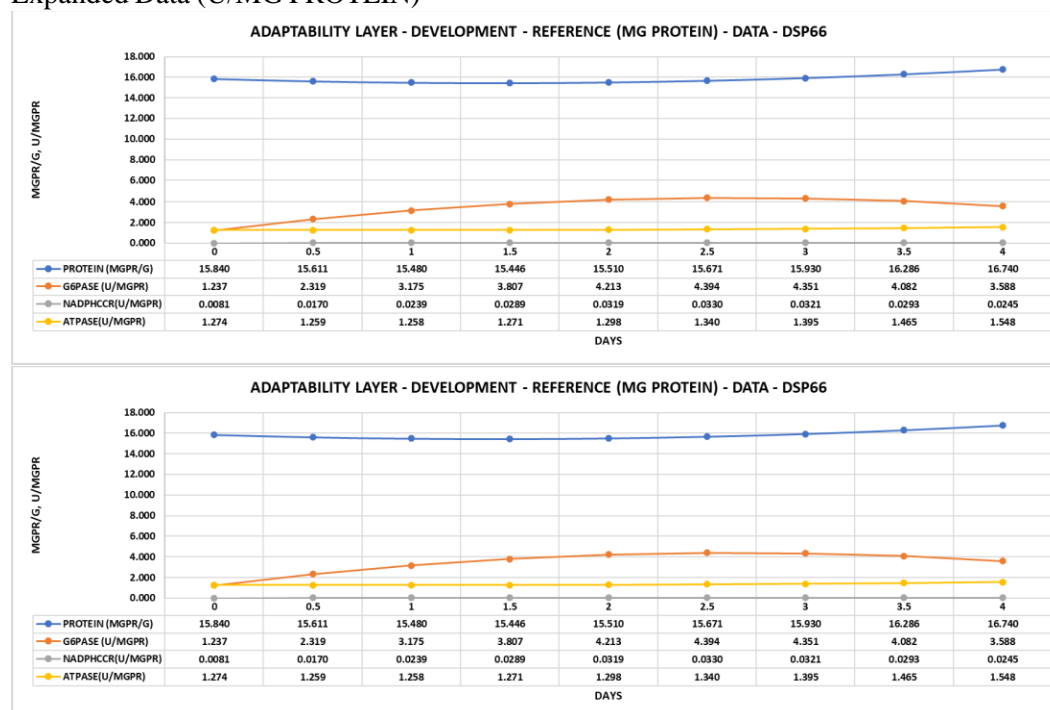
Figure 5.57 Since the ratio plots showed no duplicates at day eight, solving the phosphatases remained a work in progress.

Phosphatases (Data References): Although limited (just three data points), we'll use the data from Figure 5 of the publication to see how changes in the mg protein/gram and U/mg protein compare when normalized. This will allow us to check our earlier assumption that the two references change roughly in parallel. First, we'll expand the original data to generate enough points to allow the normalization. However, the key piece of information we missed now appears in Figure 5.58. The protein content per gram of liver remained essentially constant (only three data points), which suggests that changes related to a mg protein parallel changes related to a gram of liver. When calculating enzyme densities with normalized data, mixing gram and cm³ of cytoplasm references routinely produces results identical to those calculated with the same references (see case study 10).

Original Data



Expanded Data (U/MG PROTEIN)



Normalized Data (U/MG PROTEIN-NORM1)

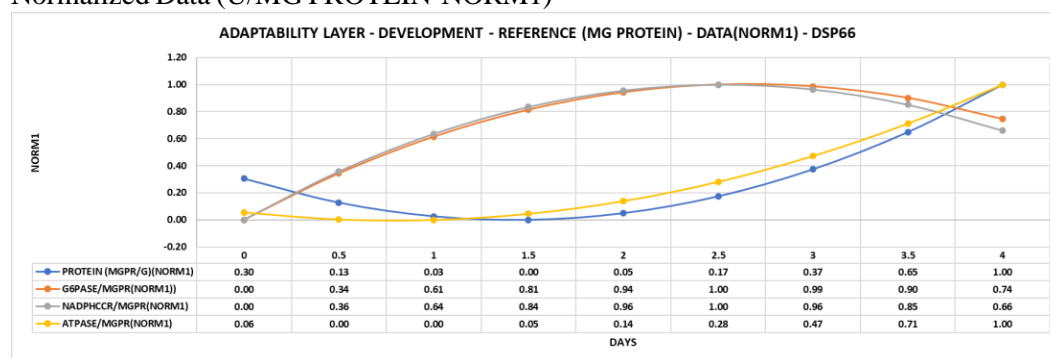


Figure 5.58 The original data expanded and normalized. The mg protein per gram of liver tracked with ATPASE, but not with G6PASE and NADPHCCR.

Plotting data pairs in the adaptability and rules layers generated patterns of change (Figure 5.59). The first pair (G6PASE vs NADPHCCR) showed that they change at the same rate (0.5:0.5), whereas the two remaining pairs (G6PASE vs ATPASE, NADPHCCR vs ATPASE) displayed roughly similar patterns of change.

Adaptability and Rules Layers (U/MG PROTEIN)

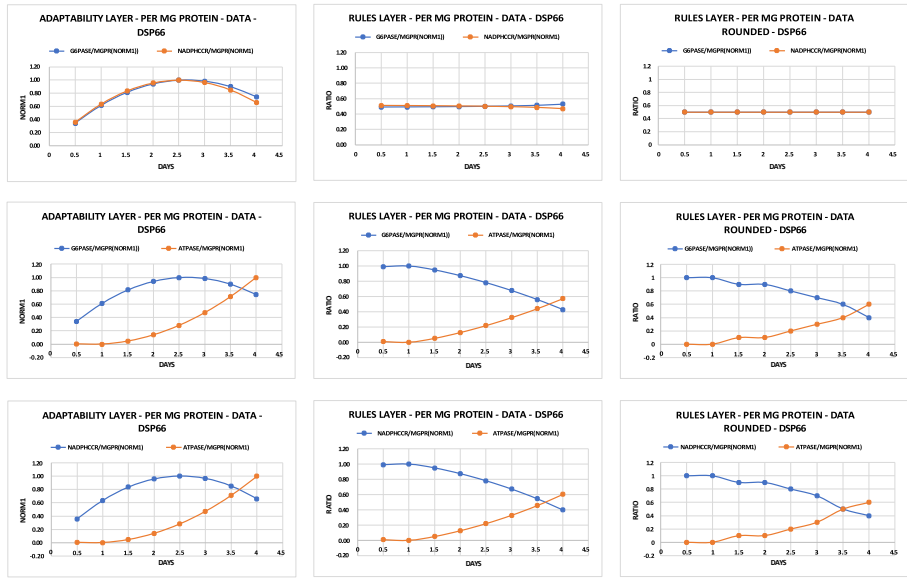
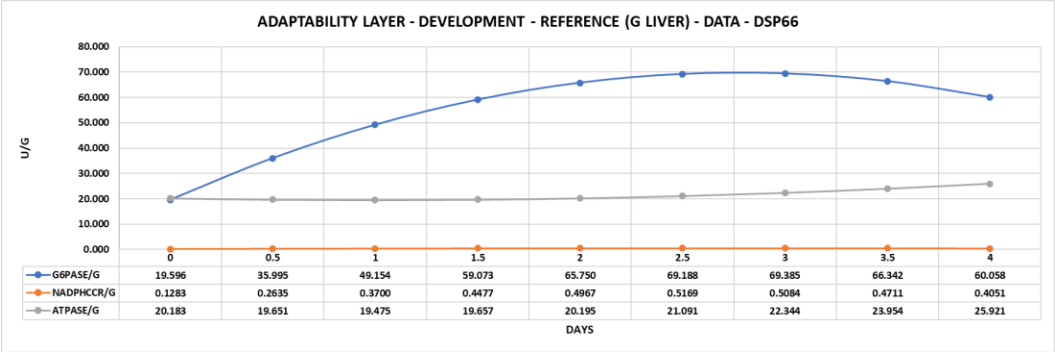


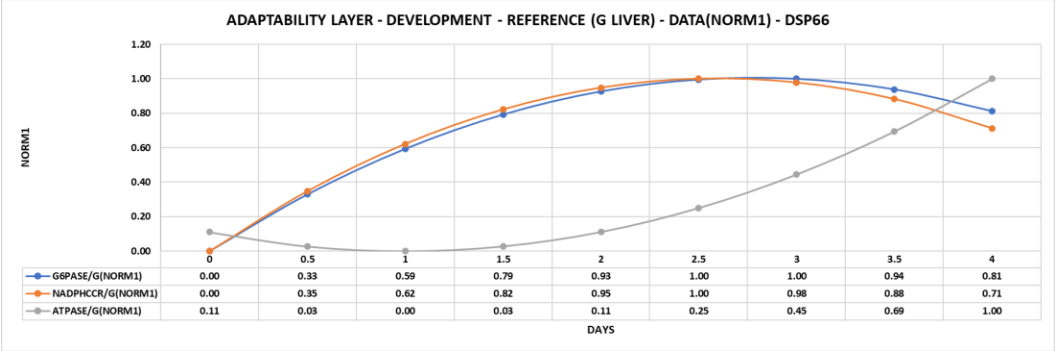
Figure 5.59 The first data pair (G6PASE vs NADPHCCR) showed the same rate of change (0.5:0.5), whereas the two remaining data pairs (G6PASE vs ATPASE, NADPHCCR vs ATPASE) displayed roughly similar patterns of change.

Next, we can repeat the calculations using the gram of liver reference (Figure 5.60). As expected, the results were similar because the protein per gram of liver showed little change.

Expanded Data (U/G LIVER)



Normalized Data (U/G LIVER-NORM1)



Adaptability and Rules Layers (U/G LIVER)

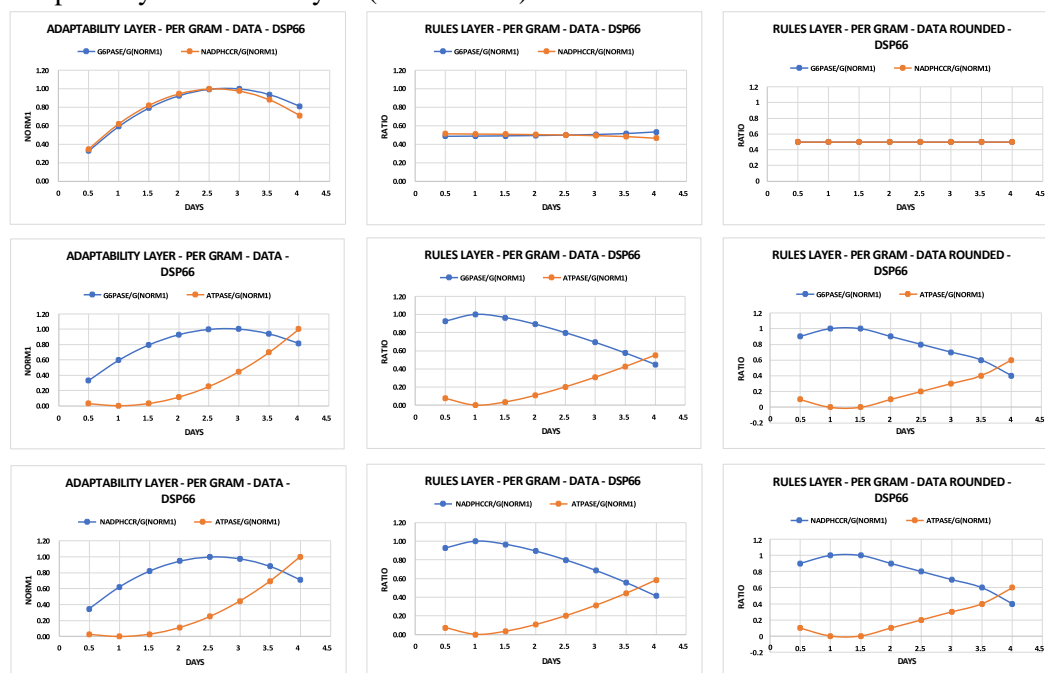


Figure 5.60 Changes in data pairs related to a gram of liver resembled those related to a mg of protein.

Although both liver references detected similar results, superimposing the curves gives a stronger test. The results in Figure 5.61 showed that both references gave reasonably similar results for the normalized data. However, mixing data references should not be part of an experimental design.

Adaptability and Rules Layers (U/MG PROTEIN VS U/G LIVER)

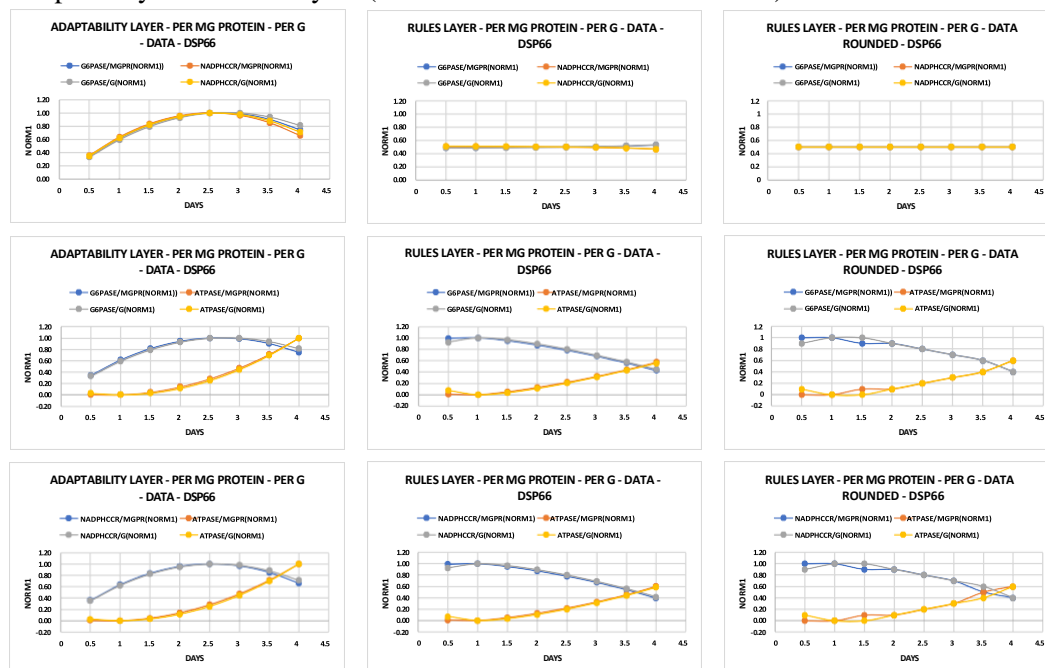


Figure 5.61 The test (superimposed curves) suggested that the mg protein and gram references can produce similar results when normalized. In this case, mixing the data references produced roughly comparable results.

5.3.7 Case Study 7: Development (Glucose-6-Phosphatase) – LSP71

Source: Update applied to original data from Leskes A., Siekevitz P., Palade G. E. (1971) Differentiation of endoplasmic reticulum in hepatocytes. Glucose-6-phosphatase distribution in situ. J Cell Biol 49: 264-287. ER membranes: Herzfeld et al., (1973);HFG73.

Topic: Pre and postnatal development of the hepatocytic ER using Glucose-6-phosphatase and ER surface area.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, calculate enzyme densities [$ED = [(U/G)/(S/G)]$], $ED-NORM1 = [(U/G-NORM1)/(S/G-NORM1)]$, $ED-NORM2 = [(U/G-NORM1)/(S/G-NORM1)] \rightarrow NORMALIZED$], analyze patterns, and report biological solutions (recipes).

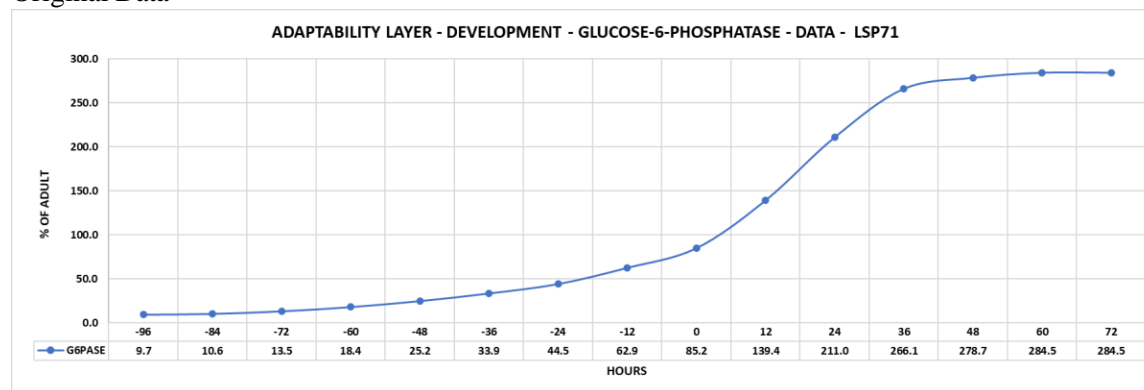
Dataset: Glucose-6-phosphatase activities and ER membrane surface areas.

The study follows the development of glucose-6-phosphatase during the pre and postnatal periods with data collected from liver homogenates and *nonfasted* animals (Leskes et al., 1971; LSP71); ER surface areas came from Hertzfeld et al., (1973; HFG73; *fasted* animals). The normalized enzyme densities (NORM2) used mixed references (U/g, ER(m²/cm³ hepatocyte cytoplasm) - see 5.3.10. We'll use this case study to compare four different estimates for enzyme densities to see what happens when we use mathematically incompatible data references. First, a brief description of the update.

ED = [(U/G)/(S/G)] = U/S: When reporting the results of an experiment, the change detected comes from two sources - biology and the methods used to measure the change. Recall that units of enzyme activity per gram of liver can change as well as the number of cells filling the gram. Given the expected differences in animals and methods across laboratories, reproducing enzyme densities based on data coming from the adaptability layer becomes challenging because of the many variables in play.

Normalizing data addresses this problem by removing the data units and converting the changes to just numerical values ranging from zero (representing the smallest value) to one (representing the largest). Although we can normalize enzyme activities and membrane surface areas separately and compare them globally, we also need enzyme densities to see how the hepatocytes change relationships of structure to function to solve problems. Since hepatocytes can change the enzyme activities and/or the membrane surface areas to change the enzyme densities, the relationships of each enzyme density to many others (the recipe) becomes the prized piece of information (the change). It appears that the best solution for the hepatocytes exists when they become the solution (expressed as a phenotype) and for the liver when it accommodates a new situation or reestablishes its original (normal) capacity. For both outcomes, success would seem to imply optimization of resources ranging from smallest to the largest parts (across the hierarchy of size). Figure 6.62 shows the original and expanded data.

Original Data



Expanded Data (Postnatal)

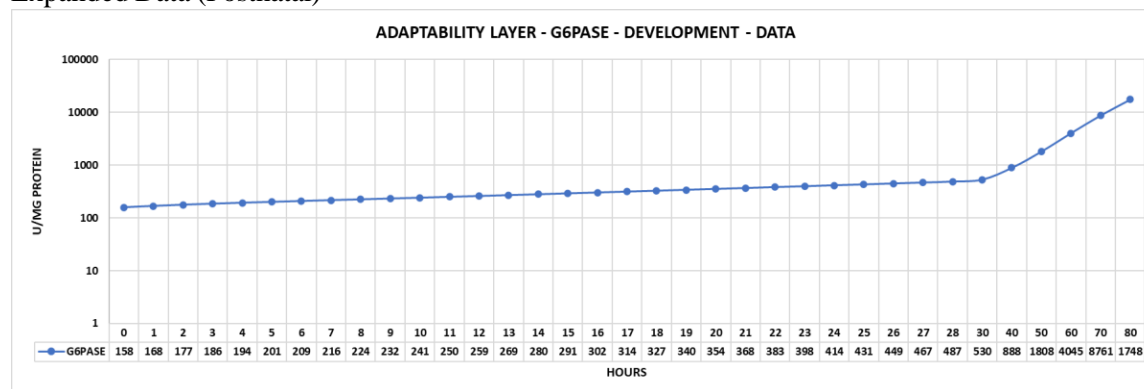


Figure 5.62 Glucose-6-phosphatase activity originally expressed as a percentage of the adult (original data) but related to a mg of protein (expanded data).

Since the enzyme data used the mg protein as a reference and the ER data a gram of liver, calculating an enzyme density using different references $[(U/mg \text{ protein})/(m^2/cm^3 \text{ cytoplasm})]$ doesn't work algebraically. By making the enzyme density calculations we're breaking the rules by dividing two incompatible data references merely to see what happens. For convenience, we'll focus on the first developmental event (hours 1 to 28), as shown in Figure 5.63.

$ED = [(U/MGPR)/ S/CM^3] \neq U/S \rightarrow \text{Dividing G6PASE/MGPR by } M^2/CM^3 \rightarrow \text{ERROR!}$

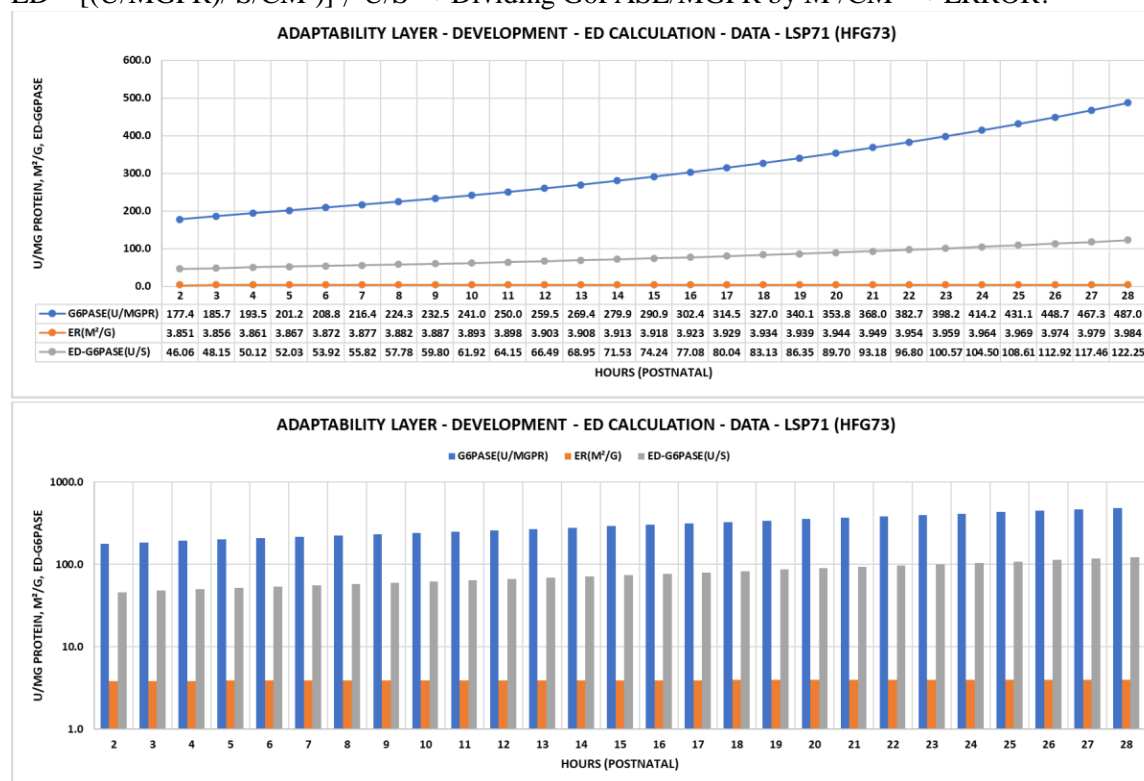


Figure 5.63 Calculating enzyme densities using incompatible enzyme and membrane data to generate incorrect results.

Next, in Figure 5.64 we'll normalize the G6PASE and ER data, calculate the EDs from the original enzyme activities (U/mg protein) and the membrane surface areas (S/cm³) and normalize the results (ED-G6PASE(NORM1)). Notice in the histogram that the G6PASE activity tracked with the enzyme densities.

Normalized Data (NORM1) $ED \neq [(U/mgpr)/(S/cm^3)] \rightarrow \text{NORMALIZED} \rightarrow ED(NORM1)$

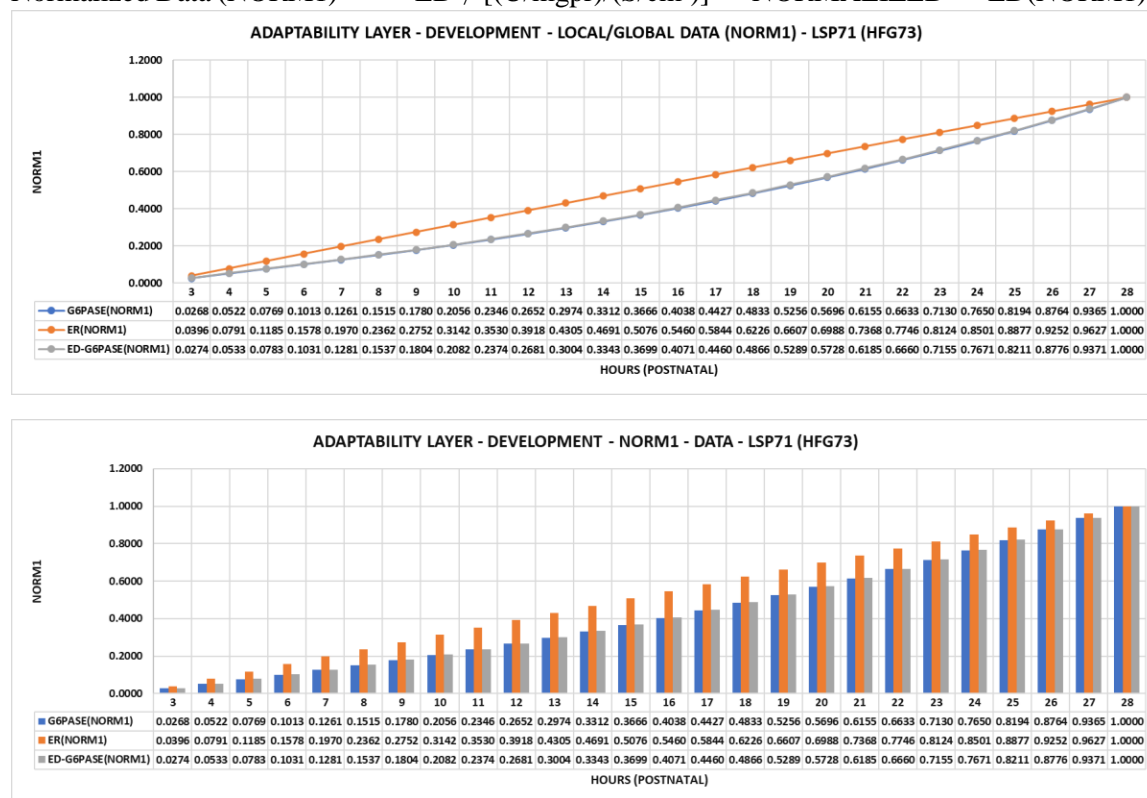


Figure 5.64 Normalizing the original data (G6PASE-NORM1, ER-NORM1). Calculating enzyme densities from the original data (G6PASE(U/mgpr), ER (m²/cm³), and normalizing the enzyme densities (ED-NORM1) produce incorrect results.

In Figure 5.65, we use the normalized enzyme and membrane data to calculate the normalized enzyme density (NORM2). In this case, we must assume that the results parallel those calculated with the correct data (U/g and S/g). The point of the exercise is to see what happens.

$ED-NORM2 = [(U/G-NORM1)/(S/G-NORM1)] \rightarrow \text{NORMALIZED}$

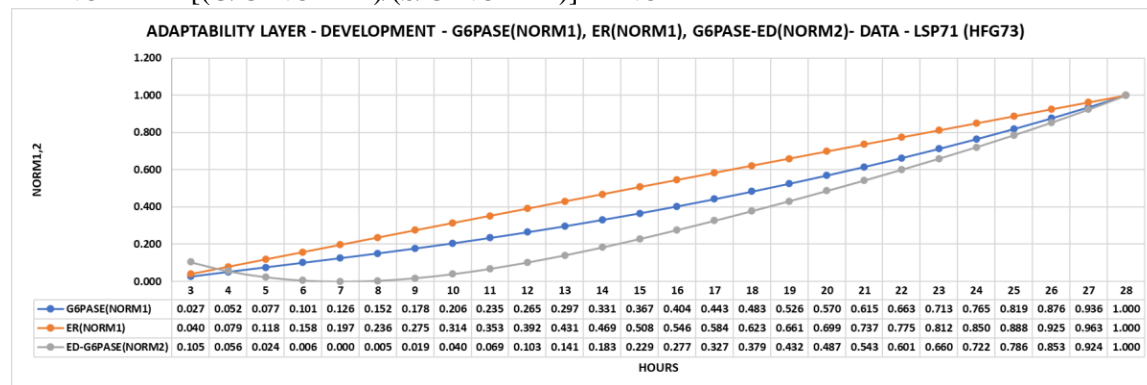


Figure 5.65 Normalized data (NORM1) used to calculate enzyme densities (ED-NORM2).

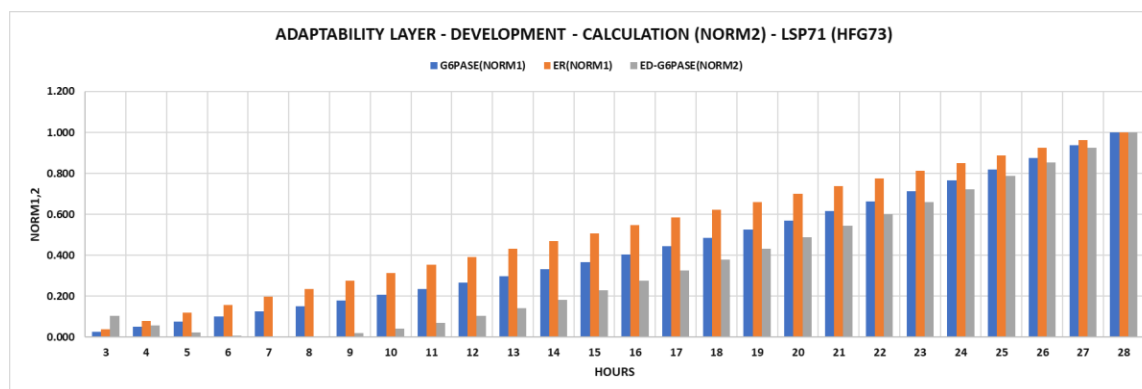


Figure 5.66 Normalized data used to calculate the enzyme densities. Changes displayed by the three data types converged at day twenty-eight. Recall that 1.0 identifies the maximum value.

We may have a problem, however, as shown in the figure below. The estimates of ED-G6PASE(NORM1) and ED-G6PASE(NORM2) don't agree. Why not? Because we calculated them from different starting points of the original data. How close were the changes detected with the NORM1 NORM2 enzyme densities? Figure 5.67 shows substantial differences and similarities.

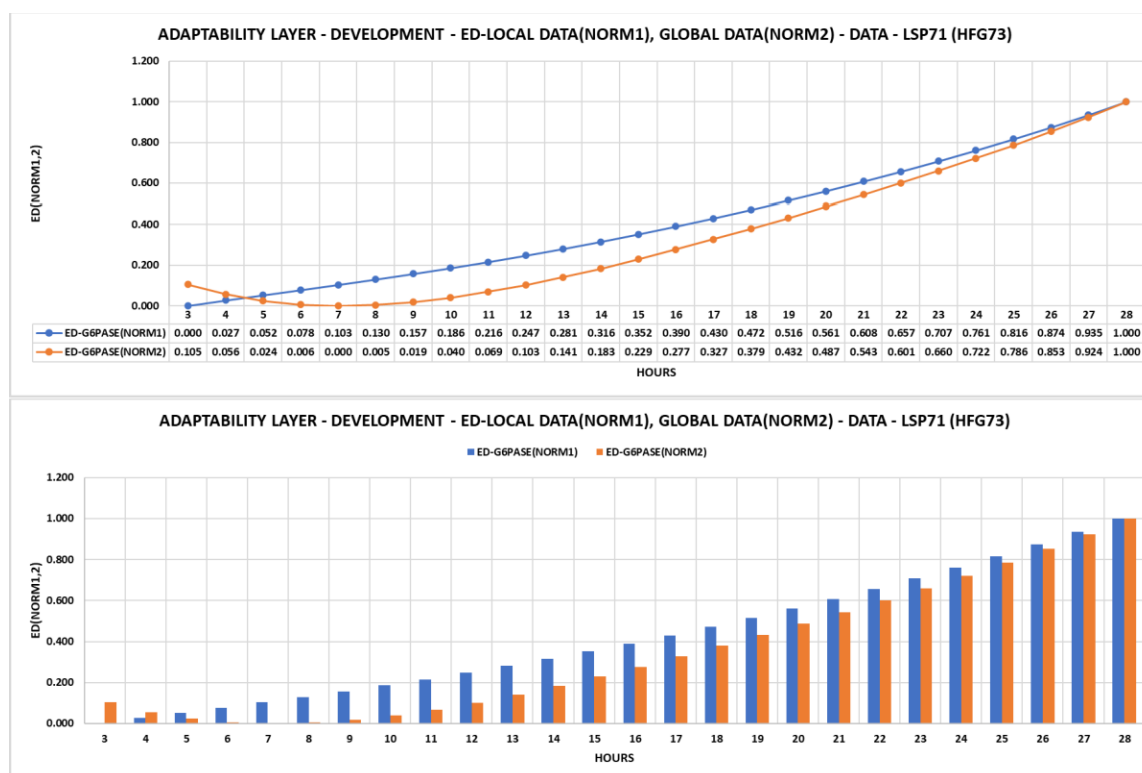


Figure 5.67 The enzyme densities calculated locally (ED-NORM1) differ from those calculated from normalized data (ED-Norm2).

Rules Layer: By shifting to the rules layer, we can see how the two EDs (ED(NORM1) vs ED(NORM2) shown in Figure 5.66 changed relative to one another by calculating their ratios (Figure 5.67).

PATTERNS [ED(NORM1) VS ED(NORM2)]

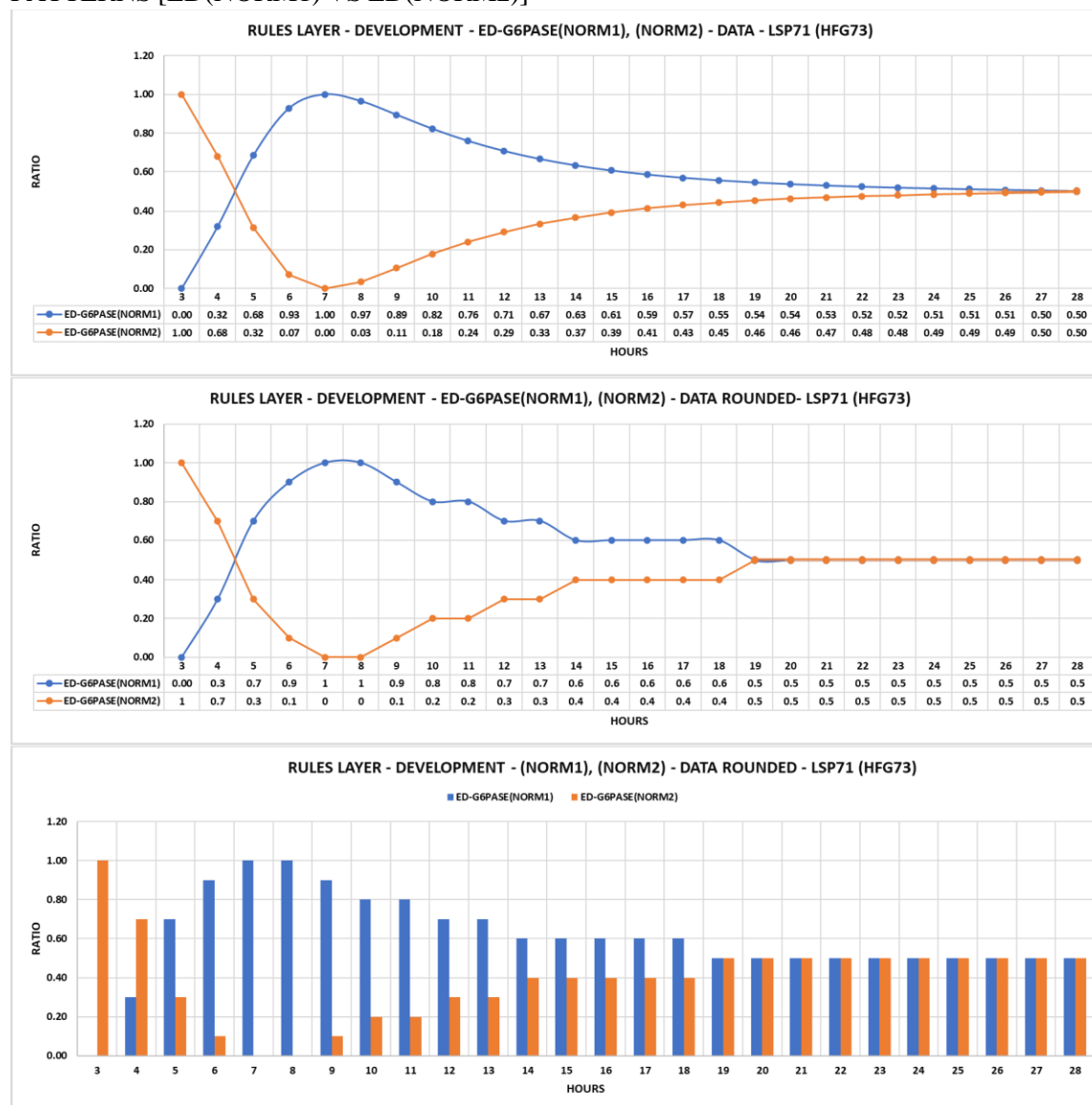


Figure 5.68 The relative changes in enzyme densities differed in amounts until hour nineteen when they became the same (data rounded). The original assumption that the mg protein reference paralleled changes related to a CM^3 was incorrect for days 3 \rightarrow 18 but correct for days 18 \rightarrow 28.

Comment: In other case studies, mixing some data references (e.g., U/g vs S/cm³ cytoplasm) worked consistently (detected parallel curves with data rounded), but not here. Mixing U/mg protein vs S/cm³ cytoplasm displayed parallel curves for the data rounded ratios less than half the time (hours 19 to 28). The point? When mixing references becomes the only option, use the ED-NORM2 estimates but run a backup test. The mg protein reference repeatedly carries the highest risk. By applying a first principles approach to biology, standardization soon follows and such tedious workarounds become unnecessary.

5.3.8 Case Study 8: Development (G-6-Phosphatase, Tyrosine Aminotransferase) - G69

Source: Update applied to original data from Greengard O. (1969) *The hormonal regulation of enzymes in prenatal and postnatal rat liver effects of adenosine 3',5'-(cyclic)-monophosphate*. *Biochem J* 115: 19-24. ER membranes: Herzfeld et al., (1973);HFG73.

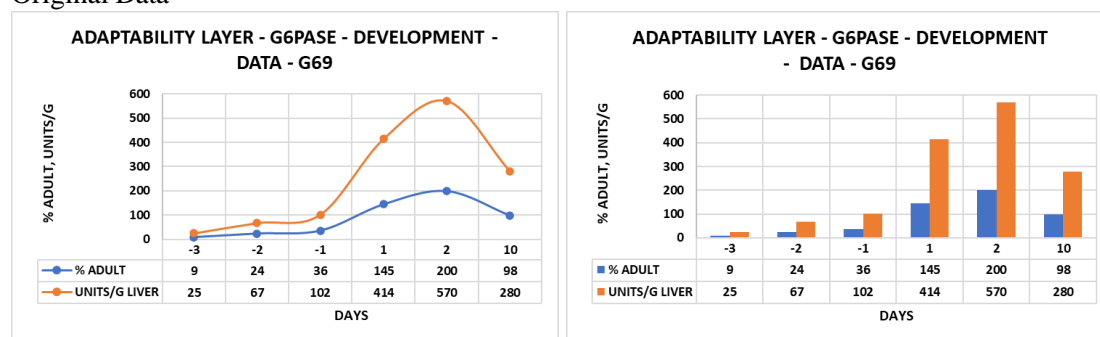
Topic: Enzyme activities (glucose-6-phosphatase, tyrosine aminotransferase) and ER surface areas during pre and postnatal development.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, calculate enzyme densities [ED(NORM2)], analyze patterns, and report biological solutions (recipes).

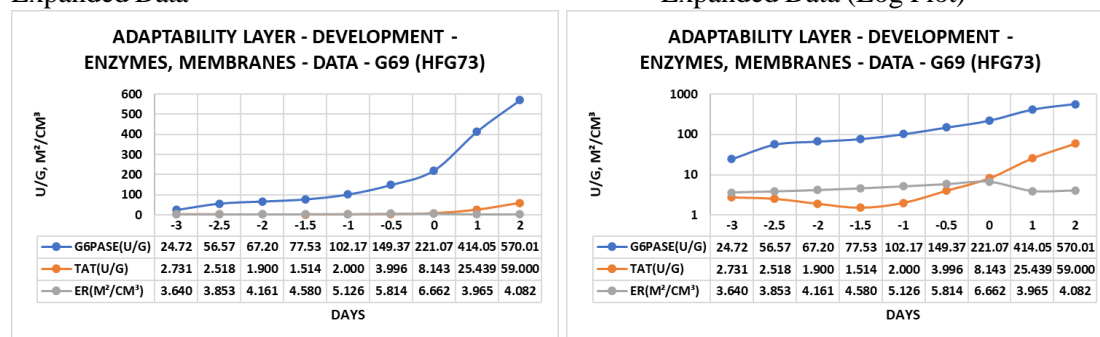
Dataset: Glucose-6-phosphatase, tyrosine aminotransferase, and ER surface area. The normalized enzyme densities (NORM2) used mixed references (U/G of liver, S/CM³ of hepatocyte cytoplasm) - see 5.3.10 and checks provided throughout the chapter.

Greengard (1969; G69) studied the development of glucose-6-phosphatase (G6PASE) and tyrosine aminotransferase (TAT) activities during pre and postnatal development. The update revisits these changes by normalizing the results and calculating enzyme densities using the ER surface areas published by Herzfeld et al., (1973;HFG73). Figure 5.69 includes the original, expanded, and normalized data.

Original Data



Expanded Data



Normalized Data (NORM1)

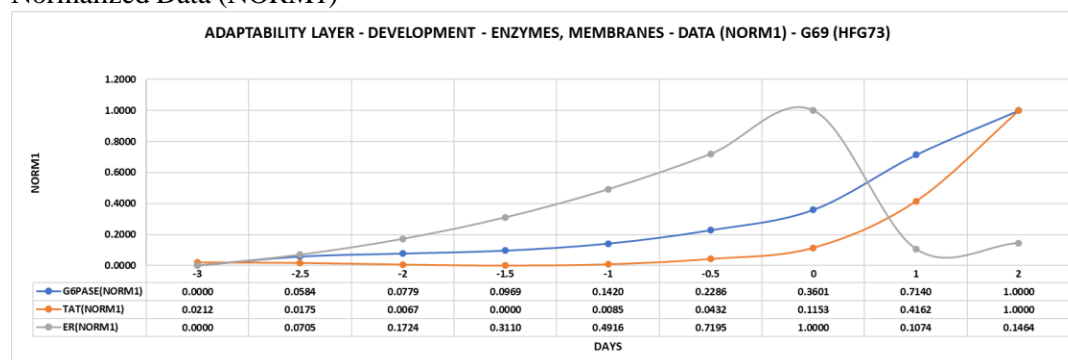


Figure 5.69 Original data, expanded, and normalized (NORM1). Notice that the data bins had different spacing.

In the rules layer (Figure 5.70), three normalized parts taken two at a time (G6PASE(NORM1), TAT(NORM1), ER(NORM1)) generated the following set of patterns.

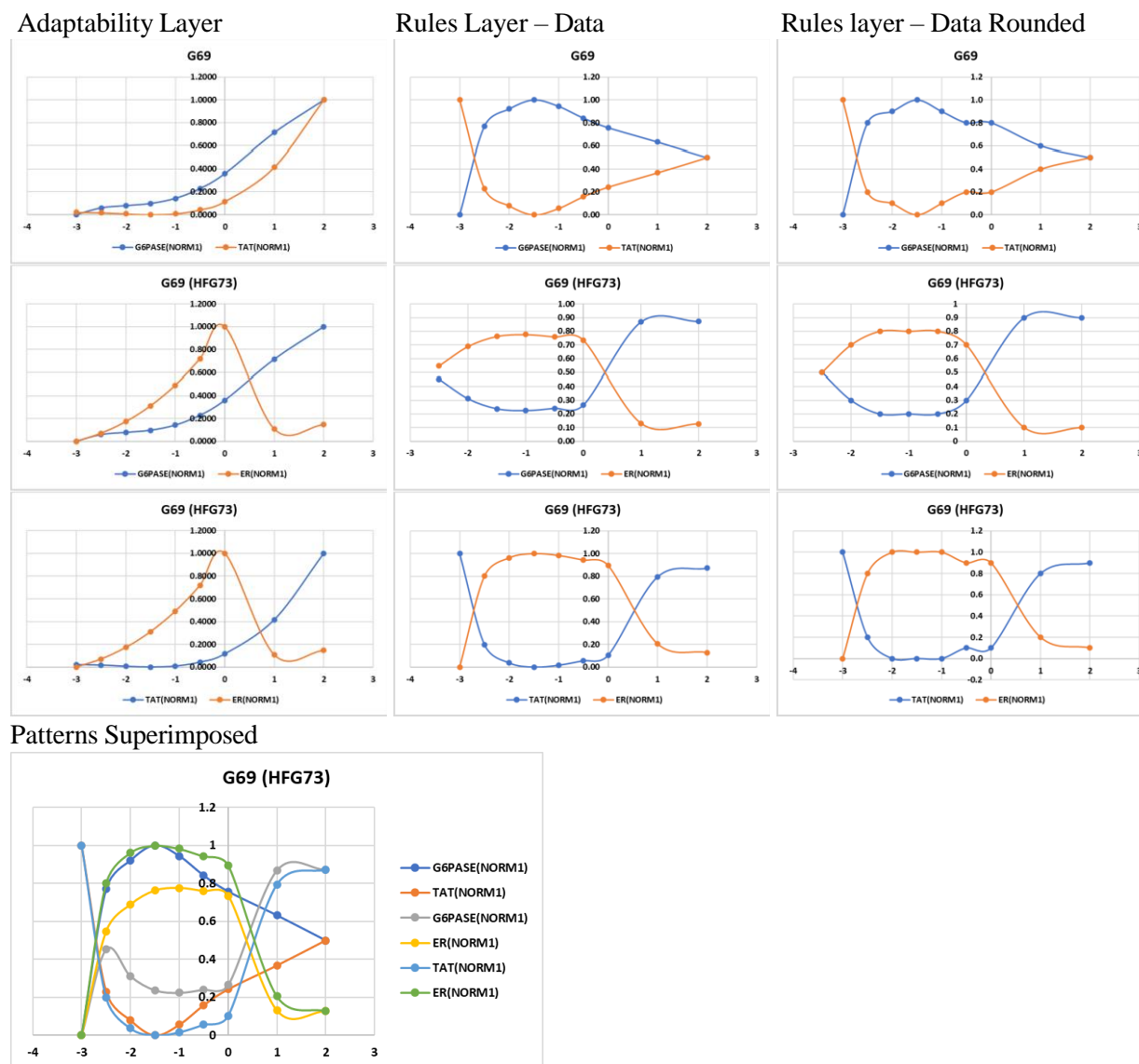
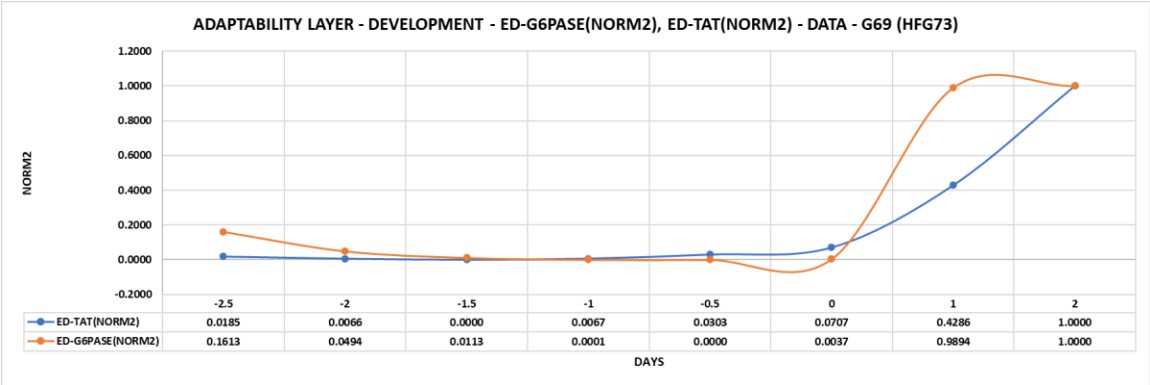


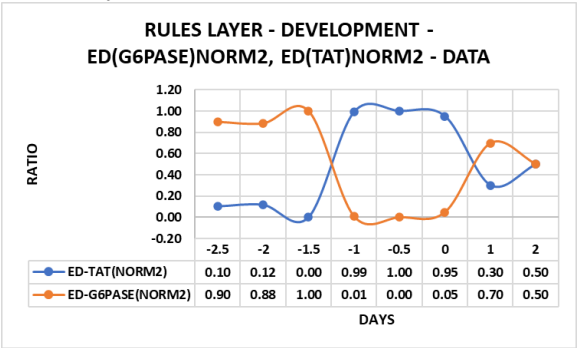
Figure 5.70 Changes in normalized data pairs all showed different patterns.

The normalized enzyme densities for G6PASE(NORM2) and TAT(NORM2) gave a pattern of largely independent changes, perhaps influenced by the smaller values characterizing TAT (Figure 5.71). Such a pattern would be consistent with enzymes belonging to different metabolic subgroups. Note that G6PASE serves as an ER marker enzyme whereas TAT occurs as a soluble enzyme in the hepatocyte cytoplasm. However, we can interpret changes in both the soluble (TAT) and membrane bound enzyme (G6PASE) as normalized enzyme densities because they share the same membrane reference (ER).

$ED-NORM2 = [(U-NORM1)/(S-NORM1)] \rightarrow NORMALIZED$



Rules Layer – Data



Rules layer – Data Rounded

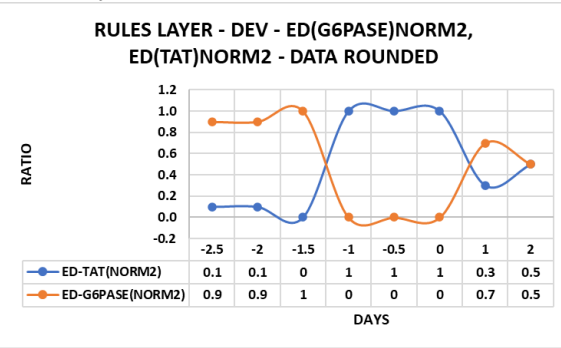


Figure 5.71 Normalized enzyme densities (NORM2) of TAT and G6PASE displayed distinct proportional changes, a pattern not suggested by the original data (Figure 5.62).

Comment: By referencing both soluble and membrane bound enzymes to the same membrane reference (ER), the interpretation and reproducibility of results improves because the two physically separated datasets become connected. Since the methods routinely turn data references into variables during a change, soluble enzymes need a constant reference just as much as the membrane bound enzymes.

5.3.9 Case Study 9: Development (Exposure of cAMP to Glucagon) – VHCC76

Source: Update applied to original data from Vinicor F., Higdon G., Clark J. F., Clar, C. M. Jr. (1976) Development of Glucagon Sensitivity in Neonatal Rat Liver. *J Clin Invest* 58: 571-578. ER membranes: Herzfeld et al., (1973);HFG73.

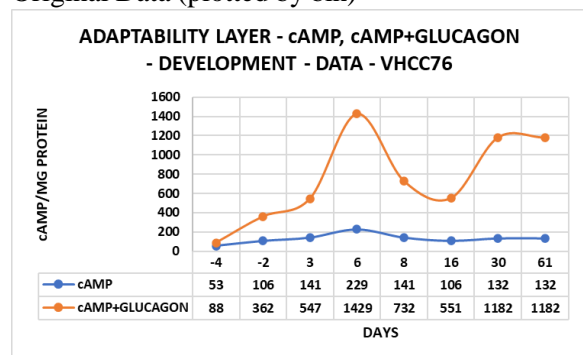
Topic: Enzyme activities (adenyl cyclase) and morphological membranes (ER) of hepatocytes during development including responses of cells to hormones.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, calculate enzyme densities [(ED = ED(NORM2)], analyze patterns, and report biological solutions (recipes).

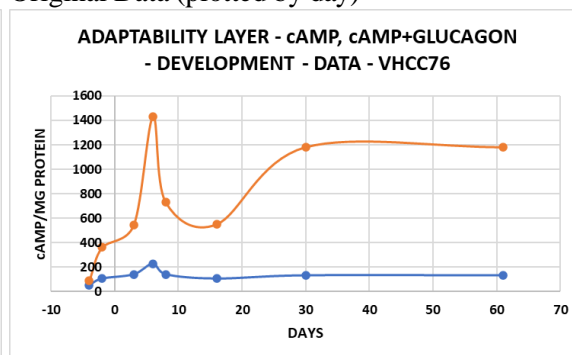
Dataset: Adenyl cyclase activities and ER surface area. The normalized enzyme densities (NORM2) used mixed references (U/mg protein and S/CM³ of hepatocyte cytoplasm) - see 5.3.10 and checks provided throughout the chapter.

By updating the study (Vinicor et al., 1976; VHCC76), we can follow the effect of glucagon on the formation of cAMP from adenyl cyclase activity. Figure 5.72 includes the original and expanded data.

Original Data (plotted by bin)



Original Data (plotted by day)



Expanded Data

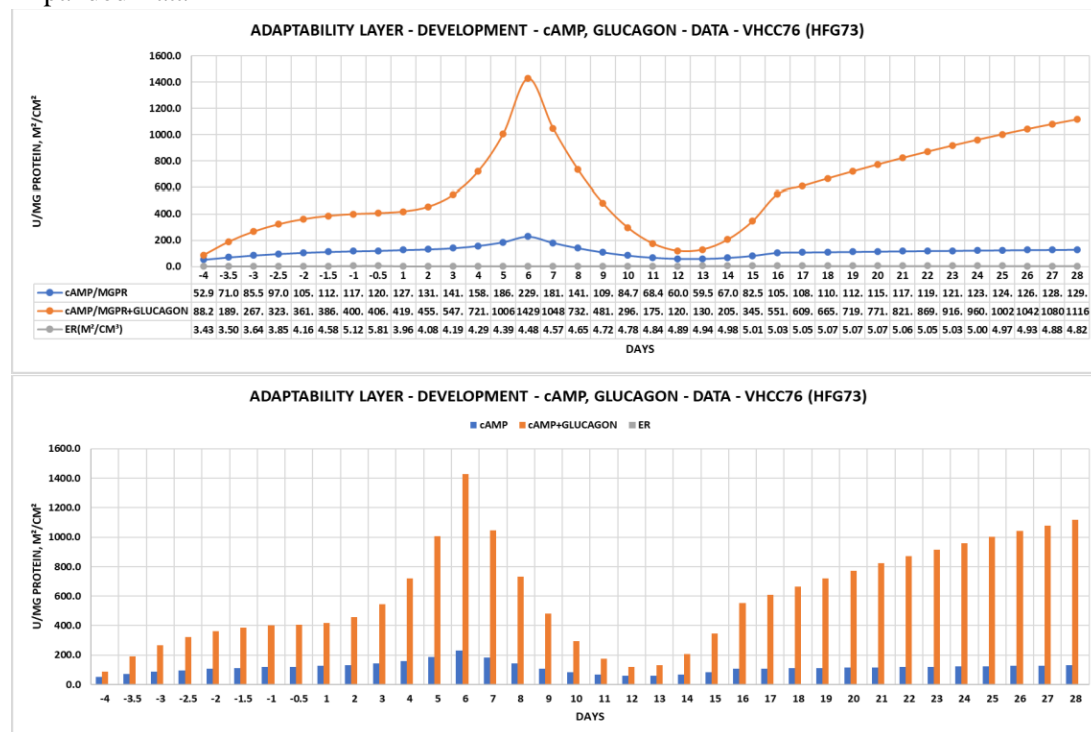


Figure 5.72 Original and expanded data show the effects of glucagon on the formation of cAMP from adenyl cyclase.

For this update, we have a mixed data reference problem but no workaround. We can deal with U/G vs M^2/CM^3 of hepatocyte cytoplasm, and u/mg protein vs cm^3 of hepatocyte cytoplasm when the protein content per g of liver remains constant, but not with just u/mg protein vs cm^3 of hepatocyte cytoplasm. To assume that the protein content per gram of liver remained constant throughout the developmental period was too high a risk. However, to assume a parallel relationship between the changes in the mg protein and gram references for the last three experimental days, reduced the risk enough to continue the calculations (see Figure 5.68). We can test the assumption somewhat by looking for duplicate data pairs containing ED-adenyl cyclase-NORM2 in the global developmental phenotype. Figure 5.73 includes the normalized data.

Normalized Data (NORM1)

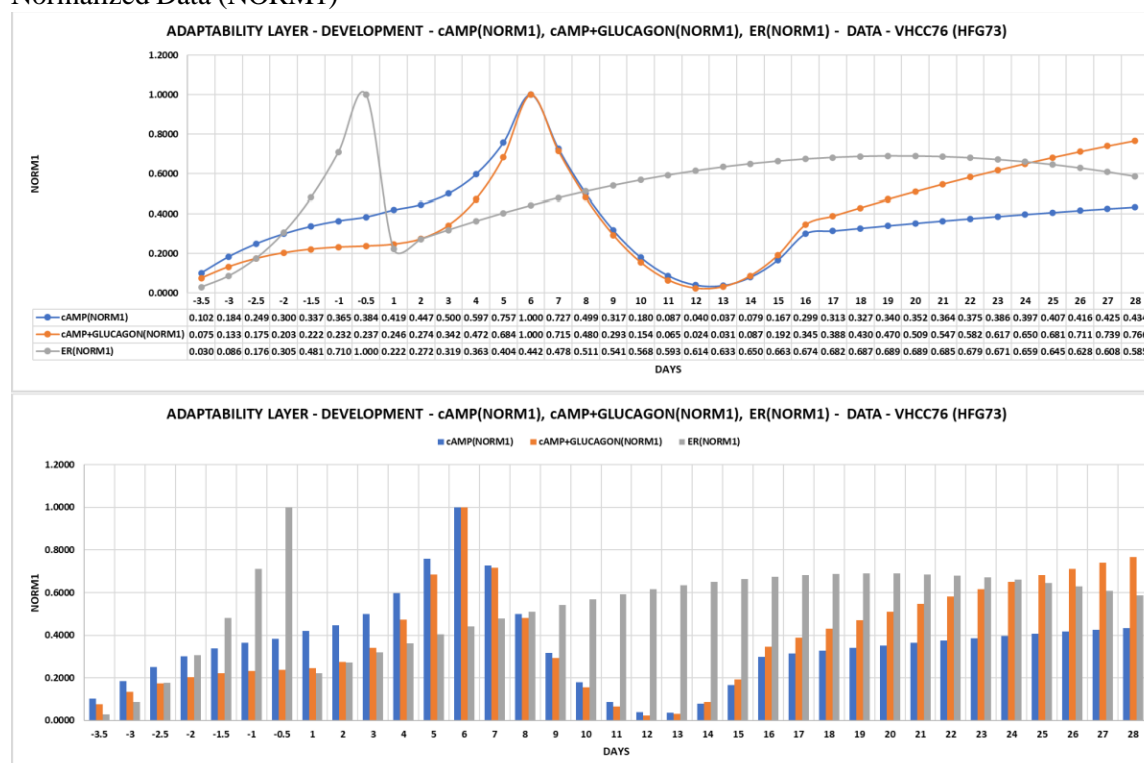


Figure 5.73 Normalized enzyme data (with or without glucagon) displayed both similar and different responses during pre and postnatal development. In any case, the cAMP+glucagon combination produced all possible results (\uparrow , \downarrow) during pre and postnatal development.

The normalized enzyme densities (ED-NORM2) in Figure 5.74 show the effect of glucagon on the enzyme activity. Starting at postnatal day four, glucagon began to influence the amount of cAMP associated with a unit of ER, decreased during days eleven to thirteen, and then increased steadily for the remainder of the experiment. The updated results show the effect of detecting and interpreting the same changes with local (Figure 5.73) and global data (Figure 5.74). The point? Hepatocytes change by changing complex relationships of structure to function.

$$\text{ED-NORM2} = [(U\text{-NORM1})/(S\text{-NORM1})] \rightarrow \text{NORMALIZED}$$

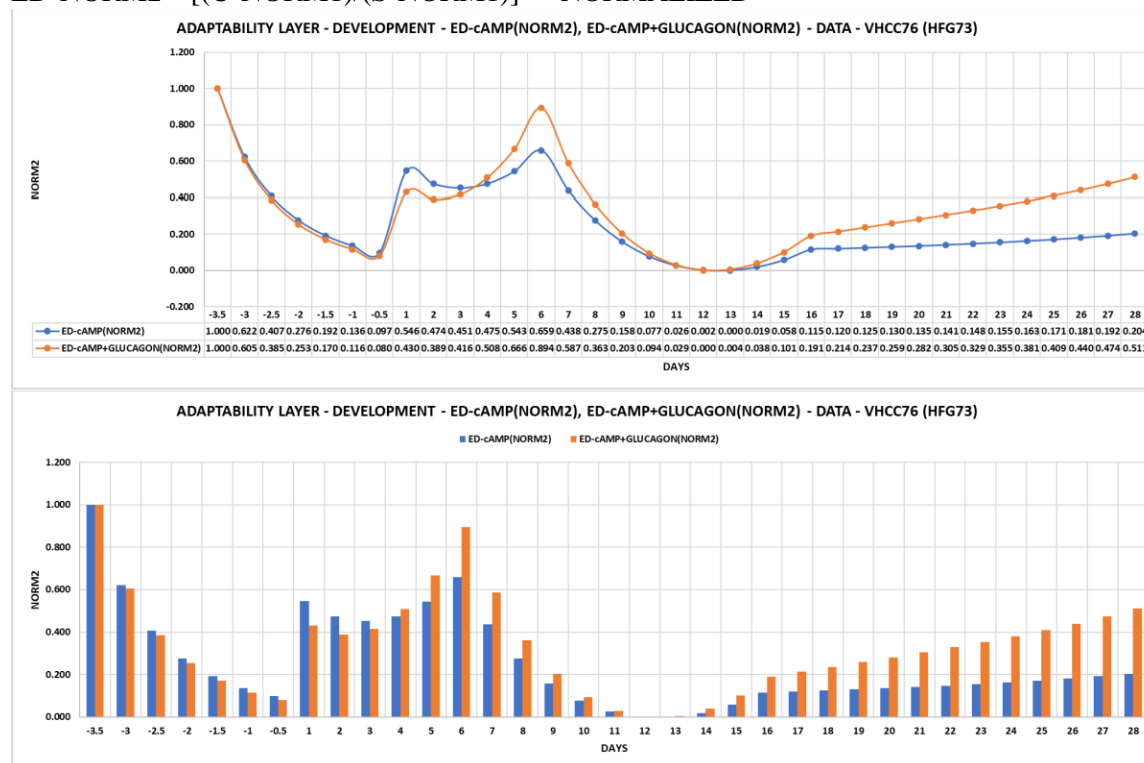
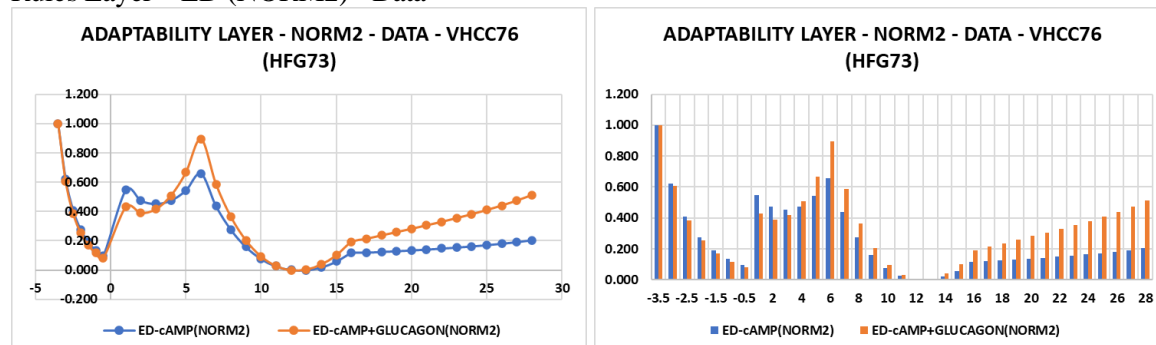


Figure 5.74 The normalized EDs (NORM2) revealed the effects of glucagon on the packing of enzyme activity in ER membranes during pre and postnatal development.

By moving the enzyme density data from the adaptability to rules layer, we can generate data pair ratios to identify the effects of the glucagon on cAMP (Figure 5.75). The Figure shows that cAMP increased with and without glucagon according to the ratio of 7:3. In other words, glucagon more than doubled the output of cAMP. However, the relationship between the two experimental states went from 0.5:0.5 (days -3.5 to -0.5) to 0.6:0.4 (days 5 to 9) to 0.7:0.3 (days 18 to 28). How does one interpret such results? The rounded data suggests the presence of a switching mechanism, wherein 0.5:0.5 = the off position, 0.6:0.4 = level 1 on position, and 0.7:0.3 = level 2 on position. Are the “switches” receptors, gene expression, or something else?

Rules Layer – ED (NORM2) - Data



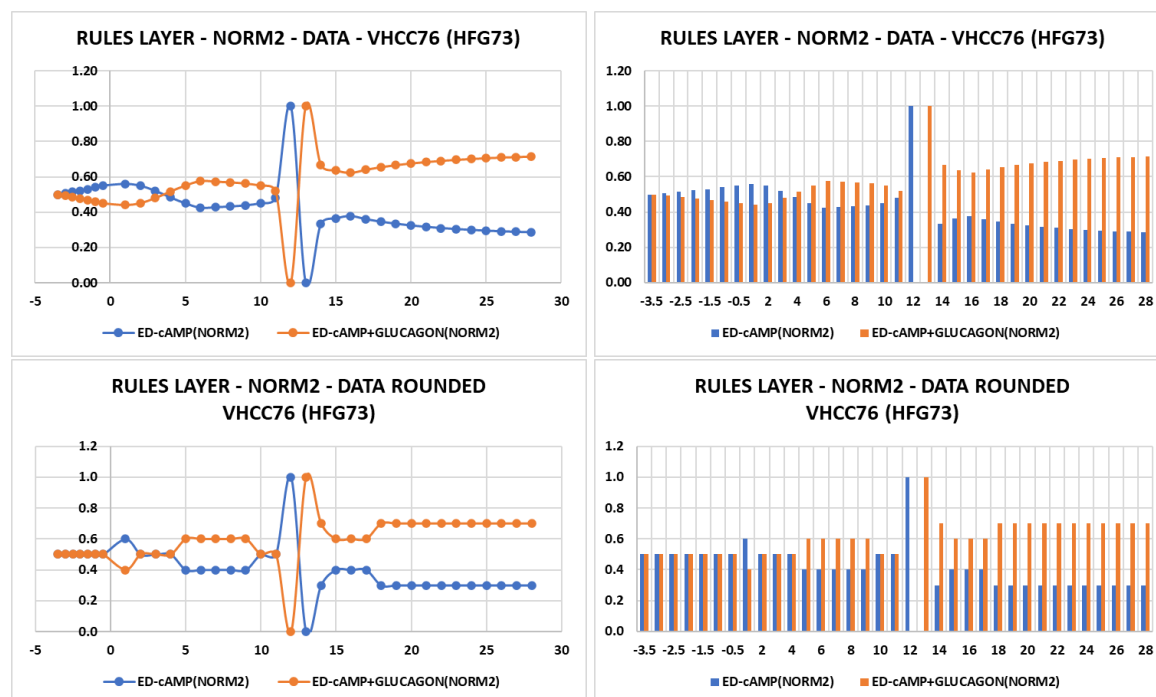


Figure 5.75 When analyzed as data pairs in the rule layer (data rounded), the enzyme densities detected a parallel pattern of change (days 18-28) for cAMP with and without glucagon (0.7:0.3). This differs from the results shown in Figure 5.72 that detected nonparallel changes in the adaptability layer. Changes in individual parts do one thing whereas changes in the relationship of one part to another do something else. The principal focus of the cells was on the sequential outcomes (the enzyme-membrane recipes) because they were working out solutions to the ongoing development.

Comment: Given the absence of a confirmed workaround for the mixed data references, one should treat the above results based on the enzyme densities with caution. Nonetheless, the results shown in Figure 5.75 provided an opportunity to see how one could interpret the increasing changes in the adaptability layer as ratios in the rules layer. By providing new information in the form of patterns, the rules layer contributes importantly to defining and understanding the complexities of a change.

5.3.10 Case Study 10: Development (Hexokinase and Glucokinase) – JG70

Source: Update applied to original data from Jamdar S.C., Greengard O. (1970) Premature formation of glucokinase in developing rat liver. J Biol Chem 345:2779-2738. ER membranes: Herzfeld et al., (1973);HFG73.

Topic: Development of enzymes (hexokinase, glucokinase) and membranes (ER) largely during postnatal development of the liver.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, calculate enzyme densities [ED(NORM2)], analyze patterns, and report biological solutions (recipes).

Dataset: Enzyme activities (hexokinase, glucokinase) and ER surface areas. The normalized enzyme densities (NORM2) used mixed references (U/G of liver, S/CM³ of hepatocyte cytoplasm) - see 5.3.10 and checks provided throughout the chapter.

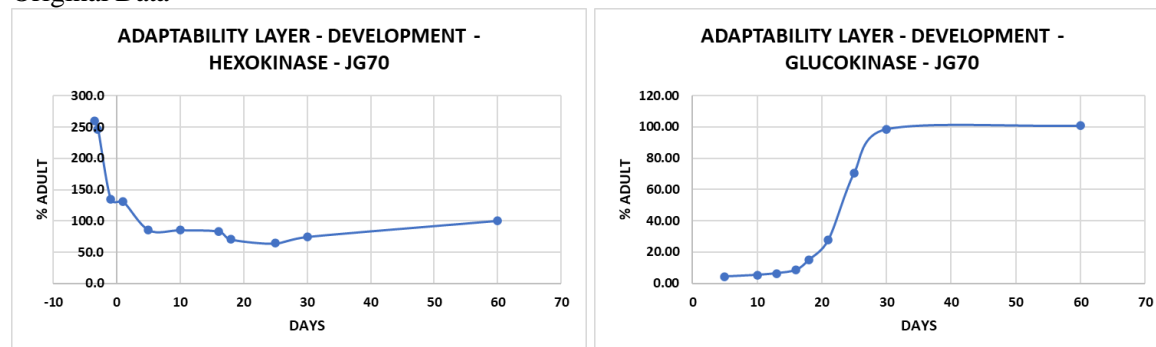
Recall that the challenge when updating published data includes generating patterns that we can use to detect changes, apply biology's rules, and unravel - empirically - the way biology solves problems. This involves two strategies, one for publishing new research data (local) and another for combining data from many publications (global).

When hepatocytes change, they apply a tactic that allows them to solve a problem by changing the mix of biochemical activities and membrane surface areas to arrive at a solution (a new membrane recipe). In effect, a change solves problems by redefining relationships of structure-function in cells, which extends to an organ as a new capacity. This means that detecting and interpreting a change in hepatocytes requires two results, one occurring in the cells and the other in the liver.

But how do we detect changes in these two soluble enzymes (HEXOKINASE, GLUCOKINASE) when practically everything becomes a variable in an experimental setting? If we relate glucokinase activity to a constant amount of ER (one meter squared) and do the same for hexokinase, can we detect and interpret the way they change in both the adaptability and rules layers? Yes.

Starting with the originally published data, we'll follow the developmental changes in these two enzymes associated with carbohydrate metabolism. Specifically, we want to see how they change when related to the same data reference (the ER) before (Figure 5.76) and after (Figure 5.77) normalization.

Original Data



Expanded Data (Days 4 to 28).

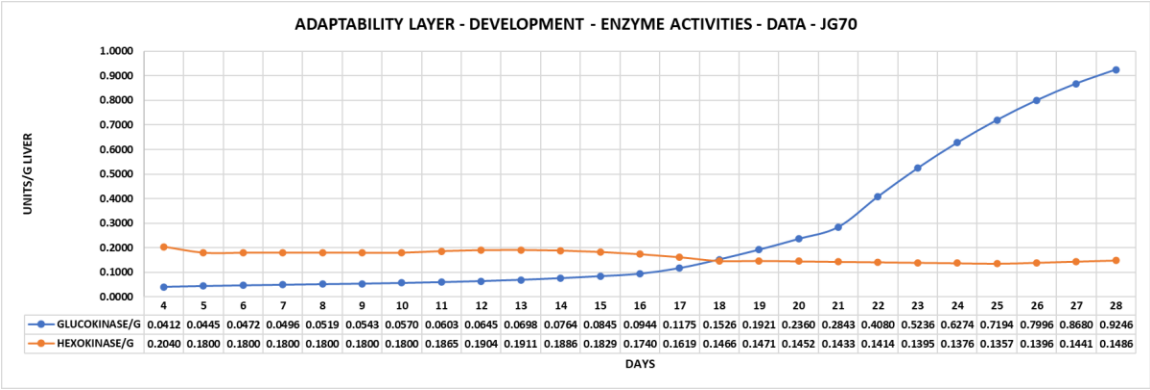
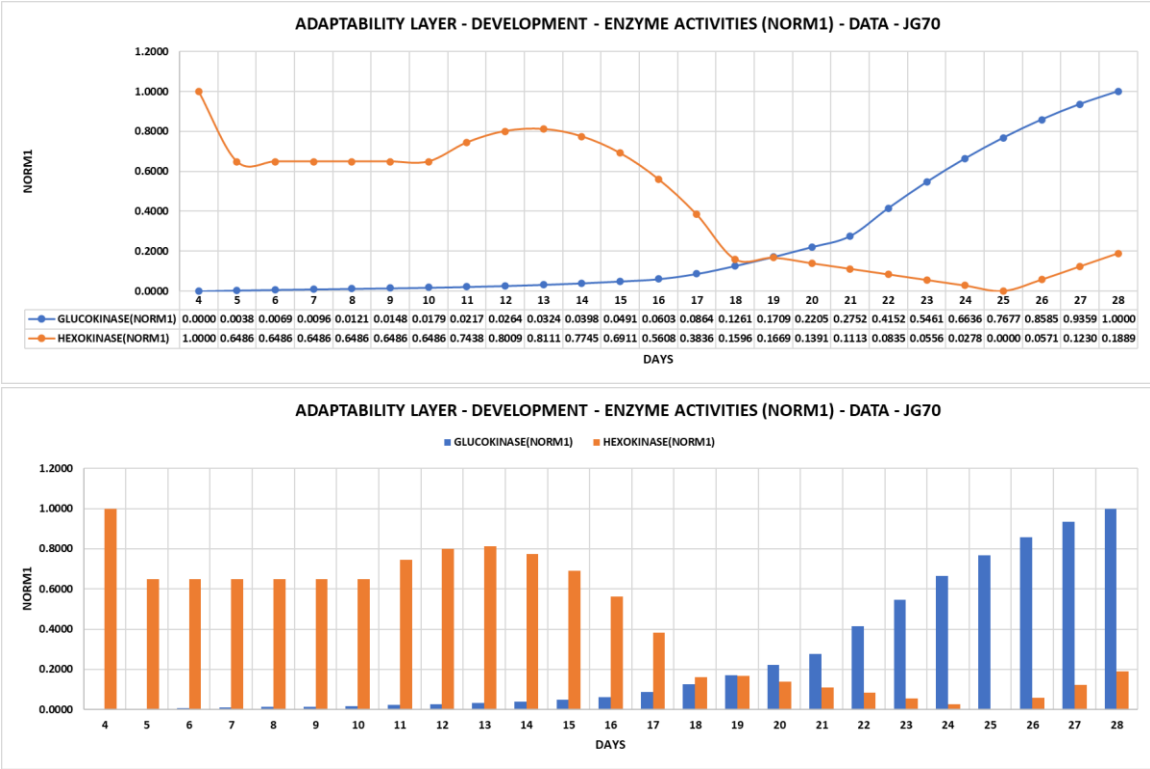
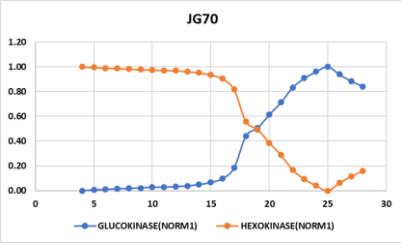


Figure 5.76 Expanded data expressed as units of activity related to a gram of liver.

Normalized Data (NORM1)



Rules Layer – (NORM1) - Data



Rules Layer - (NORM1) - Data Rounded

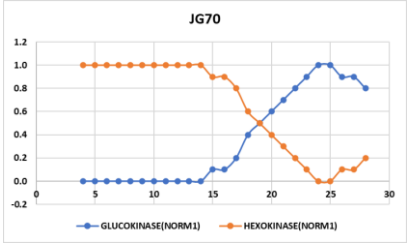


Figure 5.77 Normalized data (NORM1) show the relationships of the changes in the adaptability and rules layers. As one enzyme increased or decreased its activity the other did the opposite – proportionately. While the changes viewed in the adaptability layer appeared nonsymmetrical, the same changes viewed in the rules layer as data pairs became symmetrical. By

employing this strategy, cells can maintain the constancy of rules while at the same time allow for environmentally driven variability. In effect, cells have figured out how to solve problems by creating paradoxes. In this example, the same change can be both a variable (multiple solutions in the adaptability layer) and a constant (single solution in the rules layer) at the same time. By copying this strategy, one can, for example, reverse the chaos created by our experimental methods by assembling the enzyme density as a paradox – a data reference that can change and remain constant at the same time.

Enzyme Density (NORM2): When expressed as enzyme densities, the postnatal development of glucokinase activity showed a decrease and hexokinase an increase (Figure 5.78).

ED-NORM2 = [(U-NORM1)/(S-NORM1)] → NORMALIZED

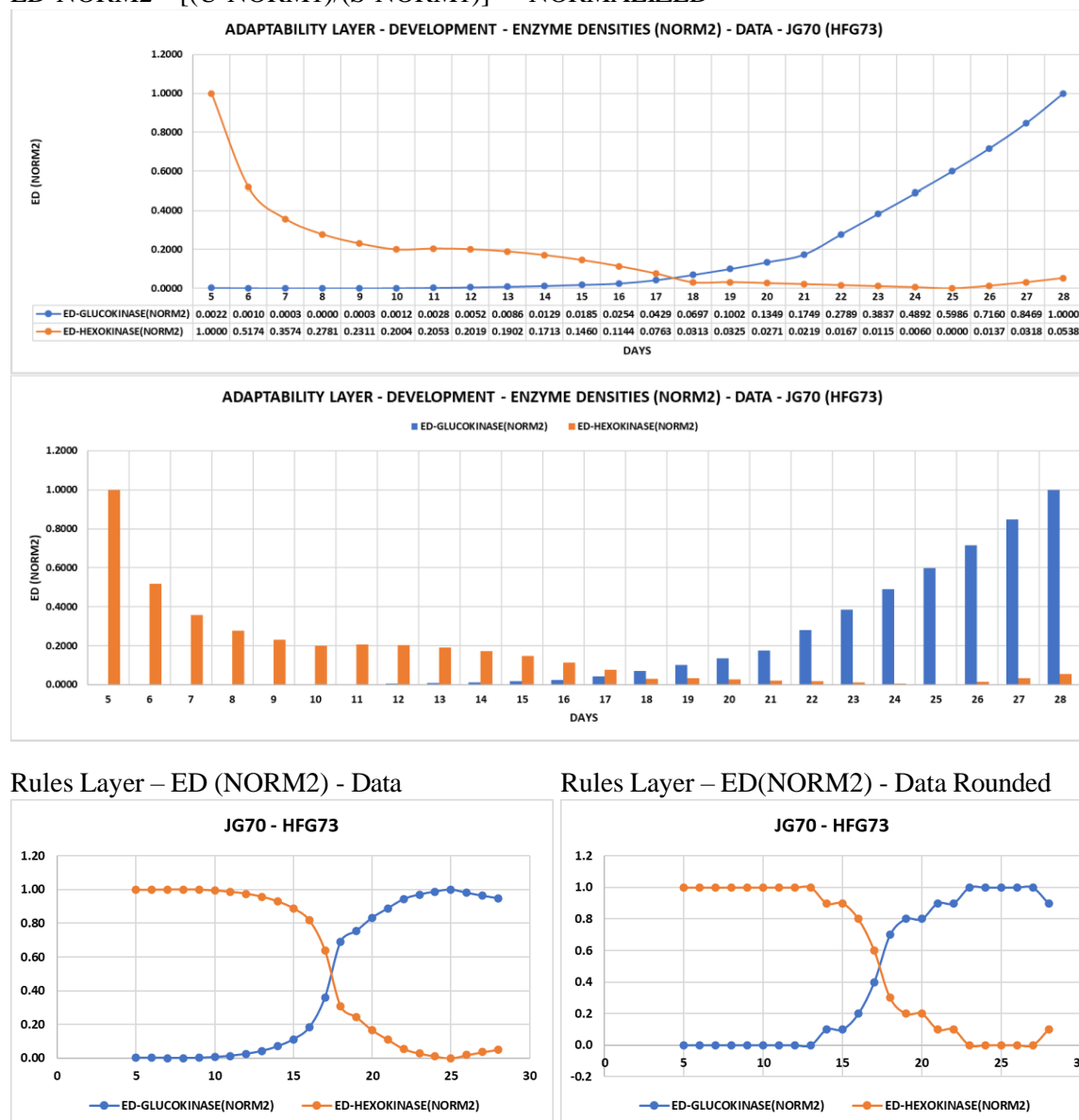


Figure 5.78 The normalized enzyme densities related two soluble enzymes (glucokinase and hexokinase) to the ER membrane. In effect, we can interpret these soluble enzymes the same way we interpret membrane-bound marker enzymes by linking them to a structure (ER). Moreover, it provides a strategy for comparing (and linking) changes in soluble enzymes to those membrane bound.

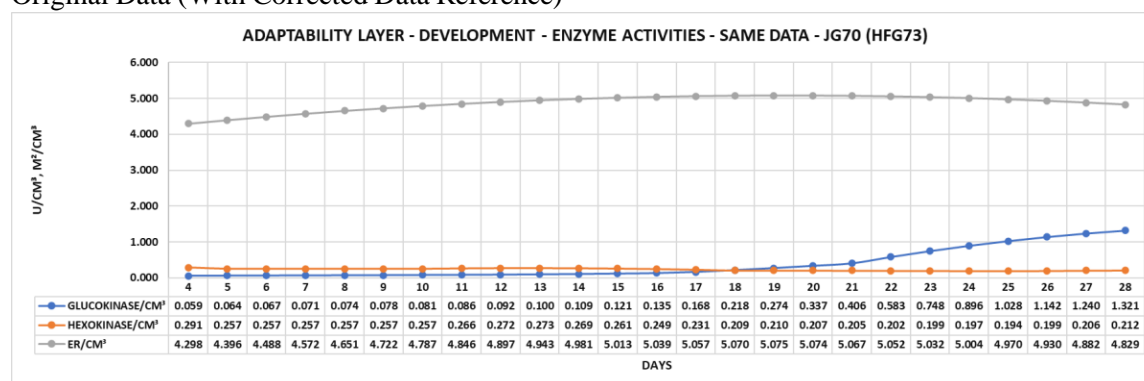
Mixed vs Same Data References: The enzyme densities included in Figure 5.78 were based on mixed data references (U/g vs m^2/cm^3 hepatocyte cytoplasm). If, however, we change the biochemical reference from a gram of liver to a cubic centimeter of hepatocyte cytoplasm, then the references will be the same and cancel out correctly. If we recalculate the enzyme densities [ED(NORM2)] based on the same data references, will the results be the same as those based on the mixed data references? We can answer the question empirically by making the two calculations.

To change a gram of liver into a cubic centimeter of hepatocyte cytoplasm, we can multiply the gram reference by a correction factor to make the conversion. Such a correction first converts a gram into a cubic centimeter, using the liver density (ρ): $\rho = \text{Weight (liver)}/\text{Volume (liver)} = 1.07 \text{ g}/\text{cm}^3$, and then multiplies the result by proportion of the liver consisting of hepatocyte cytoplasm ($V_{\text{HECY}}/V_L = 0.65$ or 65%). Multiplying a gram of liver by 0.7 (the combined conversion factor) and recalculating the enzyme activities completes the transition of the reference from gram of liver to cubic centimeter of hepatocyte cytoplasm.

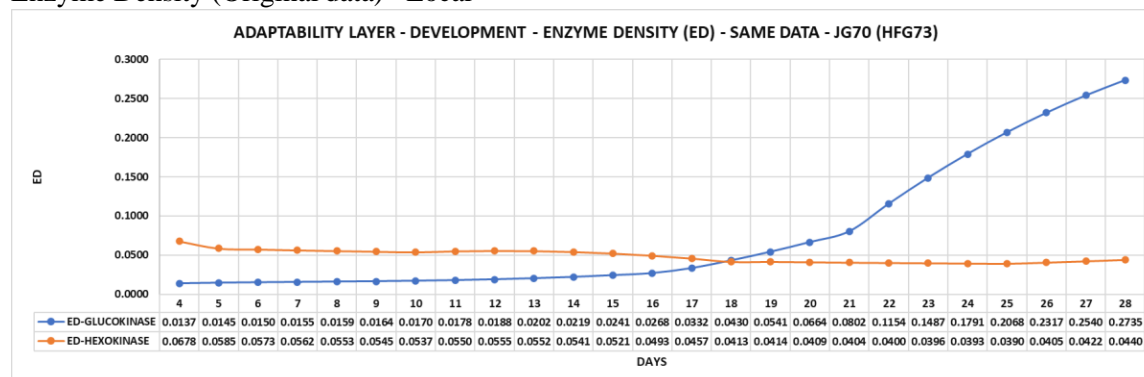
Recall that density (ρ) equals the weight (W) per unit volume (V): e.g., $\rho = W/V$ and $V = W/\rho \rightarrow V = W/(\approx 1.07 \text{ grams}/\text{cm}^3)$.

Same References (M^2/CM^3 hepatocyte cytoplasm): The enzyme activities and membrane surfaces were both related to a cm^3 of hepatocyte cytoplasm. Note that the data reference conversions assumed that the proportion of the liver attributed to the hepatocyte cytoplasm remained constant at 65% during the experiment (perhaps a generous assumption particularly for the earliest time points). First, we'll calculate the local enzyme density using the corrected data reference (Figure 5.79).

Original Data (With Corrected Data Reference)



Enzyme Density (Original data) - Local



Rules Layer

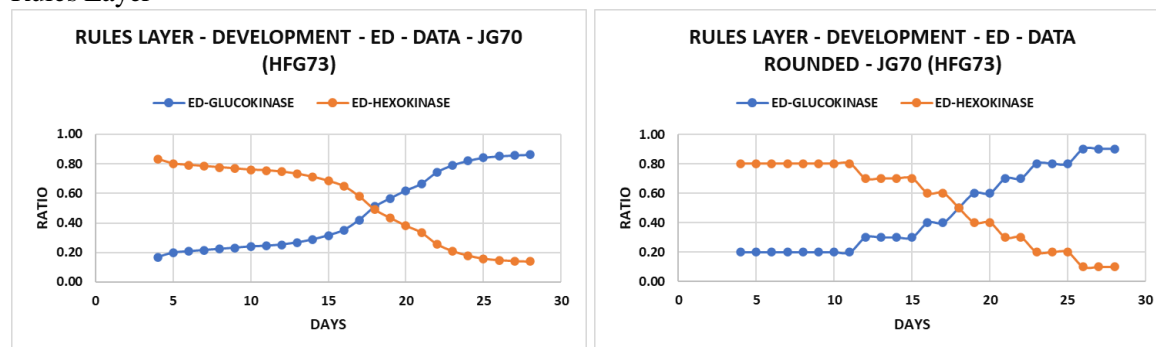


Figure 5.79 The expanded data related to the same reference (CM^3 of hepatocyte cytoplasm) and used to calculate local enzyme densities. When expressed as ratios, the changes in the two enzymes produced the expected changes.

By normalizing the data from the *mixed* (U/g vs S/cm^3) and *same* references (U/cm^3 vs S/cm^3) and calculating enzyme densities (ED-NORM2) therefrom, we can compare the results. A successful outcome would have found no difference between the two estimates [mixed(M) vs same (S)]. Unfortunately, this was not the case. The outcome was successful for hexokinase but not for glucokinase (Figure 5.80).

The Test

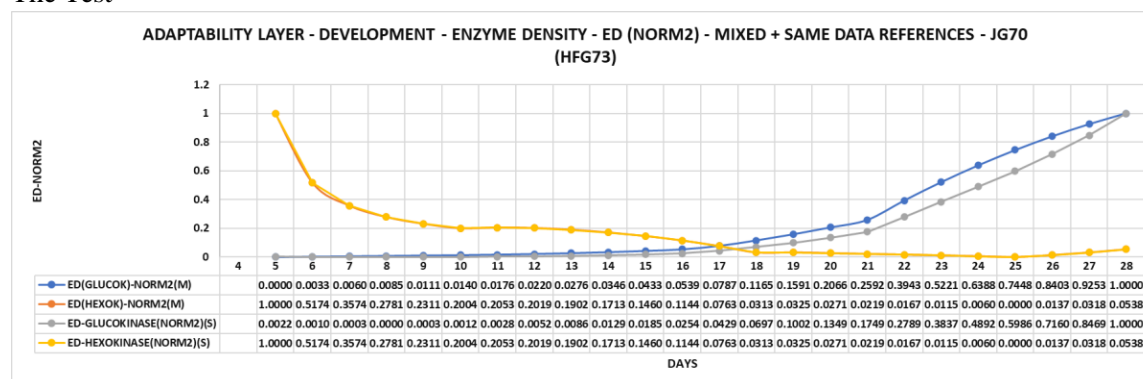


Figure 5.80 After normalizing the data and calculating the enzyme densities, the mixed (M) and same (S) datasets worked as expected for hexokinase but not for glucokinase. Why? When normalizing data, outliers can appear and change outcomes. If we go back to the NORM1 plot, the plot at day four looks suspicious. By removing the day four data and recalculating the results, we can rerun the test.

For the mixed data references at day four, the normalized data included the maximum (1.0 for hexokinase) and minimum values (0.0 for glucokinase). When used to calculate the enzyme density from the normalized data (ED-NORM2), this led to dividing values by zero. Plotting the results starting with day six solved the problem (Figure 5.81). Now both data references - mixed (M) and same (S) - produced identical results for the enzyme densities when calculated from normalized data.

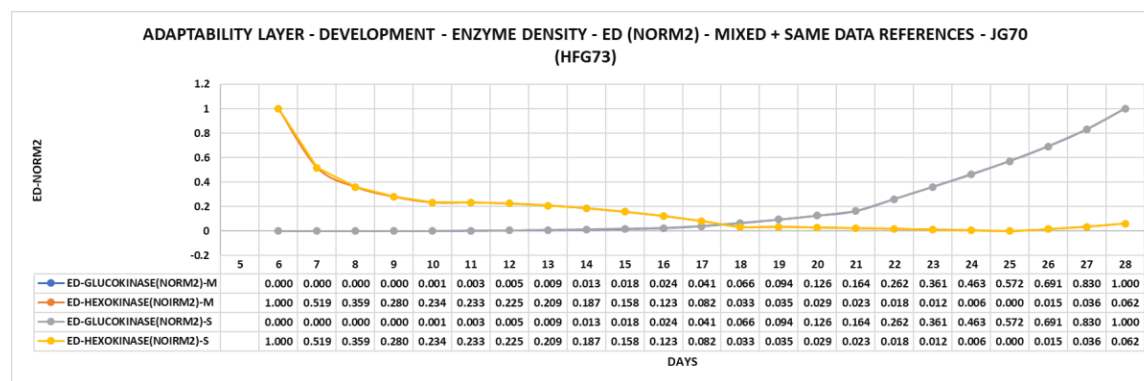


Figure 5.81 Both the mixed and same references passed the test. This supports the view that mixed and same data references can produce the same normalized results. Although this empirical confirmation applies to mixing GRAMS and CM³s, mixing the mg protein and CM³ references may require an assumption or additional information (e.g., mg protein per gram of liver).

Summary: Testing the validity of mixed references when detecting changes became a necessity because many case studies used morphological results related to a cm³ of hepatocyte cytoplasm and biochemical results to a gram of liver. By changing the biochemical reference from a gram of liver to a cm³ of cytoplasm, and running the above compatibility tests, the problem found a solution. The point? We can use mixed references to estimate enzyme densities with normalized data (ED-NORM2) but calculating local enzyme densities (ED-NORM1) from the original data always requires the same data reference. In practice, publications wishing to demonstrate the reproducibility of their results should compare ED-NORM2 changes.

5.3.11 Case Study 11: Development (Phosphoserine) – JG69

Source: Update applied to original data from Jamdar S. C., Greengard O. (1969) Phosphoserine phosphatase: Development formation and hormonal regulation in rat tissues. Arch Biochem Biophys 134:228-232. ER membranes: Herzfeld et al., (1973);HFG73.

Topic: Enzyme activities (phosphoserine) and ER surface areas during developments. Update: Apply corrections, expand data, report results in the adaptability and rules layers, normalize data, and calculate enzyme densities [(ED = [(U/G)/(S/G)]; ED = [(U/G)/(S/G))(NORM1); ED(NORM2)].

Dataset: Phosphoserine activities and ER surface areas. The normalized enzyme densities (NORM2) used mixed references (U/G of liver, S/CM³ of hepatocyte cytoplasm) - see 5.3.10 and checks provided throughout the chapter.

For this study of Jamdar and Greengard (1969;JG69), we'll update the phosphoserine activity during pre and postnatal development by normalizing the data and estimating enzyme densities (using the ER data of Herzfeld et al., (1973;HFG73). These results will become part of the global phenotype summarizing changes during early development. Figure 5.82 illustrates a graphing issue which, when necessary, one can avoid by expanding the dataset. Figure 5.83 didn't match the original data plot because the time points were not uniform (half-day vs one day).

Original Data

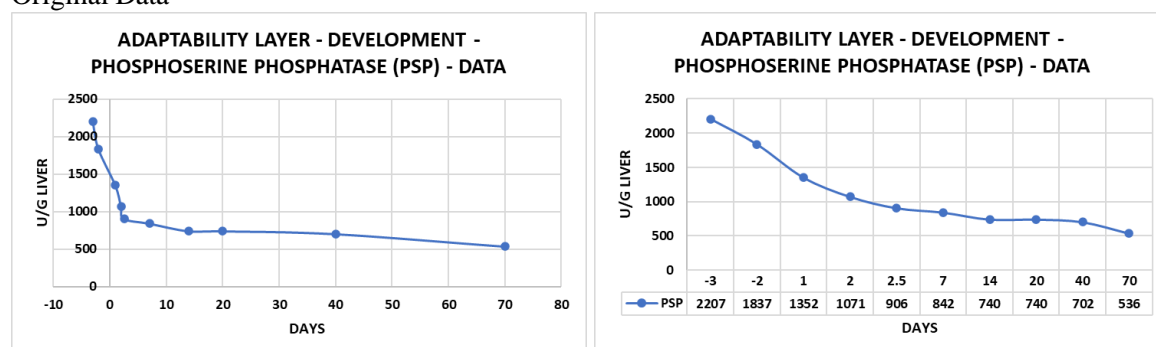
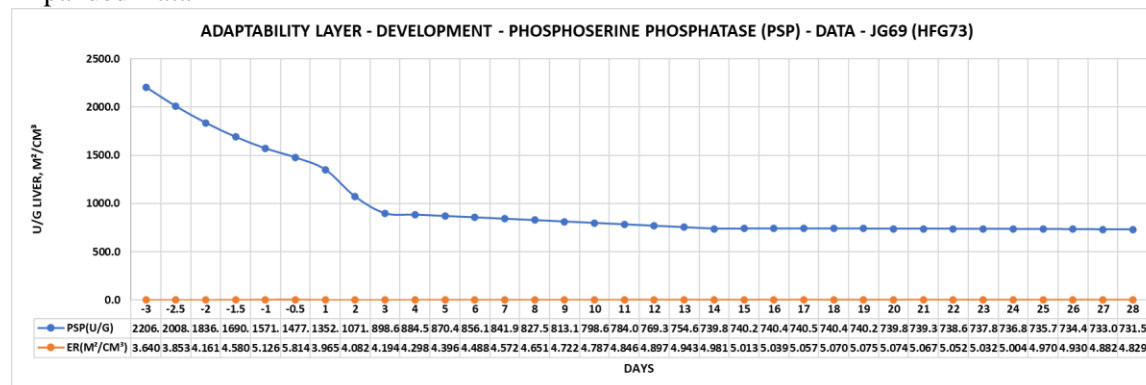


Figure 5.82 A technical note. The two plots of the original data show the differences that occur when plotting the same data in Excel without and with an accompanying data table. Notice the differences in the x axes. The one at the left shows the expected spacing between the x values, whereas the one at the right merely puts each x value in the row of bins.

Figure 5.83 includes the expanded and normalized data.

Expanded Data



Normalized Data (NORM1)

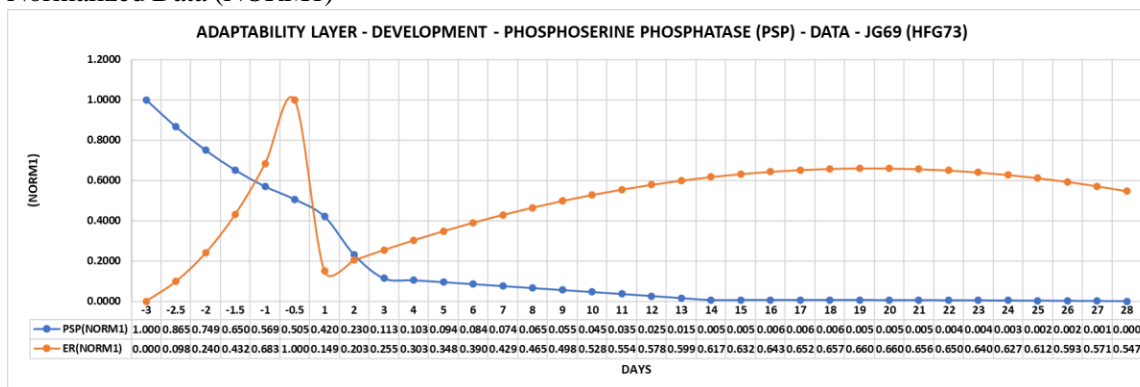
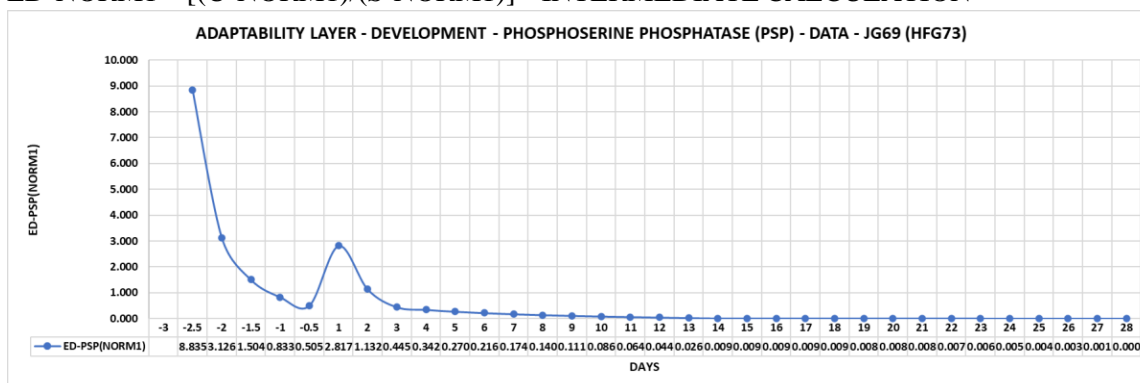


Figure 5.83 When comparing units of enzyme activity per gram of liver to M^2/CM^3 of hepatocyte cytoplasm, we can calculate local enzyme densities (ED) by converting U/G to U/ CM^3 by multiplying the gram by 0.7 and recalculating the units. Alternatively, we can remove the units by normalizing the data and then calculate the ED(NORM2). In the latter case, the mixed and same data references produced the same results (ED-NORM2).

Figure 5.84 shows (1) the results of the first calculation for the ED-NORM1 (lost 0 \rightarrow 1 range) and the second ED-NORM2 (recovered range 0 \rightarrow 1) and (2) checks the results by showing that both results run parallel.

ED-NORM1 = [(U-NORM1)/(S-NORM1)] – INTERMEDIATE CALCULATION



ED-NORM1 VS ED-NORM2 (LOG PLOT)

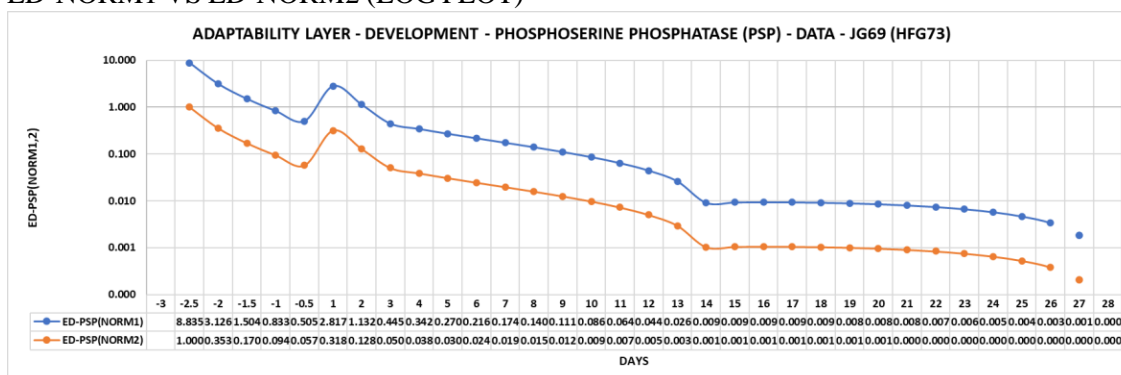


Figure 5.84 After normalizing the enzyme and membrane data, dividing one by the other to estimate the enzyme density resulted in a loss of the normalization. A second normalization recovered the zero to one range. Notice that the intermediate [ED-PSP(Norm1)] and final estimates [ED-PSP(Norm2)] produced parallel curves.

How do we show that the changes in Figure 5.85 are in fact parallel? By switching to a log plot in the adaptability layer, the parallel curves appear and by calculating the ratio of one curve to the other, we get two parallel lines in the rules layer.

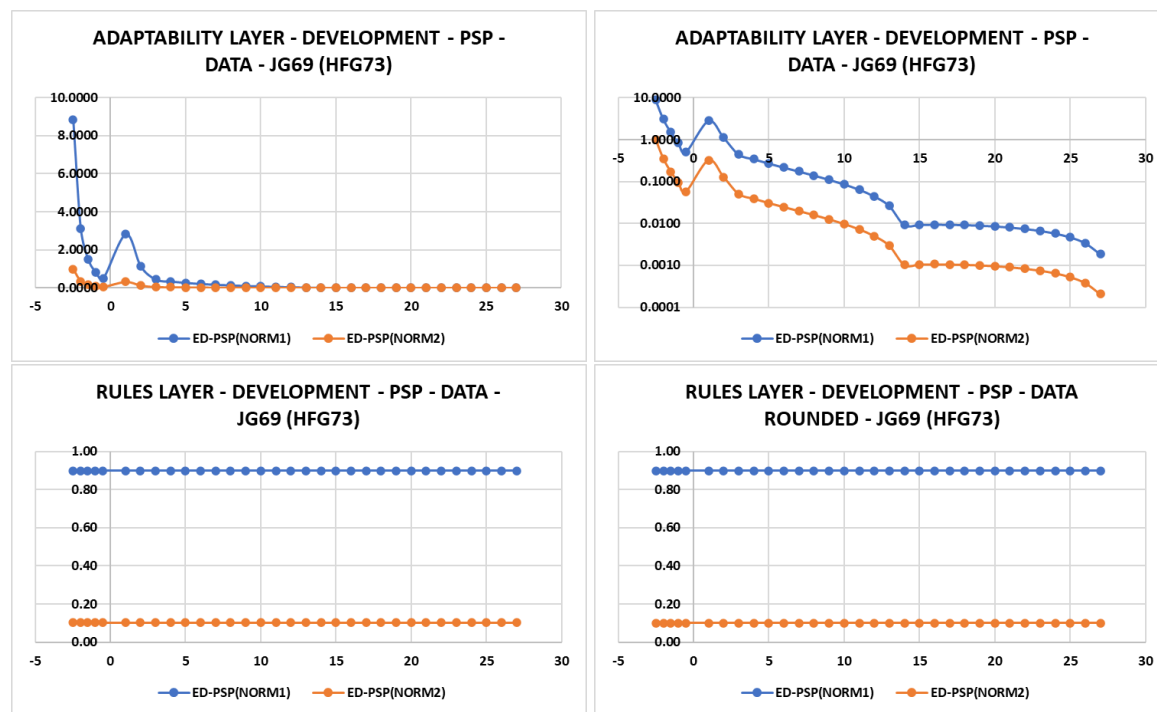


Figure 5.85 Testing the intermediate and final enzyme density estimate. Although the curves in the adaptability layer suggest differences, their ratios remain constant and parallel in the rules layer.

Comment: One can usually find ways to double check experimental results because biological changes occur as highly ordered events. Since everything in the adaptability layer operates from ratios found in the rules layer, demonstrating reproducibility often becomes an exercise based on duplicating patterns of ratios. This means that instead of just comparing new results to those already published, one now has the option of demonstrating – quantitatively - that a new study successfully reproduced previously published results.

5.3.12 Case Study 12: Development (Glycogen, Transferase, Phosphorylase, PEP) BO63

Source: Update applied to original data from Ballard F. J., Oliver I. T. (1963) Glycogen metabolism in embryonic chick and neonatal rat liver. *Biochem et Biophys Acta* 71:578-588. ER membranes: Hertzfeld et al., (1973; (HFG73).

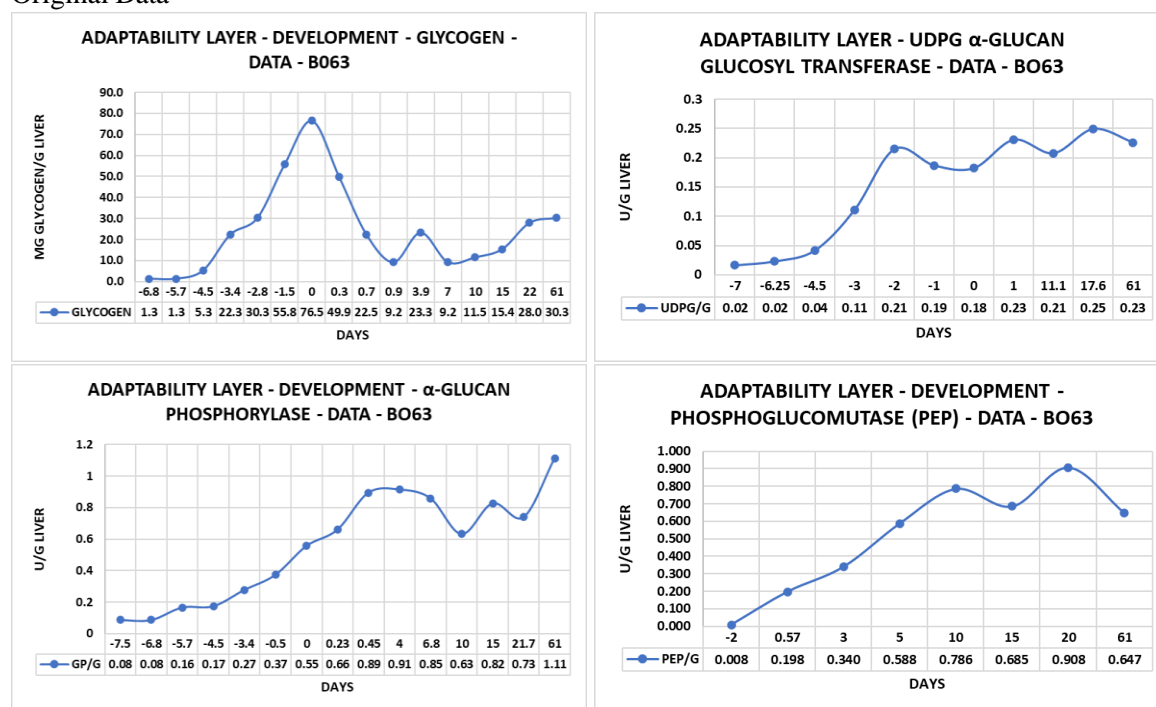
Topic: Biochemical (glycogen, UDPG α -glucan glucosyl transferase, α -glucan phosphorylase, and PEP) changes during development.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, calculate enzyme densities [(ED = [(U/G)/(S/G)]; ED = [(U/G)/(S/G)](NORM1); ED(NORM2)], analyze patterns, and report biological solutions (recipes).

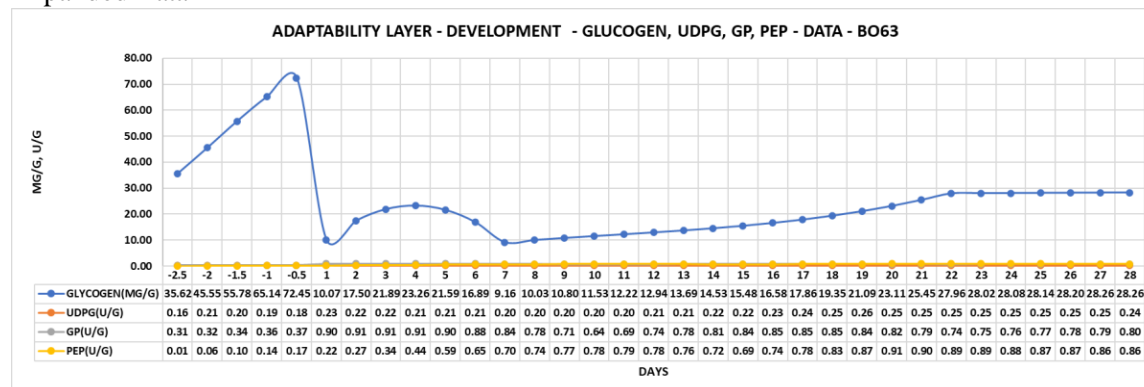
Dataset: Glycogen, and enzyme activities (UDPG α -glucan glucosyl transferase, α -glucan phosphorylase, and PEP). The normalized enzyme densities (NORM2) used mixed references (U/G of liver, S/CM³ of hepatocyte cytoplasm) - see 5.3.10 and checks provided throughout the chapter.

The update focuses on changes in glycogen and enzymes associated with glycogen metabolism during liver development (Ballard F. J., Oliver I. T. (1963, BO63); ER surface areas from Hertzfeld et al., 1973; (HFG73). Figure 5.86 includes the original and updated data, whereas Figure 5.87 adds normalized data and enzyme densities (ED-NORM2).

Original Data



Expanded Data



Expanded Data (Log Plot)

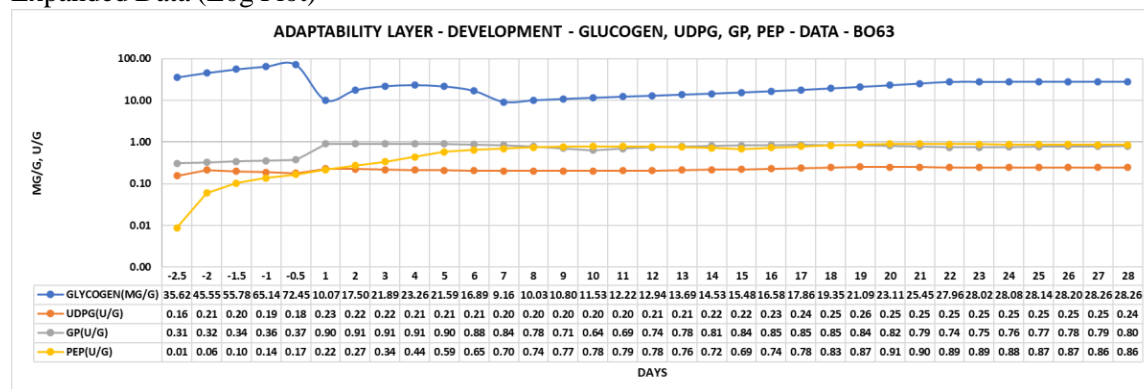
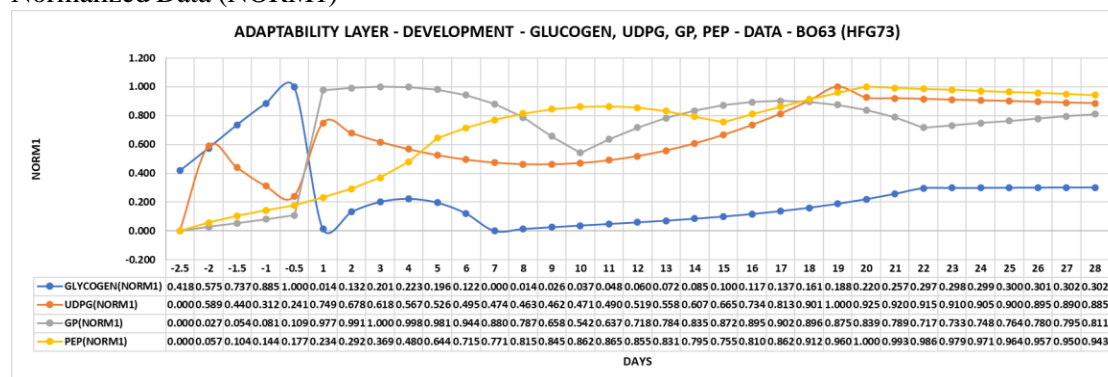


Figure 5.86 Original data expanded. The log plot shows changes for the widely ranging data.

Normalized Data (NORM1)



ED-NORM2 = [(U-NORM1)/(S-NORM1)] → Normalized

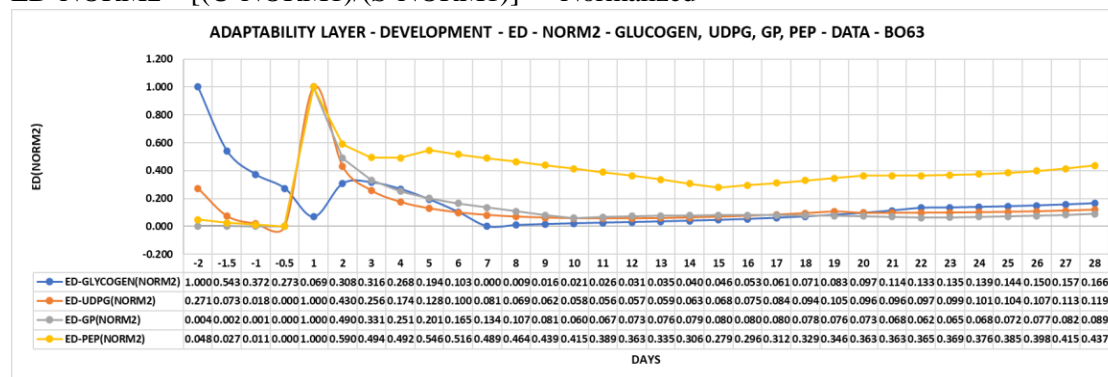


Figure 5.87 Enzyme densities (ED-NORM2) calculated from normalized data. Note that the “enzyme density” for the glycogen represents the mg glycogen per unit ER surface area. By relating everything to the same thing, everything connects, and one can see how the rules change.

Data Pair Patterns (NORM1): The two panels in Figure 5.88, include pairs of enzymes and enzyme densities (data pair ratios) expressed as patterns in the adaptability and rules layers (data and data rounded). The NORM1 data (enzymes), which represent one part of the larger change, show one set of patterns whereas the combined dataset ED(NORM2) a second set. In effect, we have one set of rule-based changes occurring within a second set of rule-based changes. By generating such patterns quantitatively, we’re progressively putting biology back together one pair of enzymes or enzyme densities at a time. When combined into data pair ratios (ED₁NORM2:ED₂NORM2), the patterns become the

developmental phenotype, which we can then reverse-engineer to see how hepatocytes orchestrate the developmental process. The point in juxtaposing the two representations of the same dataset allows the reader to see the difference between a separate biochemical solution (NORM1) and a combined biochemical-morphological solution (ED-NORM2). The point being that cells don't change one part at a time (NORM1), they change recipes based on relationships of structure to function (ED-NORM2).

NORM1 vs NORM1

ED-NORM2 vs ED-NORM2

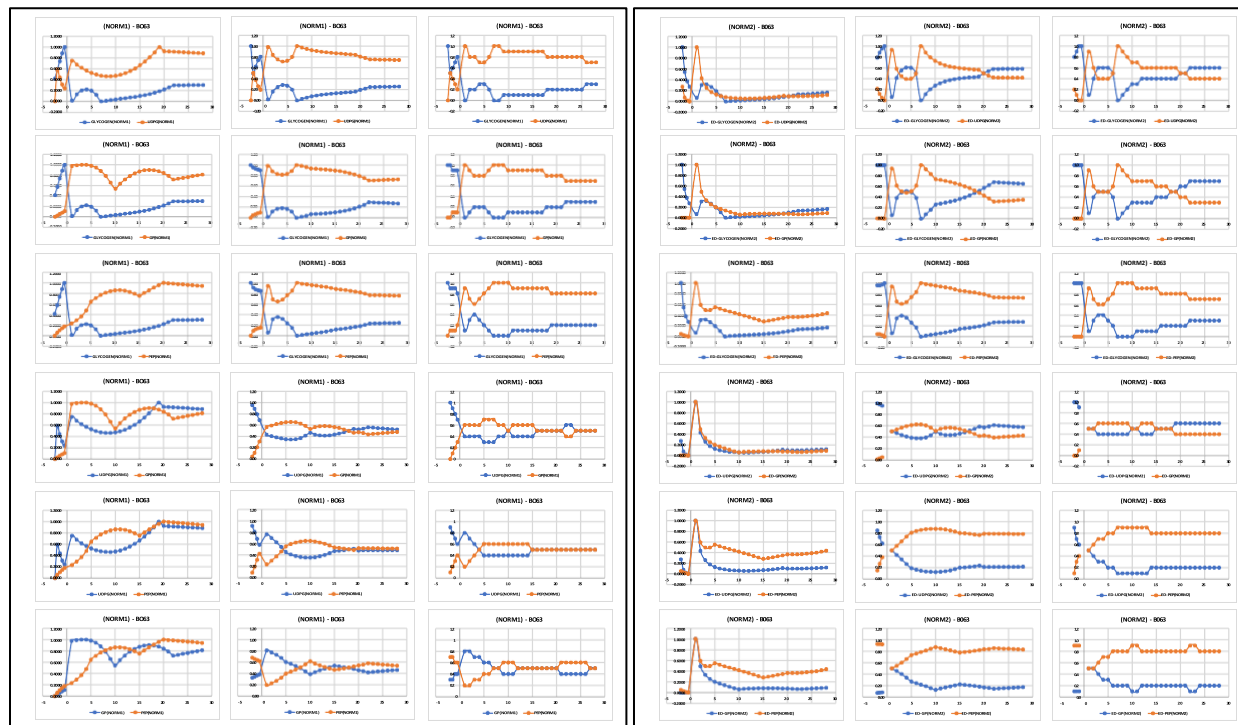
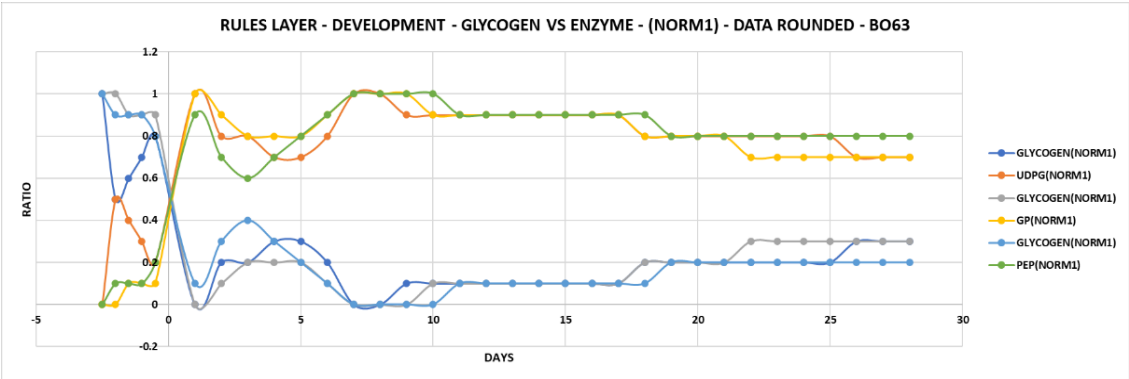
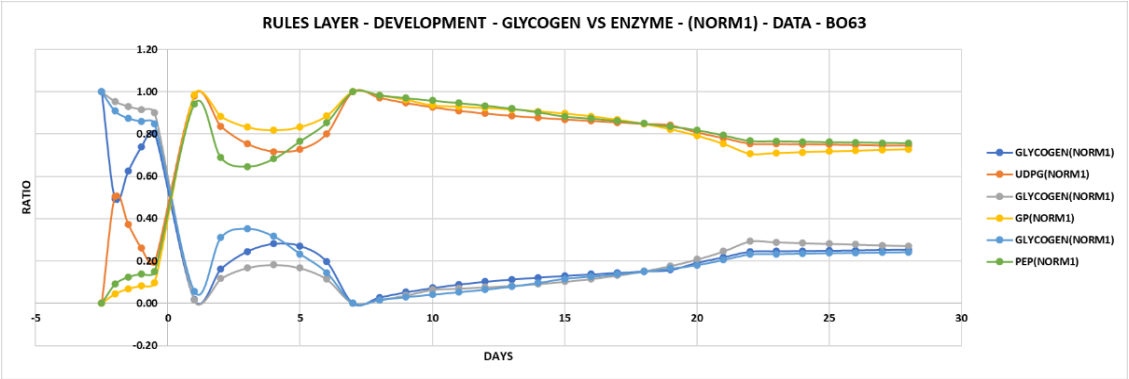


Figure 5.88 Note the differences between the biochemical changes (NORM1) and the biochemical/morphological changes (ED-NORM2) when applied to the same data pairs. The NORM1 ratios came two parts (U_a/U_b), whereas the NORM2 ratios came from four [$(U_a/S_a)/(U_b/S_b)$]. $U_{a,b}$ identifies units of enzyme activity or glycogen content for two parts and S_c membrane surface area of organelle c . Changes in the number of hepatocytes per gram of liver or CM^3 of hepatocyte cytoplasm do not affect these ratios.

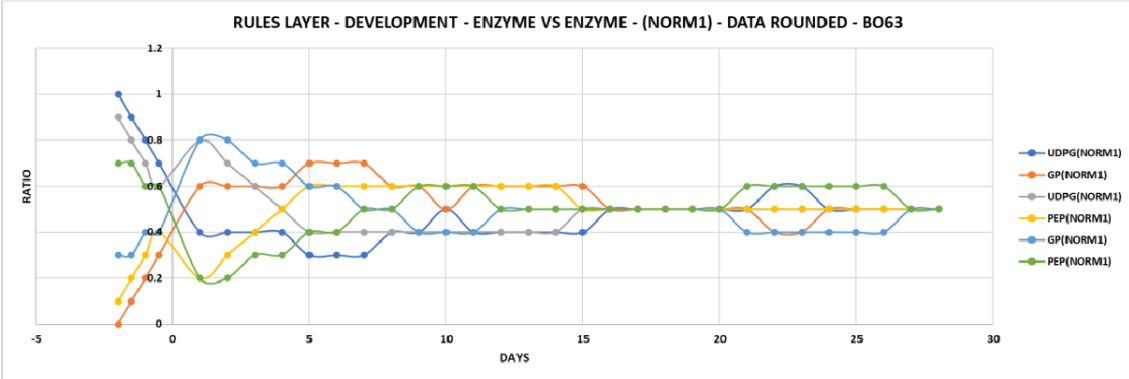
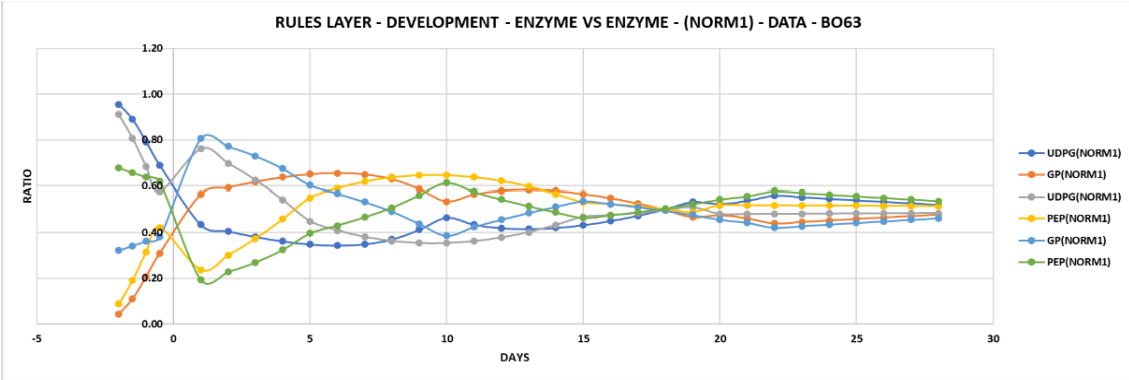
By superimposing similar patterns taken from the NORM1 and ED(NORM2) panels shown in the above figure, we can follow changes in enzymes vs glycogen, enzymes vs enzymes, and enzyme densities vs enzyme densities (i.e., relationships of structure to function). In Figure 5.89, the ratio plots give the unrounded data followed by the rounded. Note that as a part of their solution, hepatocytes regrow the relationship of their parts from complex (input) to simple (output). They start with complex relationships of data pair ratios and then translate complex problems into sets of related parts defined as subgroups sharing similar ratios (rules). For the experimental animals, the solutions (i.e., recipes) produce emergent properties. Currently, we cannot predict emergent properties from recipes.

Using the patterns in Figure 5.88, we can identify and superimpose duplicated data pair ratios and begin to sort the similarities into subgroups (outputs showing similar ratios). The point of the sorting is to identify which parts changed together for three consecutive days at the output. Notice how the relationships of the data pair ratios changed from complex to simple.

Rules Layer – Glycogen vs Enzyme (NORM1)



Rules Layer – Enzyme vs Enzyme (NORM1)



Partial Developmental Phenotype (Aggregated Data)

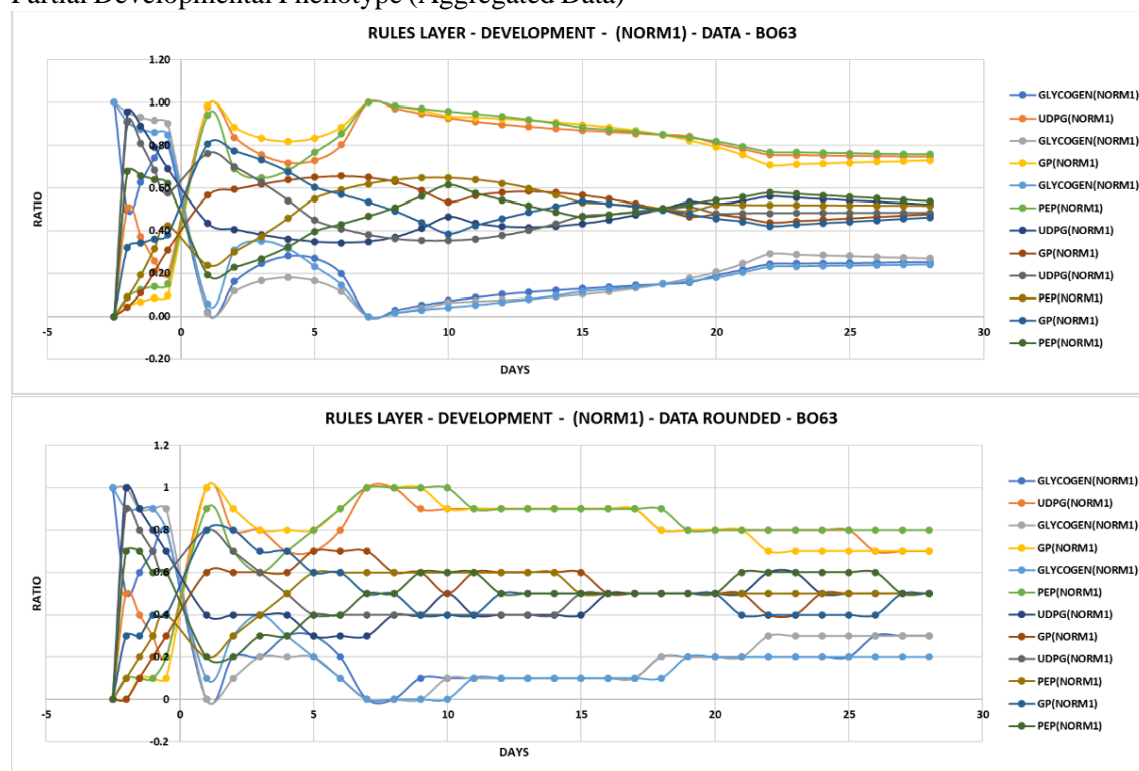


Figure 5.89 The aggregated data (rounded) identify three solutions at day 28: 0.5:0.5, 0.3:0.7, and 0.2:0.8. Taken together, they represent a working solution to a developmental problem identified by relationships of biochemical parts that define just the functional changes. The plot immediately above represents the result of putting the hepatocytes back together (forward engineering) by collecting matching patterns defined by the ratios of last three days. Reversing engineering this biochemical phenotype identifies the subgroups responsible for the changes. The point? Change in biology is a continuous event occurring in many places, directions, and subgroups. Experimentally, we use biochemistry, morphology, and enzymes densities separately to detect the same change differently – for different reasons. In contrast, biology changes by redefining everything together into new relationships of structure to function (recipes).

Summary of NORM1 Results: At day twenty-eight after birth, the current “biochemical solution” to the relationships of glycogen vs enzyme and enzyme vs enzyme appears in Figure 5.90. While UDPG and GP shared the same relationship to glycogen, PEP did not. [Note that PEP is a mitochondrial enzyme.] However, all the pairs of enzymes contributed equally to the change (50%). Since all the assays came from the supernatant fraction, we’re dealing with soluble enzymes. The point? Cells control everything by rule.

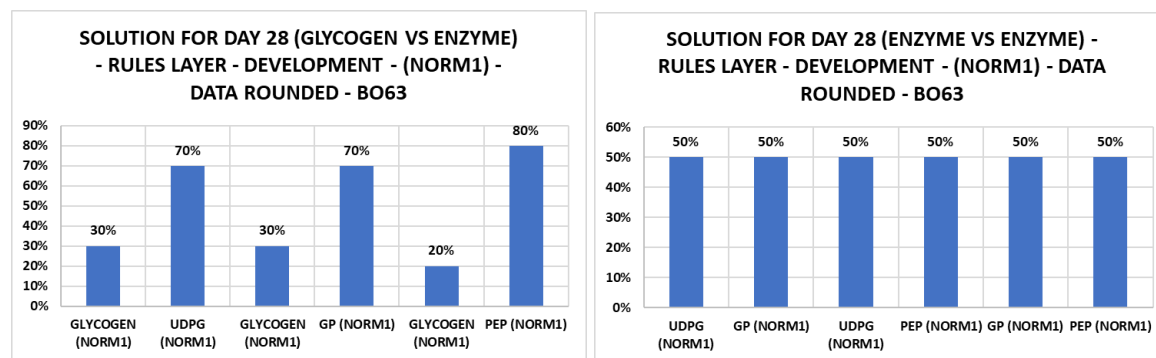
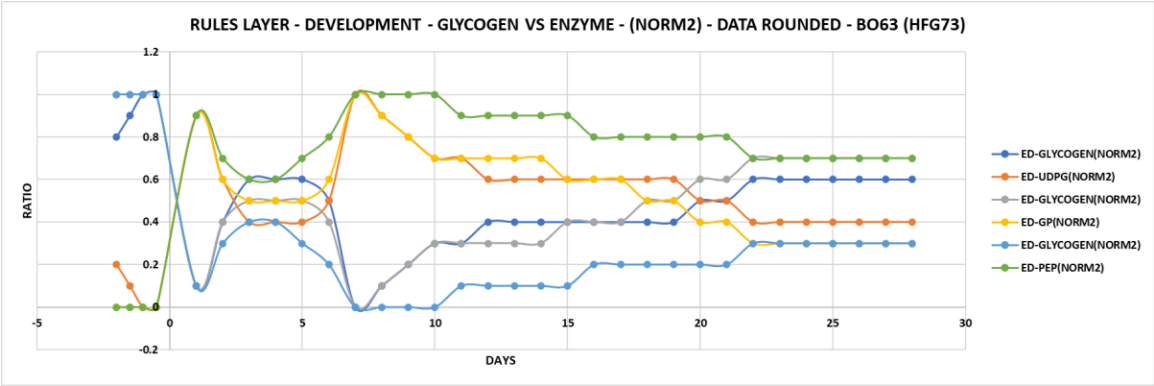
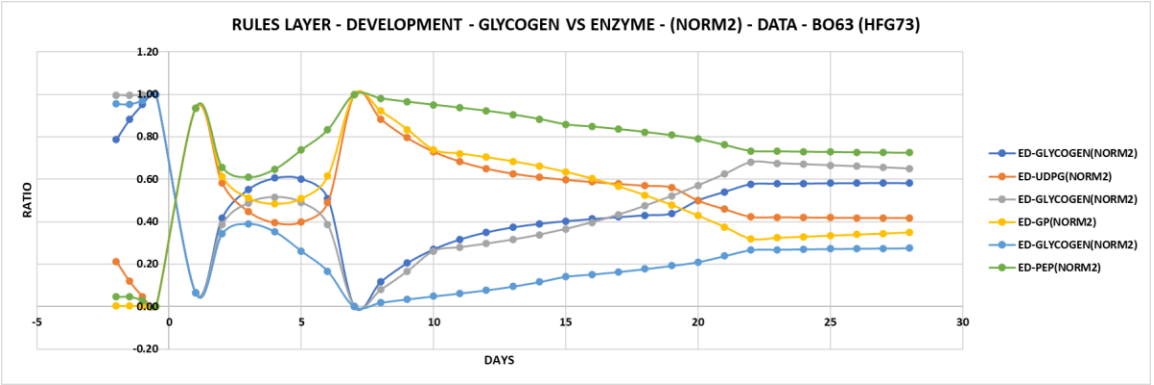


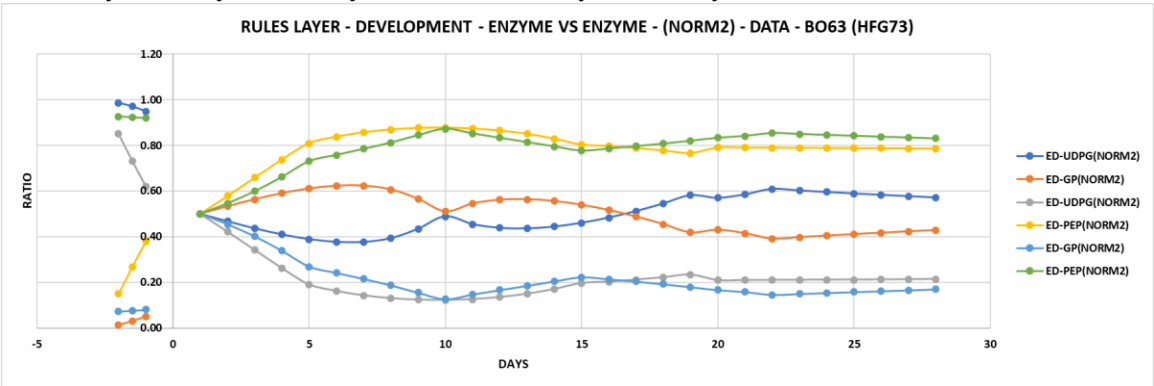
Figure 5.90 The data pair ratios were 0.3:0.7 and 0.2:0.8 for the glycogen vs enzyme and 0.5:0.5 for the enzyme vs enzyme.

Data Pair Patterns (NORM2): In turn, we can view changes in the same enzymes (NORM1) expressed as enzyme densities (ED-NORM2). Figure 5.91 shows the relationships of enzyme to glycogen and enzyme to enzyme as the hepatocytes work their way through the current developmental problem, the solution to which appears as parallel curves (data rounded) running horizontally for at least three consecutive days.

Rules Layer – Glycogen vs Enzyme Density (NORM2)



Rules Layer – Enzyme Density (NORM2) vs Enzyme Density (NORM2)



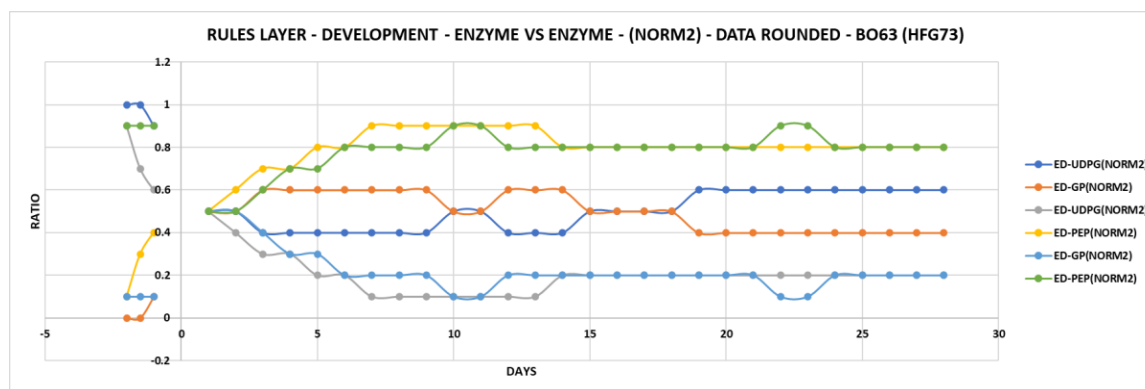


Figure 5.91 Enzyme densities (ED-NORM2) shown in the rules layer using data (two decimal places showing) and rounded data (one decimal place).

The third set of enzyme densities (Figure 5.92) summarizes the solutions of the combined dataset. Notice that the rounded data suggests that the hepatocytes solved their current developmental problem of glycogen metabolism by applying just three ratio rules: 4:6, 3:7, and 2:8.

Rules Layers Combined – Enzyme Density (NORM2)

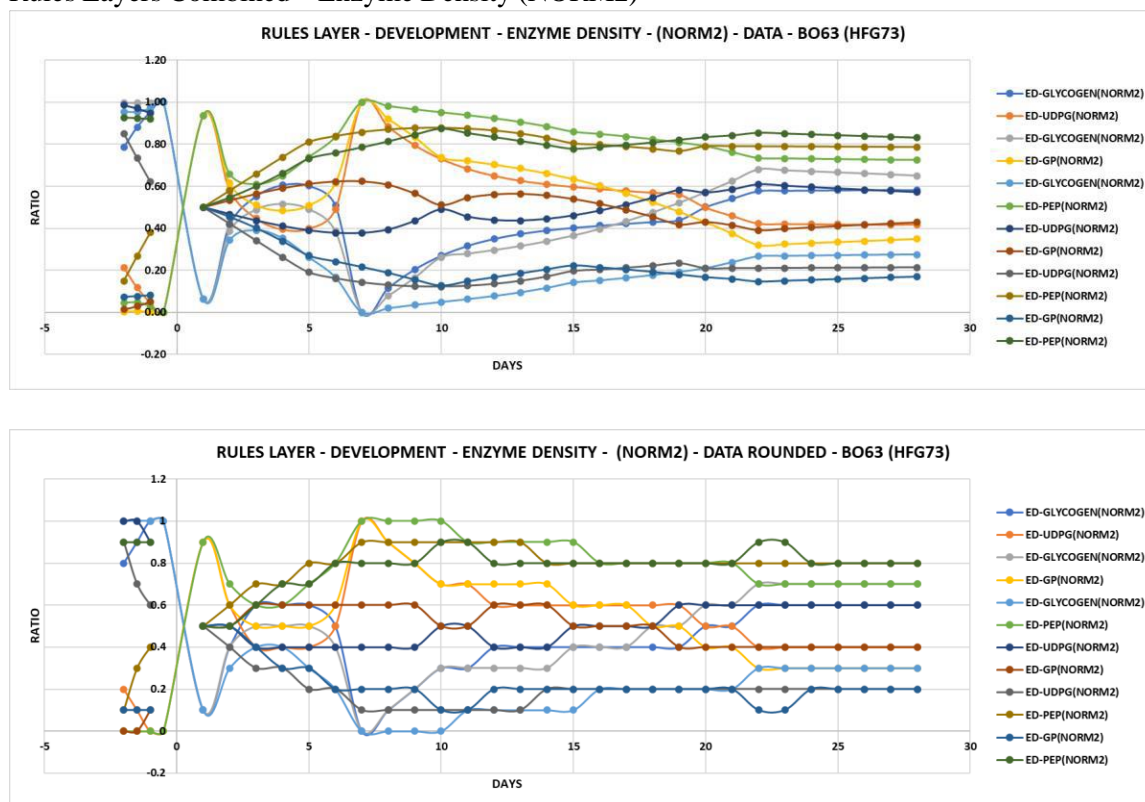


Figure 5.92 The combined dataset involved three rules expressed as ratios: 0.4:0.6, 0.3:0.7, and 0.2:0.8. Displaying the same results five days in a row qualified as a solution.

Figure 5.93 summarizes the changes to three soluble enzymes during early development – before and after relating them to a unit of ER membrane surface area .

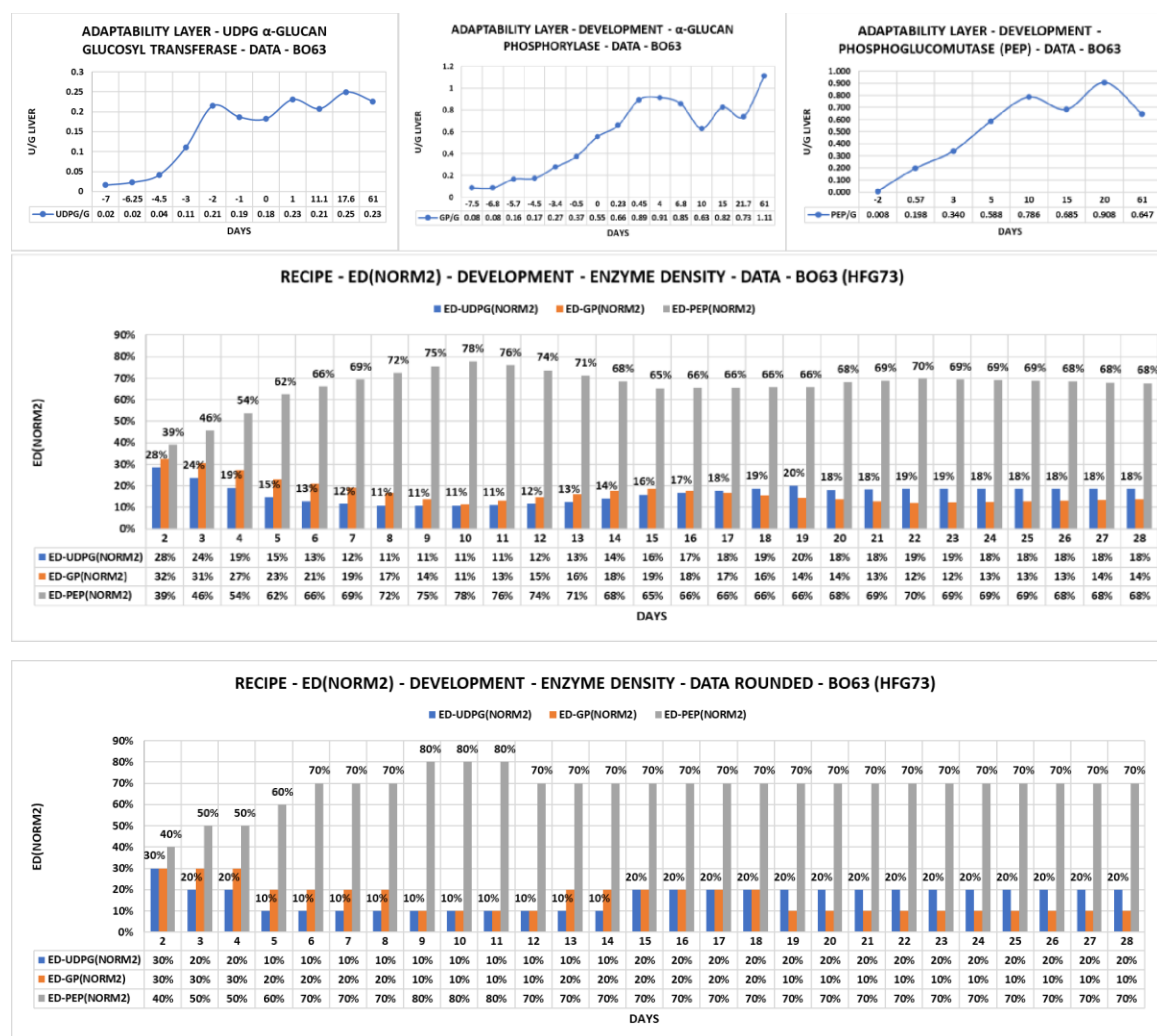


Figure 5.93 By plotting the enzyme densities as percentages, we can see how the enzyme-membrane recipe (relationships of structure to function) changed during early development. The three enzymes differentiated into the same recipe by postnatal day 19 – sooner if one accounts for the rounding error (days 15 to 18). The before shows what differentiation looked like to us, whereas the after suggests how hepatocytes would have interpreted our results by rule.

Summary: The day-by-day changes in the enzyme-membrane recipes show how the enzyme densities differentiated during early development. A developmental phenotype - consisting of hundreds of enzyme densities - may or may not predict the sequence and timing of gene expression. If it does, then hepatocytes are more likely to be manufacturing machines responding largely to inputs from genes. If not, then hepatocytes may be running their own algorithms, anticipating the needed resources, and calling for resources as needed. Although we know that many parts contribute the cell's information processing network, we still don't know as much as we might about what does what, when, where, and why. Recipes tell us what's happening in hepatocytes when and where but not how and why. While the reality of classical mechanics can tell one part of the change story, we might need a different reality to write in the missing parts.

Comment: When detecting, interpreting, and predicting changes, we can use the parts (enzymes and membranes) separately or in combination (enzyme densities). While a given experiment works with the original data, relating such data to the literature requires normalization. For example, predicting genetic expression from phenotypic changes in enzyme activities will likely involve generating polynomial curves, rule-based patterns, and normalized data for both datasets. The point to make is that the biochemical or morphological changes interpreted separately adhere to one set of rules, whereas enzyme densities (biochemistry linked to morphology) have rules of their own.

In the rules layer, we can plot the normalized relationships of glycogen to enzymes and enzymes to enzymes taken two at a time during the developmental period. Since the rules layer uses ratios, it can detect how a given part changes relative to another. Recall that the patterns shown in the figures differed importantly. The (NORM1) vs (NORM1) patterns of the rules layer plotted relative changes in two biochemical parts, whereas the ED-NORM2 figure plots the same biochemical changes related to changes in a membrane (ER) surface area. As a biological event, a change occurs in a cell as an enzyme density, but the outcome appears in the liver as a capacity to solve (or mitigate) a problem globally. When studying the liver, each problem comes with a two-part solution. We need both cell and liver data to discover how the hepatocytes rearranged the enzymes and membranes to get the best enzyme-membrane recipe. In effect, detecting a biological change becomes a puzzle containing many pieces that must fit together correctly by rule.

5.3.13 Case Study 13: Development (Glycogen Metabolism) – WG76

Source: Update applied to original data from Watts C., Gain K. R. (1976) Glycogen metabolism in the liver of the developing rat. *Biochem J* 160: 263-270. ER membranes: Herzfeld et al., (1973);HFG73.

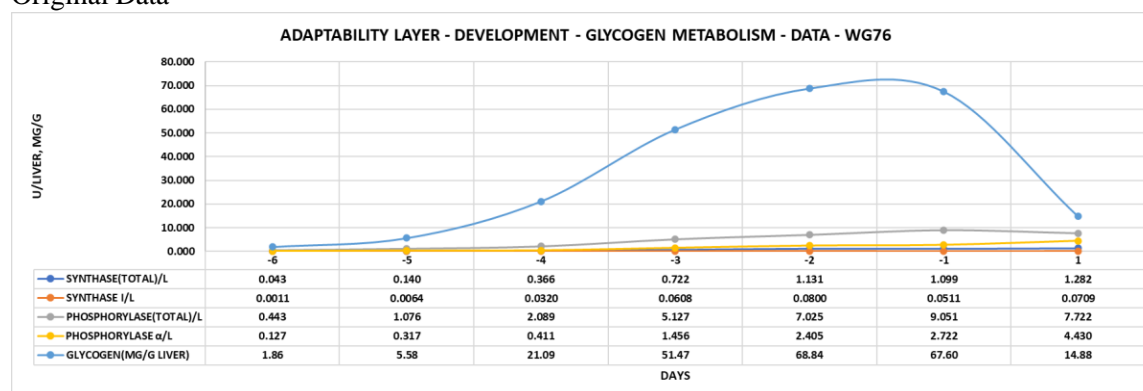
Topic: Glycogen metabolism of the developing liver.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, ED(NORM2, analyze patterns).

Dataset: Total synthase, phosphorylase α , total phosphorylase, glycogen. The normalized enzyme densities (NORM2) used mixed references (U/G of liver, S/CM³ of hepatocyte cytoplasm) - see 5.3.10 and checks provided throughout the chapter.

Since Watts and Gain (1976; WG76) included a wealth of enzyme data related to glycogen metabolism, we can use their data to reassemble part of a developmental phenotype and then reverse-engineer it. Moreover, we can calculate liver capacities with data expressed per gram and per liver. ER surface areas came from Hertzfeld et al., 1973; (HFG73). The first three figures include the original (Figure 5.94), expanded (Figure 5.95), and normalized data (Figure 5.96).

Original Data



Original Data (Log Plot)

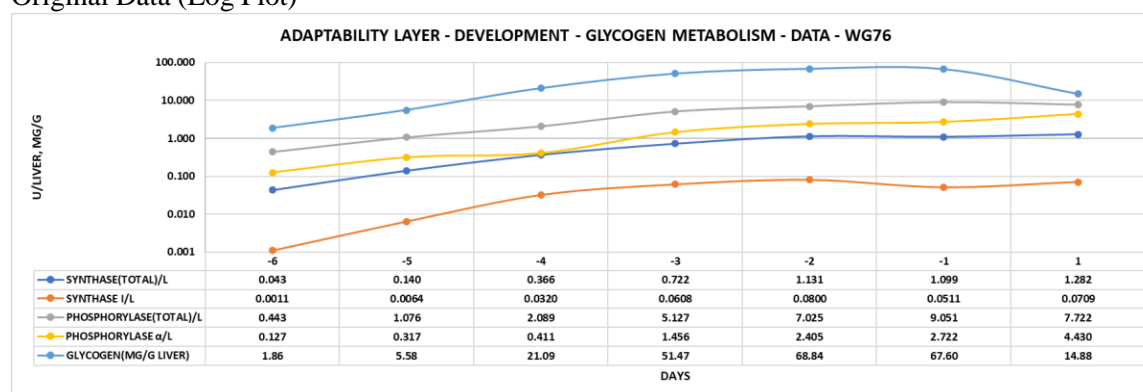
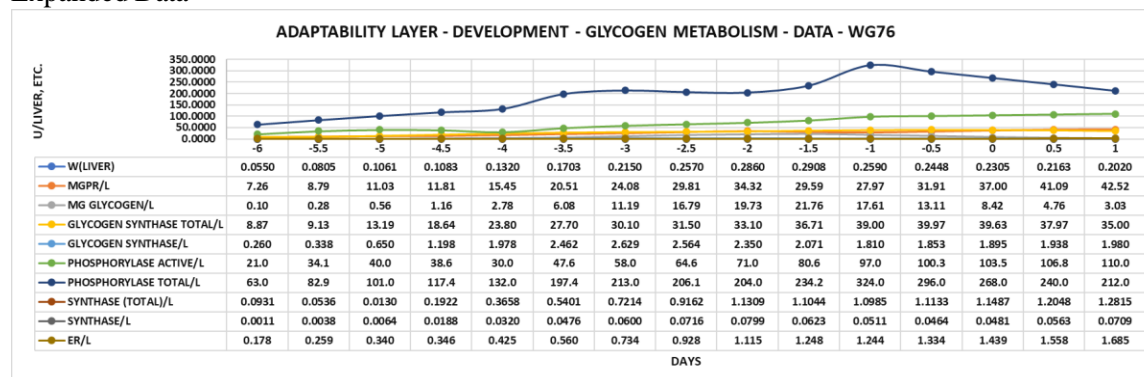


Figure 5.94 Pre and postnatal development of enzymes involved in glycogen metabolism have data related to the liver (L) and to a gram of liver (G).

Typically, most published studies would stop at this point. We would test data points for significant differences, discuss the results, and compare our new findings to those of previous investigators. By updating results to the complex setting of the hepatocytes, we can expand and normalize the data, calculate enzyme densities, identify duplicate patterns, forward and reverse engineer changes, and follow the construction of new cell recipes. Moreover, by applying the same rules used by the hepatocytes, the results become reproducible.

Expanded Data



Expanded Data (Log Plot)

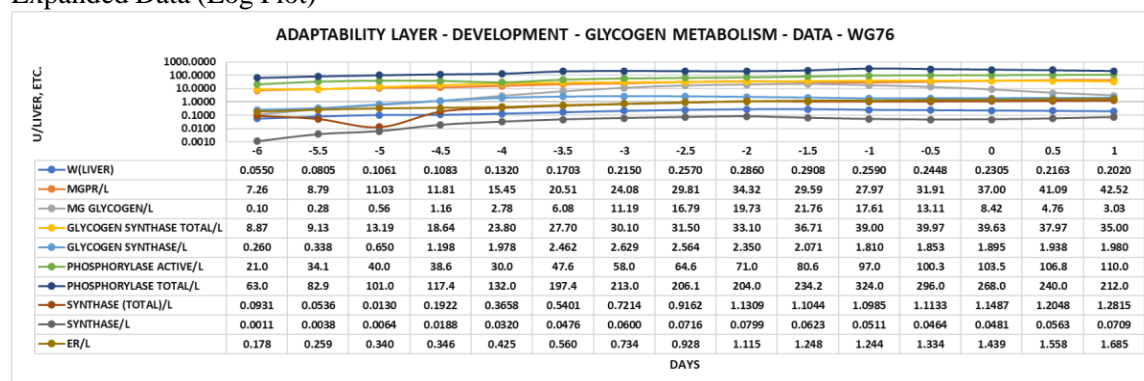


Figure 5.95 The expanded data suggest relatively small changes, most of which appear to occur in parallel.

Normalized Data: By plotting normalized results (data rounded), patterns appear (Figure 5.96).

Normalized Data (NORM1)

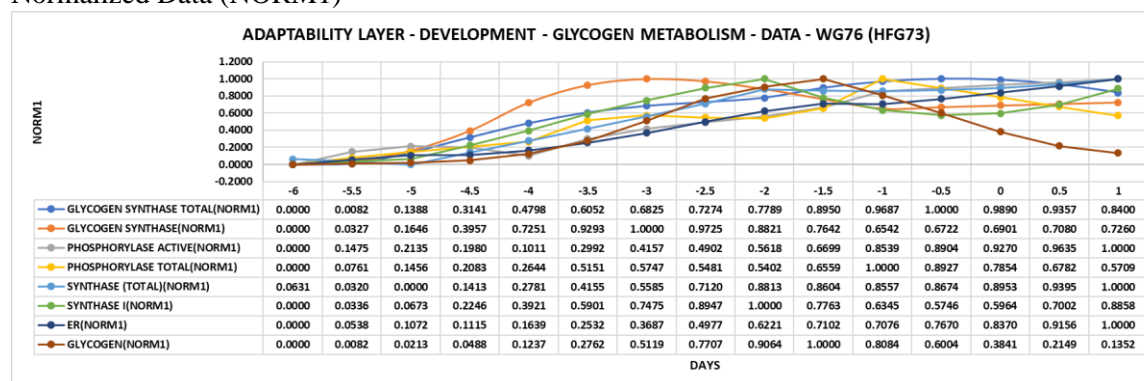


Figure 5.96 In contrast to the original data of Figure 5.94, the normalized data interpret all the changes between zero and one.

Interpreting Normalized Data in the Rules layer: Figure 5.97 plots the seven enzymes and one surface area (ER) as data pairs (eight parts taken two at a time gives twenty-eight data pairs) and expressed as ratios in the rules layer. The figure shows the developing phenotype reassembled from the data pair ratios.

Partial Phenotype: Rules Layer (Norm1)

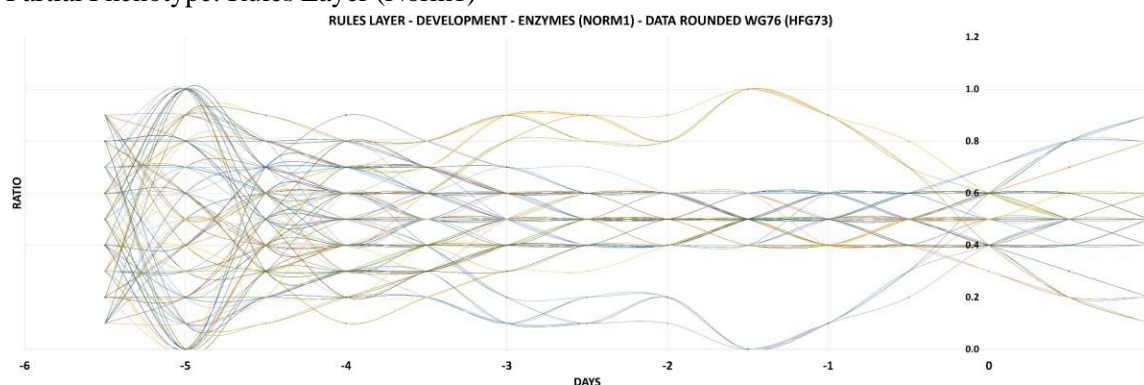
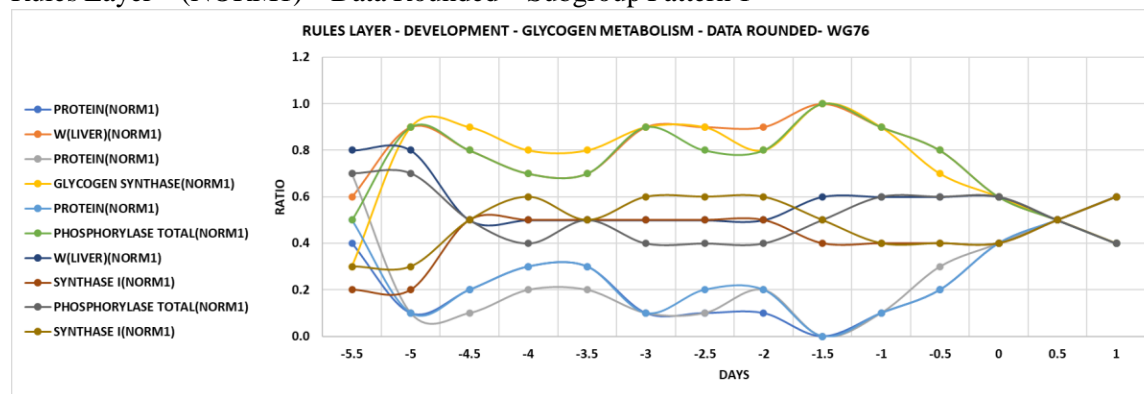


Figure 5.97 Changes in the data pair ratios during development. The plot identifies several days when crossover points occur but identifying the subgroups requires reverse engineering the phenotype.

Reverse Engineering the Phenotype into Subgroups: Recall that reverse-engineering usually involves taking something apart, understanding the purposes and relationships of parts, putting everything back together, and finally running it to see if it works. Since publications include just the parts, we must put the parts back together before we can take them apart to see how cells change.

The update applies its standard approach of generating patterns based on the way two parts expressed as ratios (data pair ratios) change relative to one another. When a data pair ratio shares the same ratio repeatedly at the far right, they identify their membership in a subgroup. In the Figure 5.98, for example, five pairs of parts shared the same ratio at day 1 (e.g., Pattern 1 = 0.6:0.4). However, the subgroup did not display three identical time points consecutively. Bear in mind that the following figures illustrate the reverse engineering of parts (enzymes, glycogen, liver weight) not enzyme densities. In effect, we're viewing changes taken out of the context of the cell's integration of structure to function - except when the ER forms one half of a data pair.

Rules Layer – (NORM1) – Data Rounded – Subgroup Pattern 1



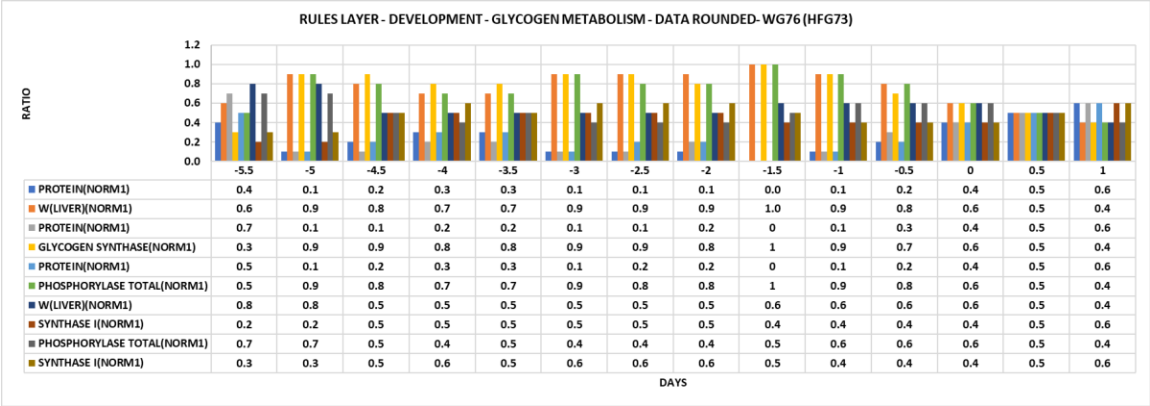


Figure 5.98 In the first subgroup, five data pairs suggested a solution by sharing the same ratio (0.6:0.4). This and other subgroups show us how change occurs by rule wherein the process of problem solving involves changing the relationships of data pairs from different, to similar, to identical.

The next subgroup pattern shows that the solution for this set of data pairs occurred three days prior to birth Figure 5.99).

Rules Layer – (NORM1) – Data Rounded – Subgroup Pattern 2

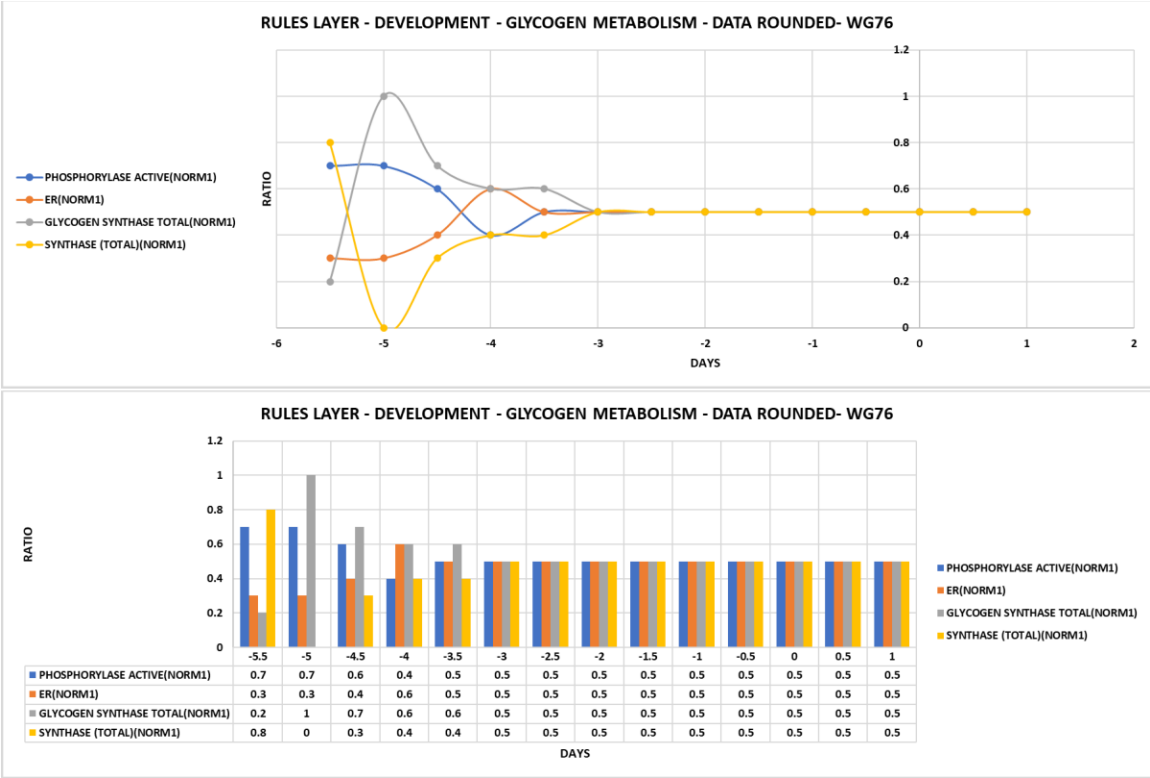


Figure 5.99 At day -3, both data pairs displayed the same ratio (0.5:0.5) By forming and duplicating data pair ratios, hepatocytes appear to orchestrate the many changes occurring simultaneously during development.

Figures 5.100 to 5.106 complete the dataset.

Rules Layer – (NORM1) – Data Rounded – Subgroup Pattern 3

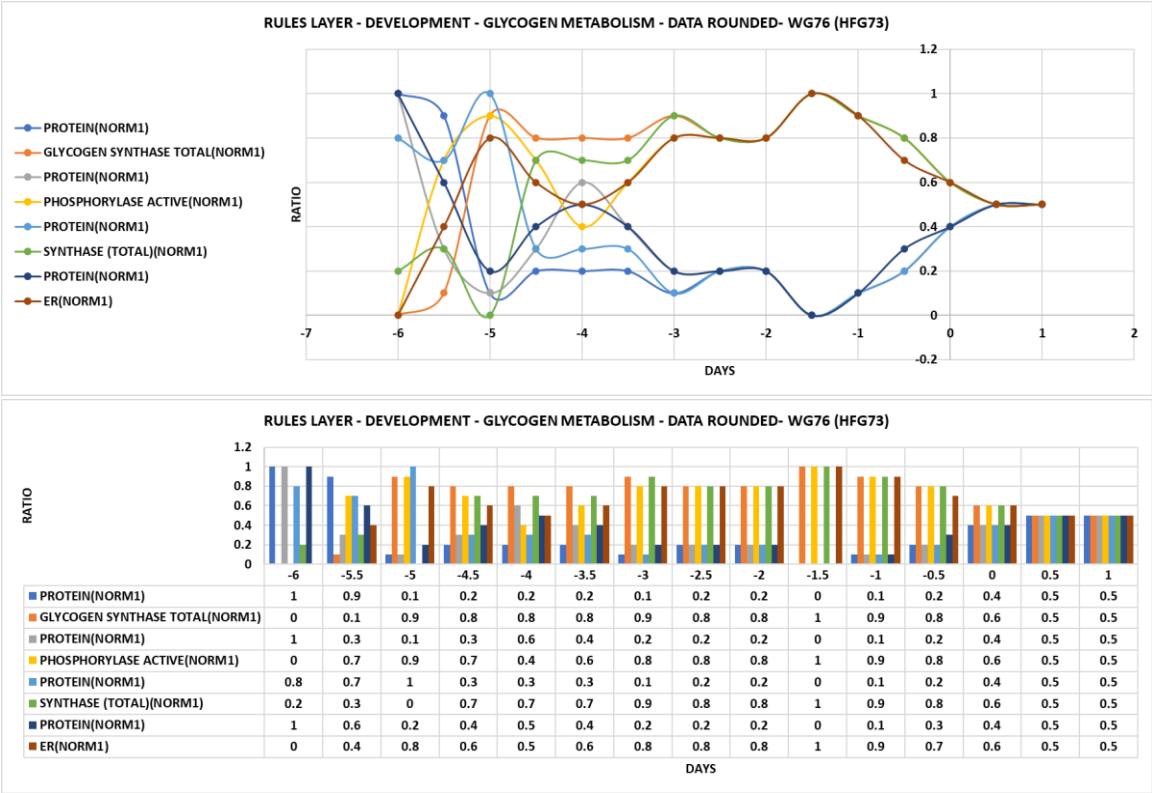
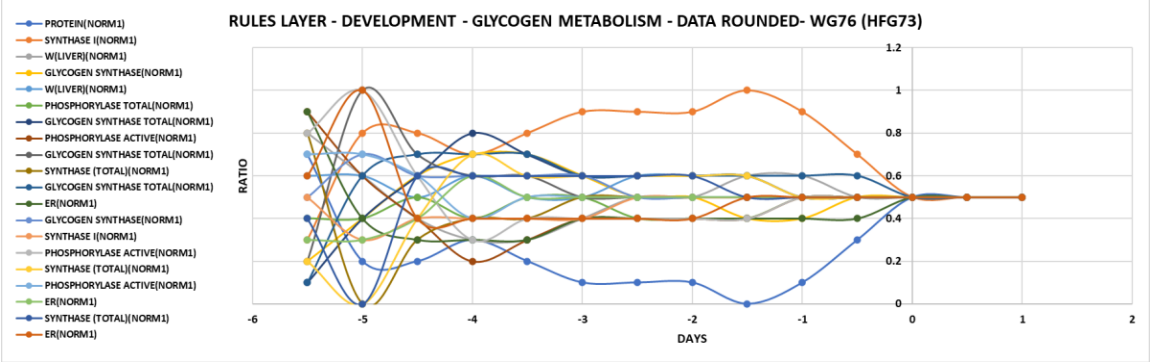


Figure 5.100 The relationship of protein concentration to enzyme activity operates by rule with solutions suggested by (0.5:0.5). By equalizing the relative amounts of both parts of the data pair one day before birth, the cells optimize their ability to change in response to the new environment.

Rules Layer – (NORM1) – Data Rounded – Subgroup Pattern 4



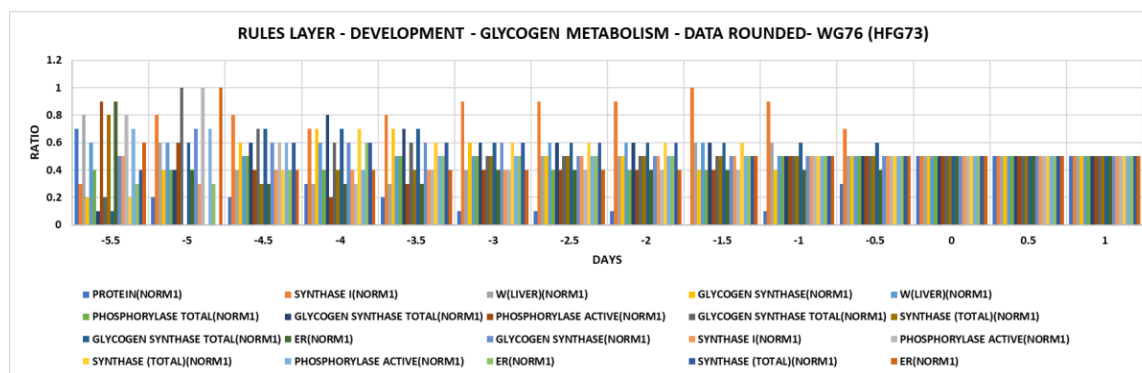


Figure 5.101 In this portion of the 0.5:0.5 subgroup, ten data pairs found a common solution at birth.

Rules Layer – (NORM1) – Data Rounded – Subgroup Pattern 5

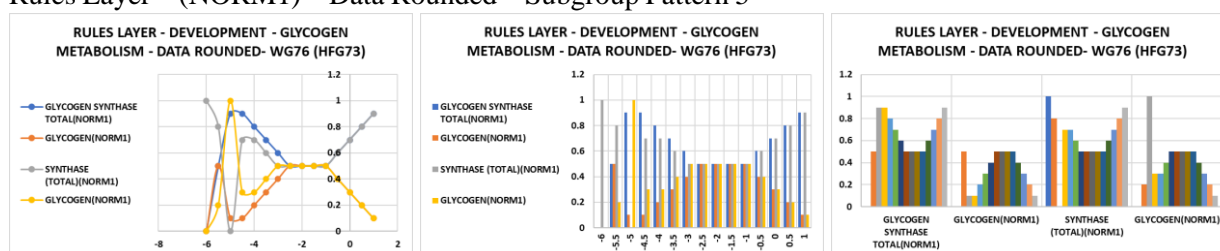


Figure 5.102 Subgroup 5 found a solution at day -2.5, but relative to glycogen increased or decreased linearly.

Rules Layer – (NORM1) – Data Rounded – Subgroup Pattern 6

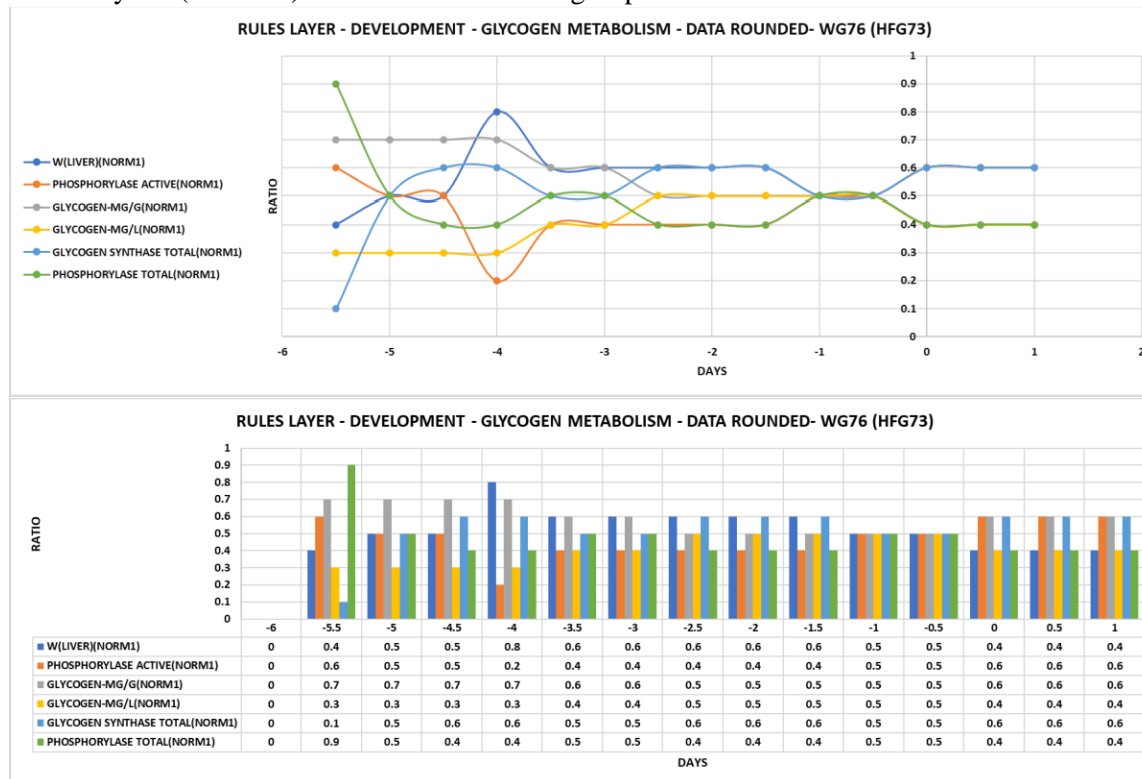


Figure 5.103 While the solution to this subgroup was 0.6:0.4, notice that the gram of liver was often larger than the liver.

Rules Layer – (NORM1) – Data Rounded – Subgroup Pattern 7

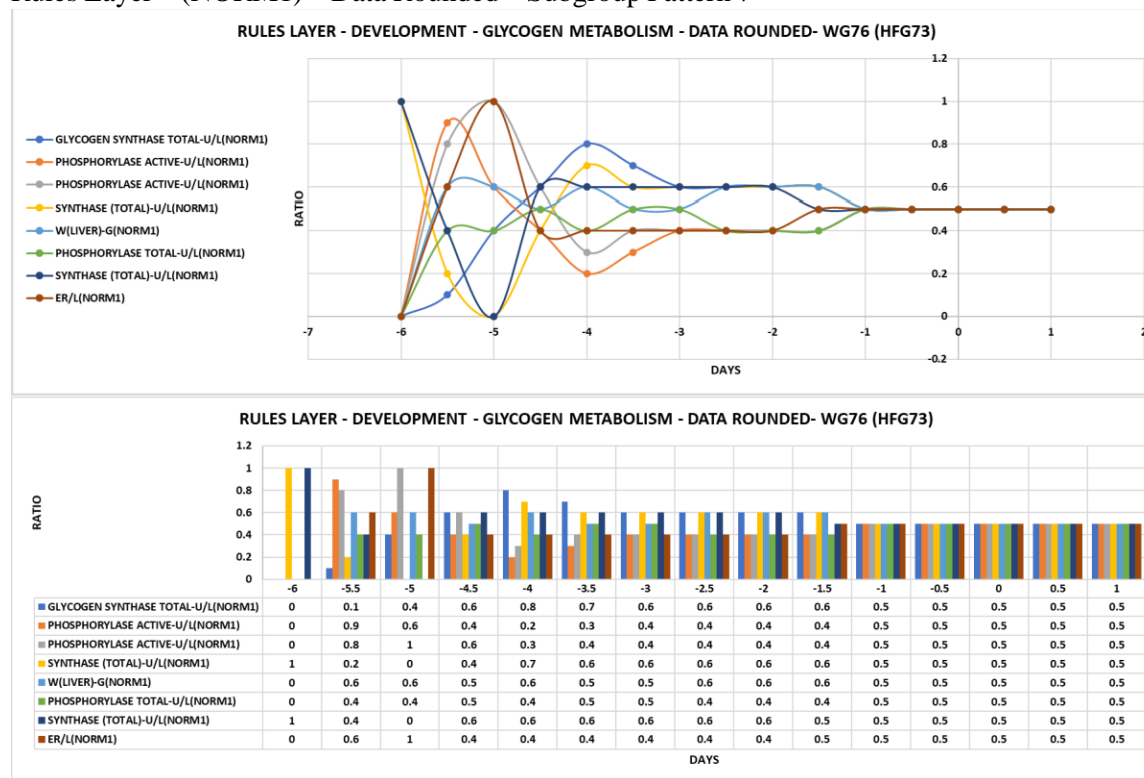
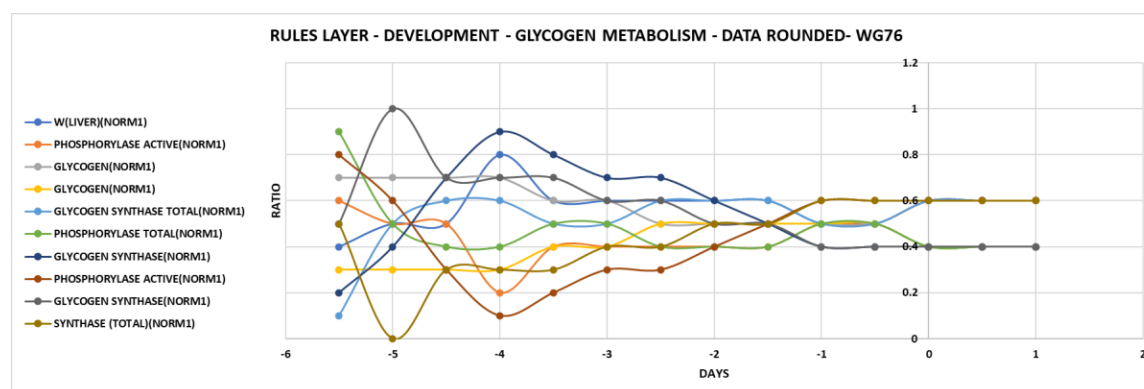


Figure 5.104 Another solution to the 0.5:0.5 subgroup occurred one day before birth. The missing values at day minus six indicate the result of dividing by zero (an error message).

Rules Layer – (NORM1) – Data Rounded – Subgroup Pattern 8



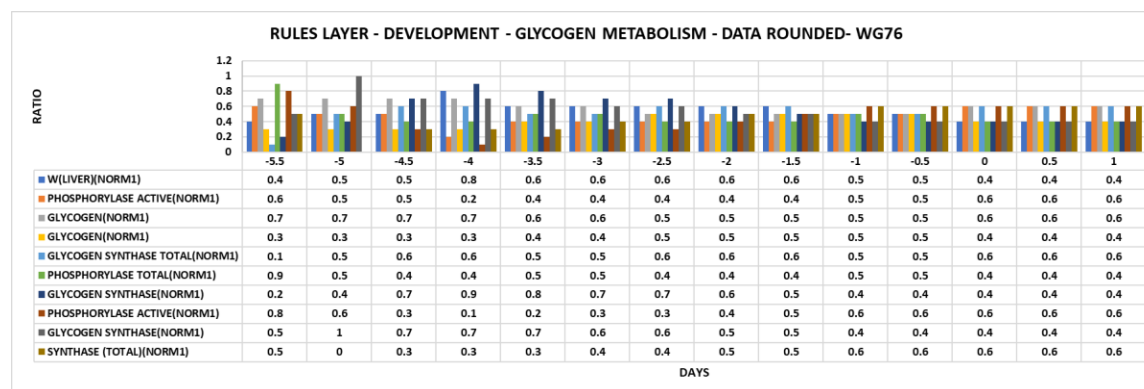


Figure 5.105 Another solution to the 0.4:0.6 subgroup occurred at birth.

Rules Layer – (NORM1) – Data Rounded – Subgroup Pattern 9

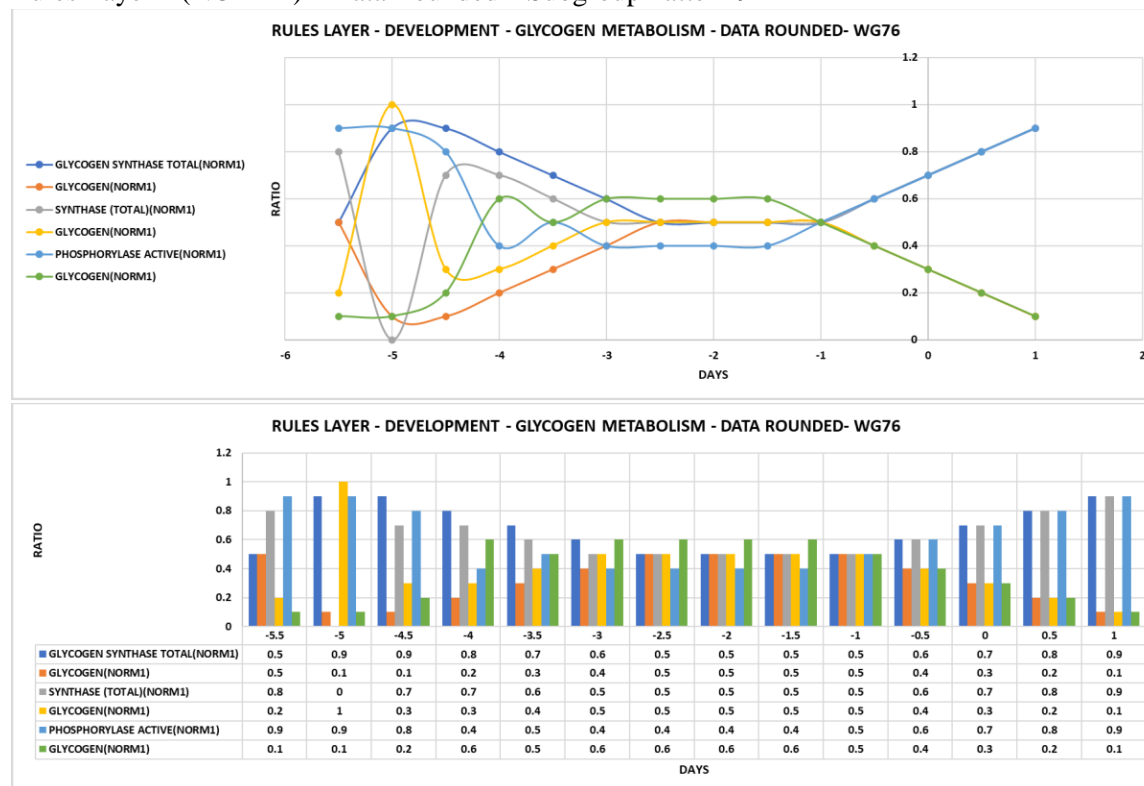


Figure 5.106 Patterns can also diverge by rule. Notice the linear pattern (days -1 to 1).

Enzyme Densities (NORM2): By calculating enzyme densities (Figure 5.107), we can see how glycogen and the enzyme activities change relative to a unit of membrane surface area. In turn, we can form data pair ratios from the enzyme densities, use them to forward engineer a phenotype (Figure 5.108), follow changes in the enzyme-membrane recipes, and reverse engineer the phenotype into subgroups (Figure 5.109).

ED-NORM2 = [(U-NORM1)/(S-NORM1)] → NORMALIZED

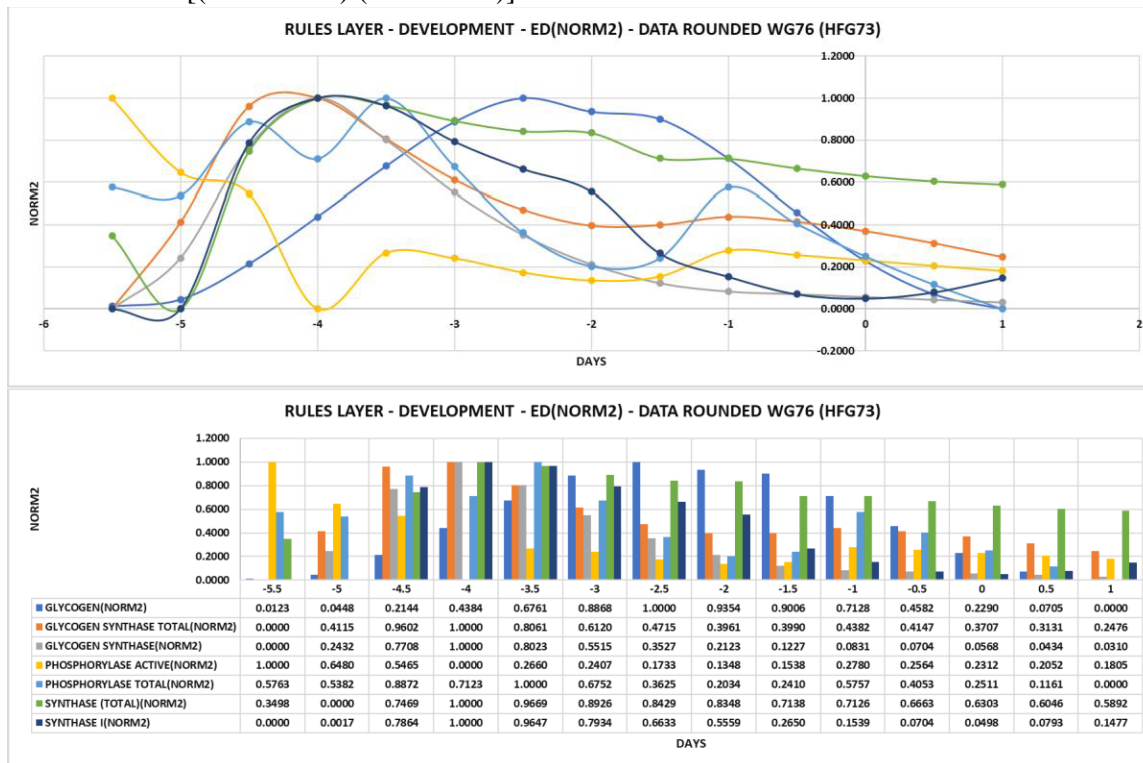


Figure 5.107 After the prenatal appearance of enzymes, they appear to wander before finding a direction.

Assembling a Phenotype: By combining the ED-NORM2 data from multiple sets of data pair ratios (data rounded), we can reconstruct a small sample taken from the larger developmental phenotype (Figure 5.108).

Phenotype: Rules Layer – ED-NORM2 – Data Rounded

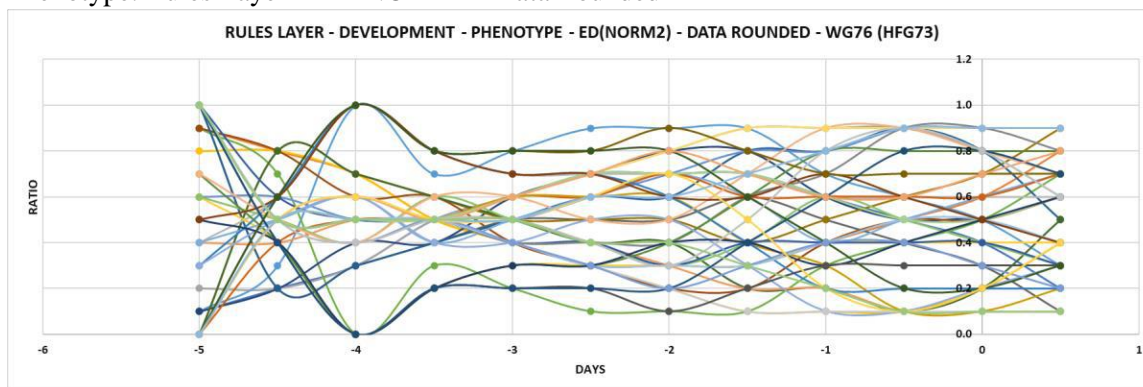
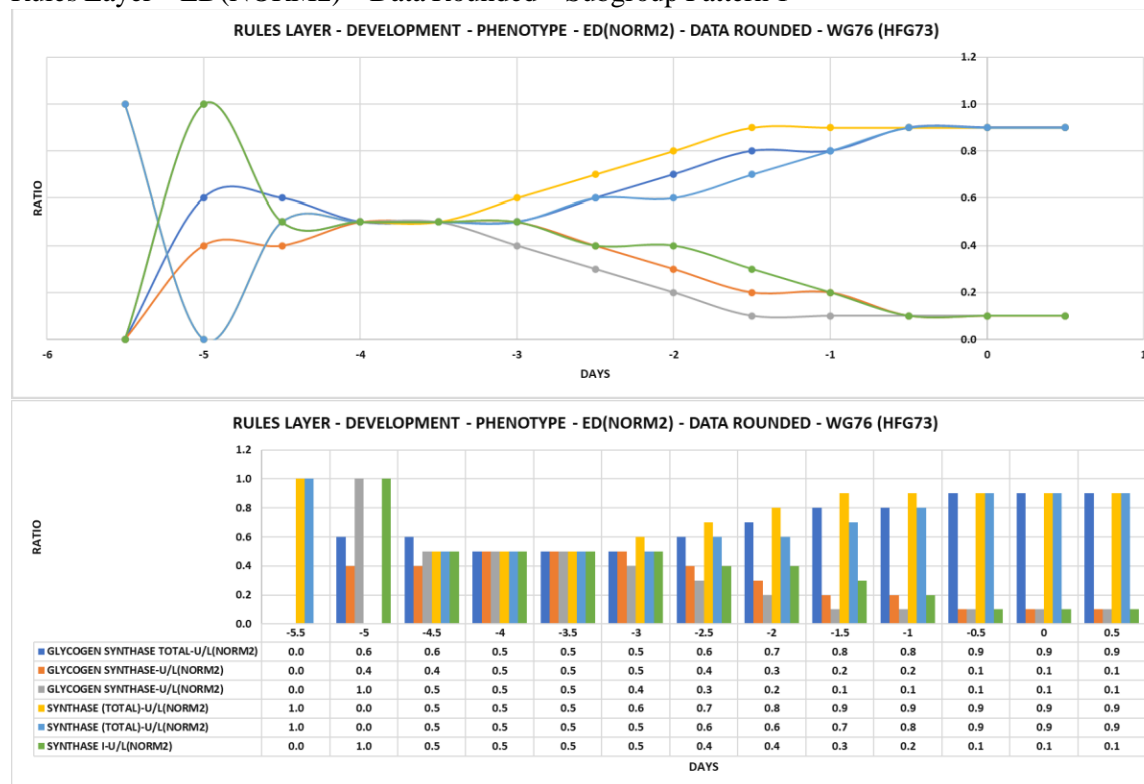


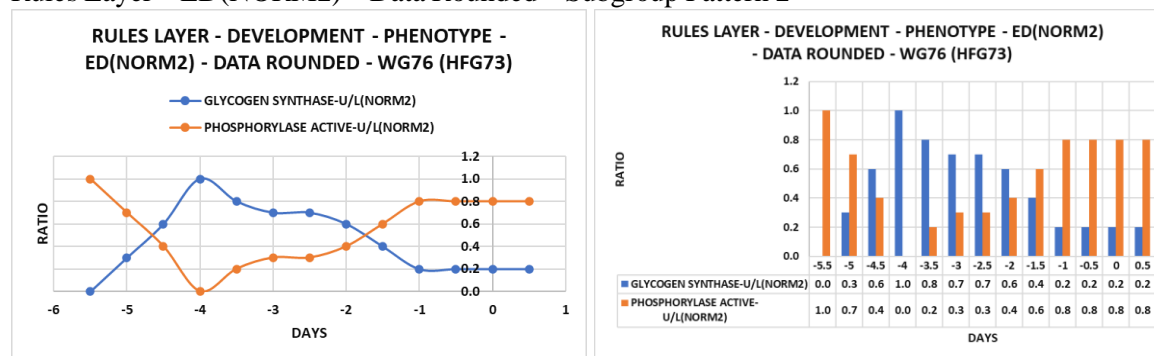
Figure 5.108 By combining the data pairs, a partial phenotype becomes forward engineered.

However, only three subgroups appeared, two without duplicates (Figure 5.109). Why? Postnatal differentiation has just begun.

Rules Layer – ED(NORM2) – Data Rounded – Subgroup Pattern 1



Rules Layer – ED(NORM2) – Data Rounded – Subgroup Pattern 2



Rules Layer – ED(NORM2) – Data Rounded – Subgroup Pattern 2

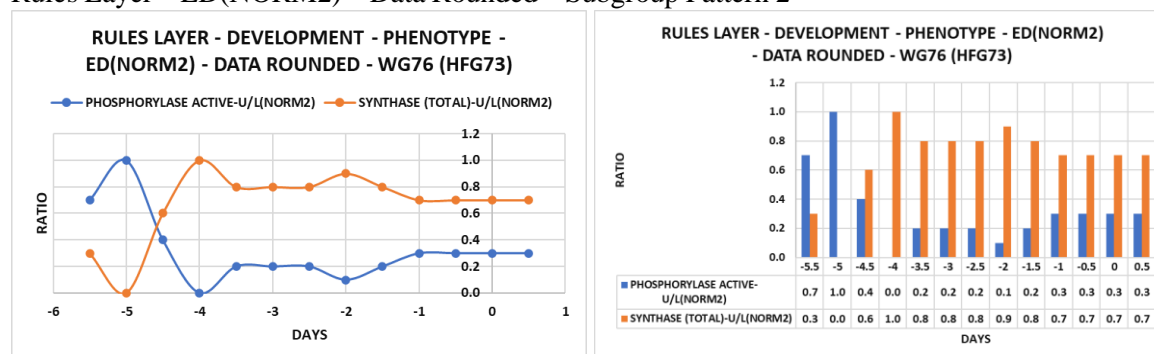


Figure 5.109 When reversed engineered, the phenotype yielded just three subgroups.

Comment: A curious pattern seems to have emerged from reverse engineering phenotypes separately from (1) enzymes and from (2) enzyme densities. By comparing the number of subgroups, prenatal development appears to focus more on relationships of enzyme to enzyme (function to function) than on those of membrane to enzyme (structure to function). This would suggest that relationships of structure to function wait for the new reality of the postnatal environment.

5.3.14 Case Study 14: Development (Glutaminase – Pre and Postnatal) – L69

Source: Update applied to original data from Linder-Horowitz M. (1969) Changes in glutaminase activities of rat liver and kidney during pre- and post-natal development. *Biochem J* 114: 65-69. ER membranes: Herzfeld et al., (1973);HFG73.

Topic: Morphological and biochemical development of the liver including responses of cells to hormones.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, calculate enzyme densities(NORM1), ED(NORM2), and analyze patterns.

Dataset: glutaminase, ER. The normalized enzyme densities (NORM2) used mixed references (U/G of liver, S/CM³ of hepatocyte cytoplasm) - see 5.3.10 and checks provided throughout the chapter.

Since learning new techniques includes working through the calculations, data tables routinely accompany the data plots and histograms. We'll use the update of the paper of Linder-Horowitz (1969;L69) and ER surface areas from Herzfeld et al., (1973;HFG73) to generate worked examples. Note that the update used mixed references (U/G, ER-M²/CM³ hepatocyte cytoplasm) to generate normalized enzyme densities (ED-NORM2).

Figure 5.110 includes the original data and two ways to fit the same points to different regression curves.

Original Data (Resembles the Published Figure) Original Data (Fails to Resemble Published Figure)

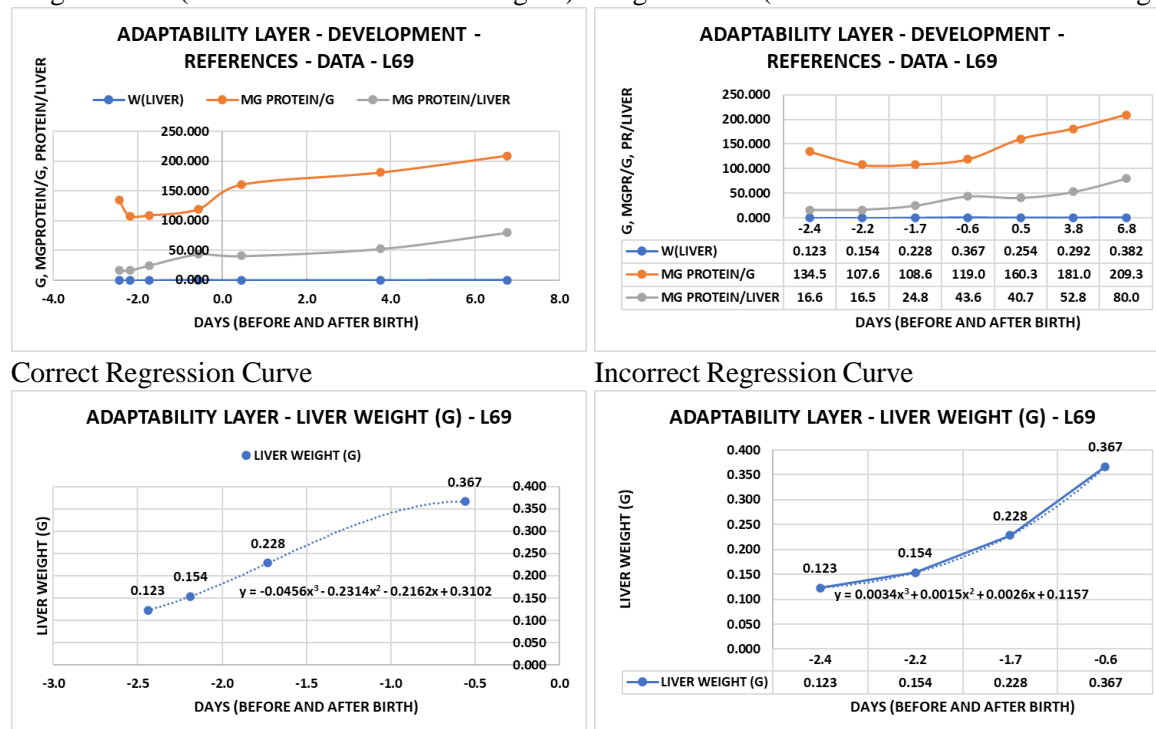


Figure 5.110 Exercising caution when including data tables with linear plots. Note the spacing of the points along the x axis.

First, let's look at the mischief being caused by one of the most often used references - the mg of protein (Figure 5.111). During development, the mix of the cells in the liver changes, the sizes of the hepatocytes change, the amount of protein in the cells change, and the number of cells filling a gram of liver change. The point? When biology changes its parts, we also change the parts according to the way we measure (estimate) them. But how much do we change things and in what directions?

Figure 5.111 shows the changes in the amount of protein per gram of liver and per liver, whereas the one at the right gives the difference (in %) between the two estimates. When using the mg protein as a reference for detecting changes in enzyme activities during early development, we can unwittingly add methodological changes to our results produced by changes (1) in the protein content of the hepatocytes, (2) in the number of hepatocytes filling a gram of liver, and (3) changes in other cell populations. To mitigate these problems, we can relate our results to the liver and interpret the results as ratios in the rules layer.

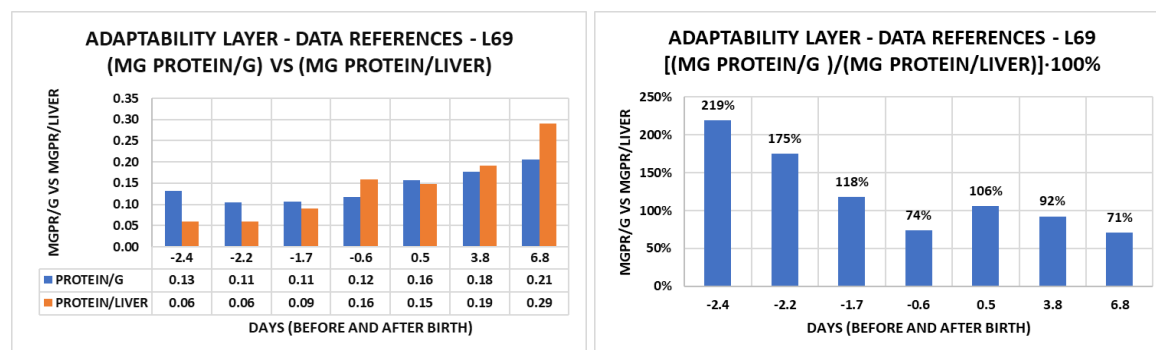
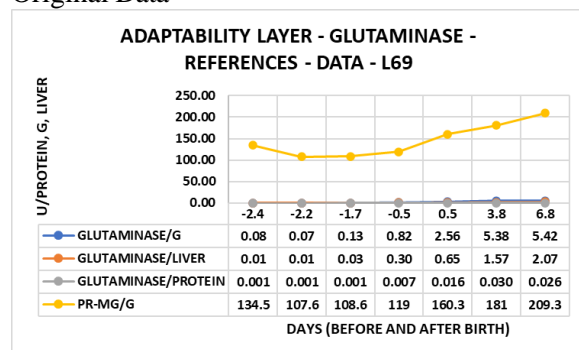


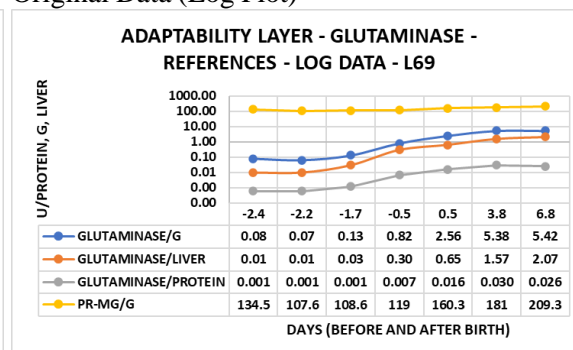
Figure 5.111 The liver detected the changes in protein correctly, but the gram of liver did not. The percentages show the extent to which protein related to a gram of liver missed the more accurate estimate coming from the liver reference. The errors are of the worst kind, big and variable. To avoid the cell packing problem, we can multiply the per gram of liver data by the weight of the liver or calculate data ratios using two parts taken from same time point.

The wide range of enzyme assays create visual problems (Figure 5.112). When working with enzyme activities in the adaptability layer, changes in small values often become reduced to straight lines. Changing the Y axis to a log scale solves the problem.

Original Data



Original Data (Log Plot)



Expanded Data

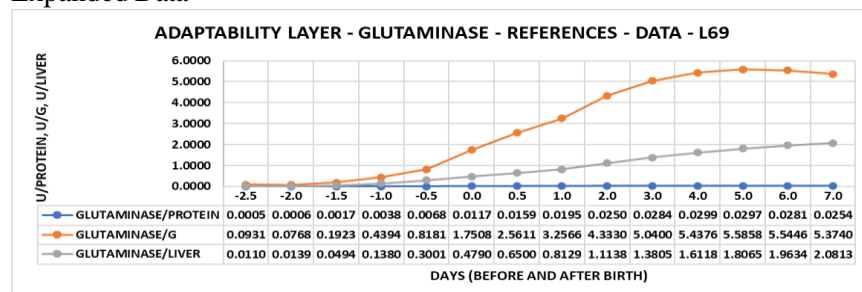
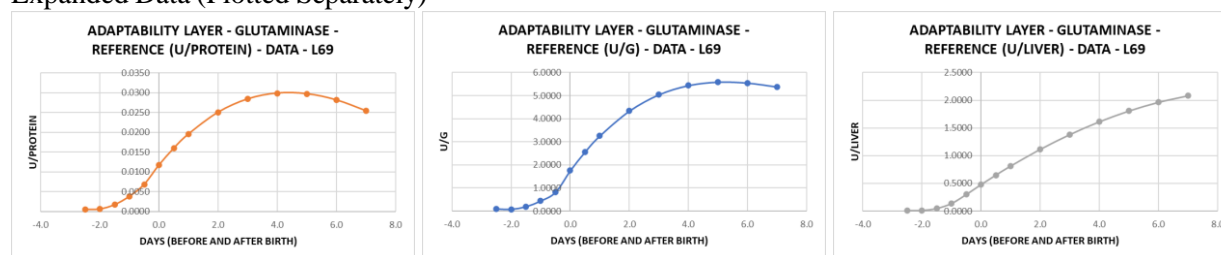


Figure 5.112 The same data related to three different references appear to change differently. Only one of the three curves, however, detects just the changes in enzyme activity. Once again, (1) The amount of protein in a cell can change, (2) the number of hepatocytes in a gram of liver can change, and (3) the number of cells in the liver can change or remain essentially constant.

Figure 5.113 shows the expanded data related to three different references before and after normalization. Using the liver as the gold standard, one can see how the other two references (protein, and gram of liver) tell noticeably different stories.

Expanded Data (Plotted Separately)



Expanded Data (NORM1)

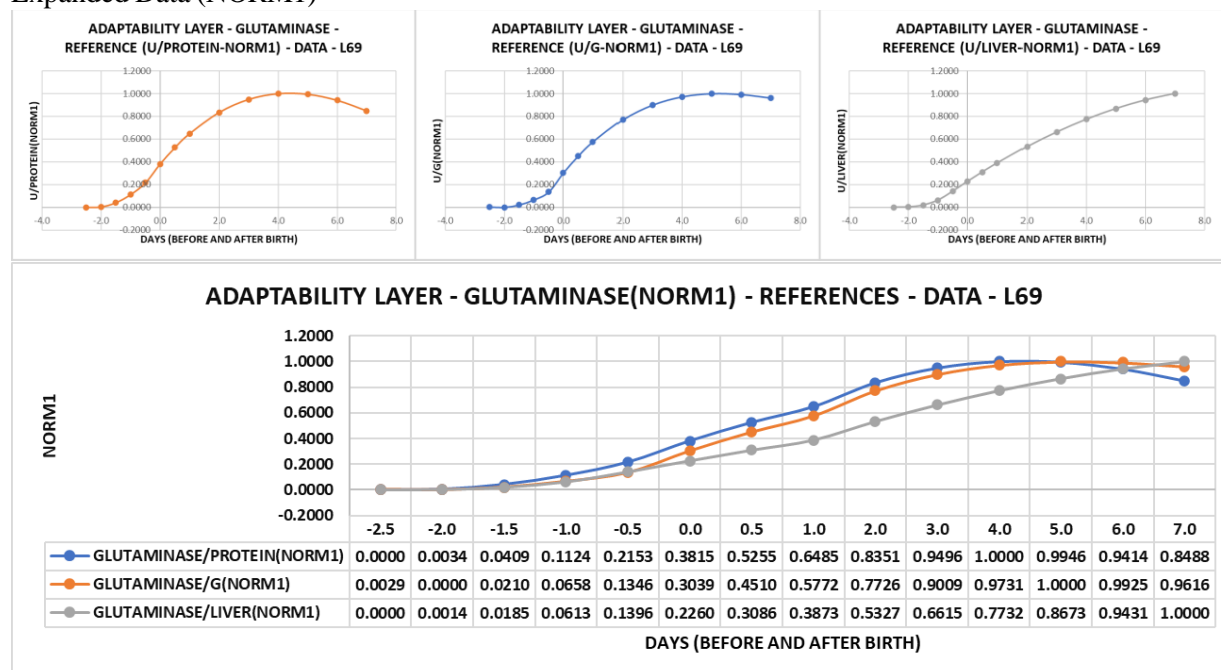


Figure 5.113 The original data expanded, normalized, plotted separately, and together. Given the variables contributing to each data reference (see previous figure), the liver delivered the best result, the gram of liver the second best, and the mg of protein the third best.

Figure 5.114 expresses the errors as percentages. Since the liver reference serves as the gold standard for detecting a biological change, we can use it to evaluate changes in enzyme activities related to the mg protein and gram of liver references.

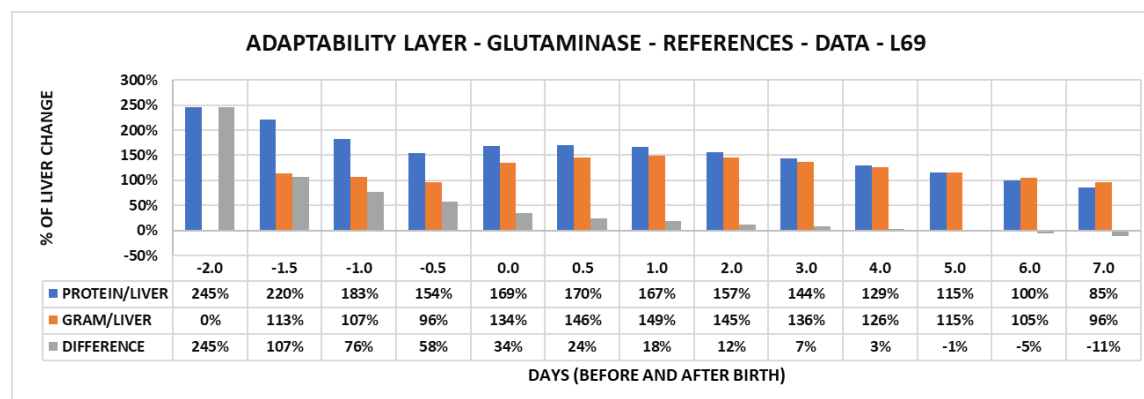
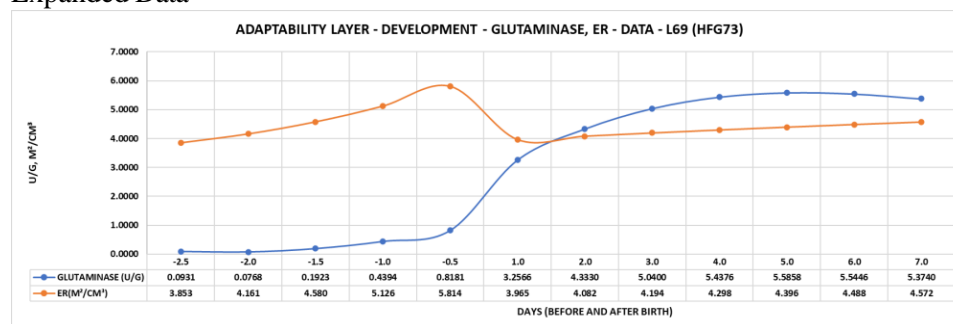


Figure 5.114 The histogram identifies changes in units of enzyme activities related to two references expressed as a percentage of the liver value $[(U/MG \text{ PROTEIN})/(U/LIVER) \times 100\%]$ and $[(U/GRAM)/(U/LIVER) \times 100\%]$. The DIFFERENCE row identifies the error associated with using the protein reference instead of the gram. Note that methodological errors produced by the choice of data reference can be substantial and extremely variable.

Now let's calculate the enzyme densities for glutaminase (a cytoplasmic enzyme) by relating its activity to the changes in the ER surface area (Figure 5.115). This creates a problem when, for example, the enzyme activity is related to a gram of liver and the ER to a cm^3 of hepatocytic cytoplasm. While this prevents a local estimate for the ED (incompatible references), we can calculate the ED(NORM2) from normalized data.

Expanded Data



Normalized Data: Enzymes and Membranes (NORM1) and ED(NORM2).

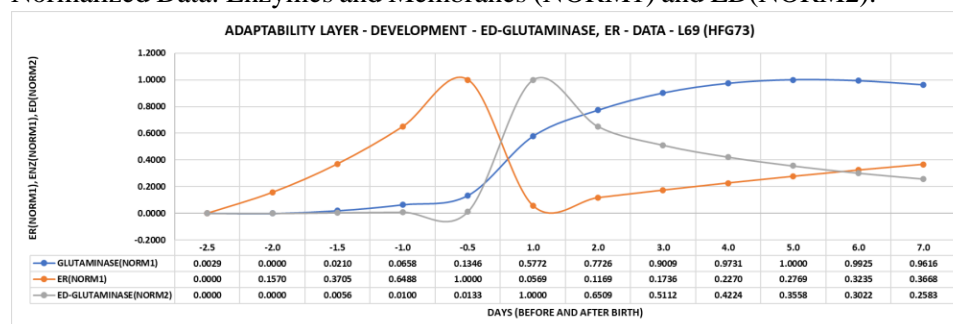


Figure 5.115 Note the crossover that occurred slightly after birth between the enzyme activity and the membrane surface area. By using the membrane as a reference for the changes in the soluble enzyme (glutaminase), we see how it changed relative to a set unit of surface area by calculating enzyme densities.

Comments: Another problem exists. Since much of the biochemical data in the case studies came from microsomes, we might be using total liver values based on roughly half the total liver activity (microsomes account for about 50% of the total ER activity). The strategy of the updating approach recognizes such shortcomings, but by using normalization we can still approximate the enzyme densities at a global level and test them for reproducibility.

5.3.15 Case Study 15: Development (Responses of Hepatocytes to Hormones) – GD67

Source: Update applied to original data from Greengard O., Dewey H. K. (1967) Initiation by glucagon of the premature development of tyrosine aminotransferase, serine dehydratase, and glucose-6-phosphatase in fetal rat liver. *J Biol Chem* 242, No. 12: 2986-2991.

Topic: Morphological and biochemical development of the liver including responses of cells to hormones.

Update: Apply corrections, expand data, report results in adaptability and rules layers.

Dataset: Tyrosine aminotransferase, serine dehydratase.

The authors (Greengard and Dewey 1967) followed the effects of glucagon and hydrocortisone on the development of two enzymes, tyrosine aminotransferase (TAT) and serine dehydratase (SD), in the fetus, newborn, and 50-day old rat. Although we can see the effects of the exposures on the enzyme activities in the adaptability layer, the updated results detected something new. Both glucagon (Figure 5.116) and hydrocortisone (Figure 5.117) triggered major changes to the rules. At 50 days postpartum, for example, serine dehydratase responded to both treatments by applying the same rule (0.5:0.5), whereas tyrosine aminotransferase used a 4:6 rule for glucagon and a 2:8 rule for hydrocortisone.

Adaptability and Rules Layers – Enzymes (TAT, SD) – Glucagon Induction

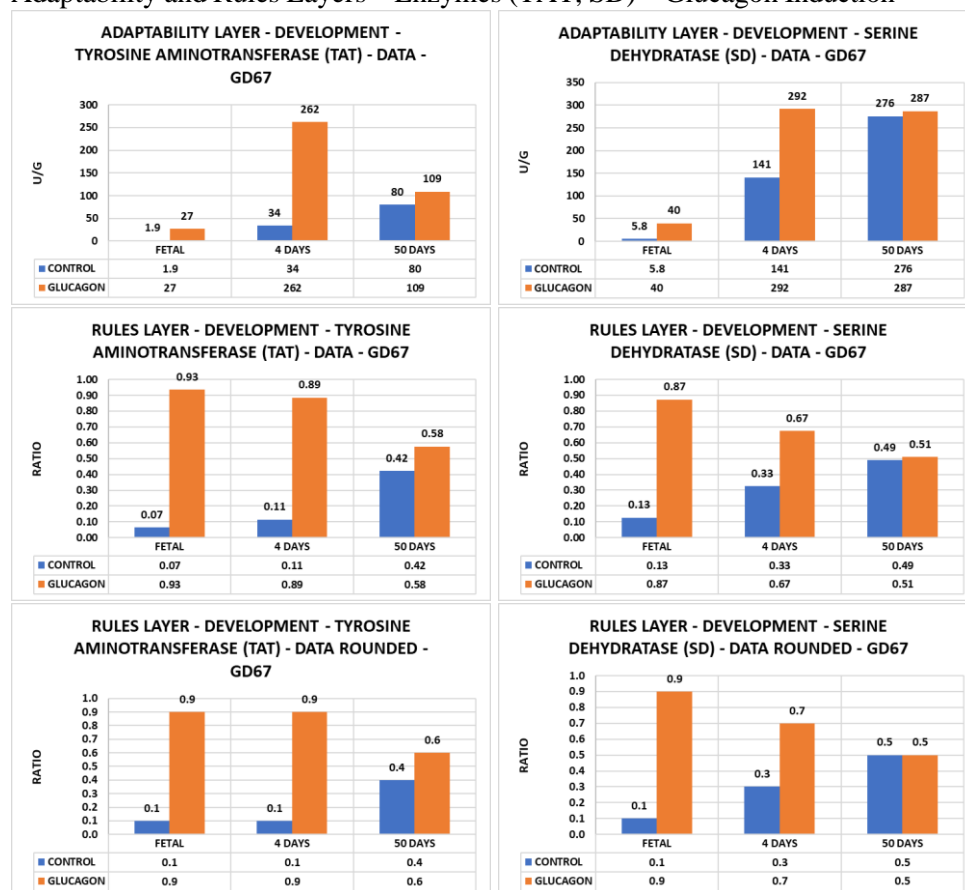


Figure 5.116 Postnatally, tyrosine aminotransferase and serine dehydratase responded differently to glucagon. By calculating ratios between two time points and not within the single data point, the cell packing problem becomes an issue. For the ratios as shown, one must assume that the number of cells remained the same per gram at days 4 and 50 (a high-risk assumption). The workaround is straightforward. Relate the data to the liver before forming the ratios. If both ratio estimates remain the same, then both came from the same number of cells. The liver-based ratios remove the risky assumption. Apply the same workaround to the hydrocortisone data.

Adaptability and Rules Layers – Enzymes (TAT, SD) – Hydrocortisone Induction

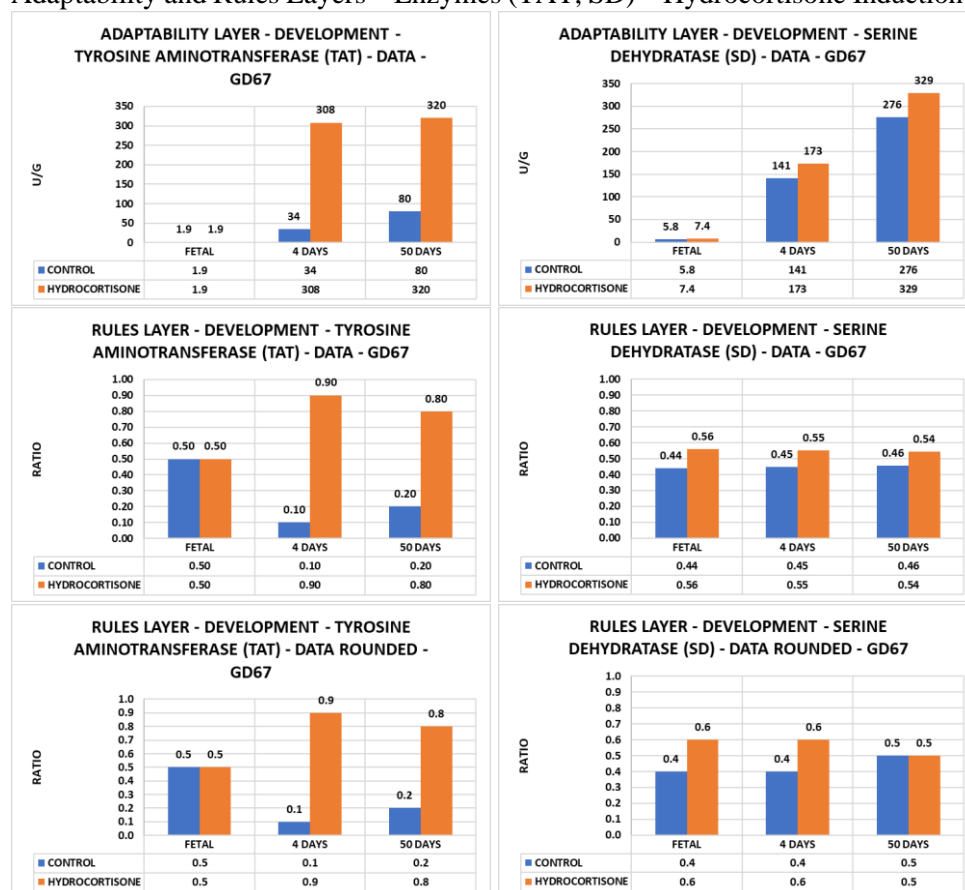


Figure 5.117 Postnatally, tyrosine aminotransferase and serine dehydratase responded differently to hydrocortisone, which once again resulted from differences in the controls. Note the ratios.

Summary: We can interpret the combined results using ratios (Figure 5.118) and changes expressed as a percentage of the control (Figure 5.119).

Summary (Ratios)

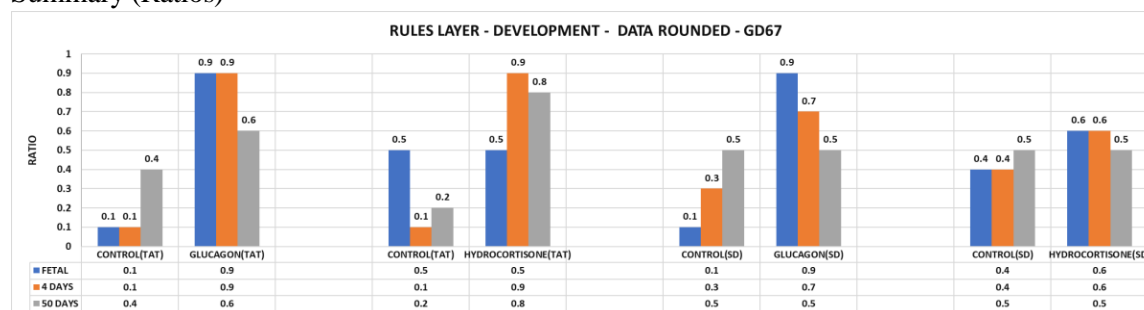


Figure 5.118 Each enzyme responded differently at different times to the hormone treatments.

Expressing the results as percentages of the controls summarizes the results wherein three distinct patterns emerge for each time point.

Summary (% of control)



Figure 5.119 Each enzyme responded differently at different times to the hormone treatments. 100% indicates no change.

Comment: The same hormones influenced the same cells (hepatocytes) differently at different times. The point? Cells can respond to the same information (hormones) differently in different developmental settings. The distributions, amounts, and sensitivities of the hormone receptors presumably played a role in the results. The methodological distractors include (1) potentially changing number of hepatocytes per gram of liver and (2) ratios calculated from two different data points (control vs experimental) containing unknown numbers of hepatocytes per gram of liver. The solution? Calculate such results per gram of liver and per liver. Such data will remove the ambiguity of the implied assumptions.

CHAPTER 6

INDUCED PHENOTYPES

SUMMARY

Hepatocytes quickly transform into an induction phenotype in response to xenobiotics. Such a transformation executes the change needed to solve the problem, which consists of upgrading (1) enzyme-membrane recipes and (2) increasing liver capacity. After removing the foreign substance, the hepatocytes and liver return to their normal state. This second change occurs when hepatocytes grow back into to their original phenotype while recycling the induced parts. After introducing the induction model, we'll go directly to the case studies and practice updating and interpreting the results. The takeaway from the chapter reinforces the point that understanding the mechanics of a change requires a basic dataset. Moreover, analyzing results begins by knowing what problem(s) the cells were trying to solve and ends by figuring out if and how they were successful.

Induction Model

The induction model duplicates the one described for developmental phenotypes. This model accommodates situations that require rapid responses (adaptability) to assure survivability. When forced to triage their resources, cells reveal their priorities. We'll follow changes in hepatocytes by (1) updating published reports, (2) using data pair ratios to reconstruct phenotypes, and (3) reverse engineering the changes to identify the subgroups of the induced phenotype. The model reinforces the view that a cellular change is not a single event but instead a complex set of changes working together to solve a problem.

Phenotypes: Recall that a local phenotype works with the original data of one experiment, whereas the global option uses normalized data to aggregate results from several publications. Figure 6.1 summarizes the information flow of the phenotype.

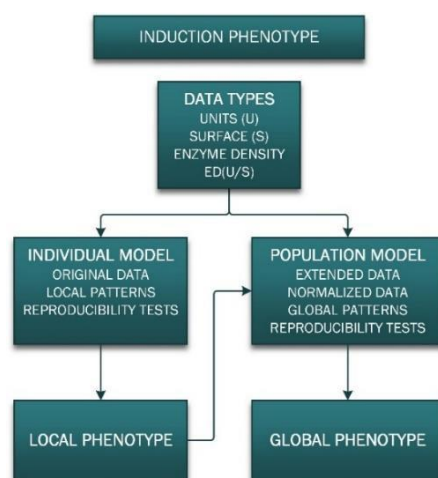


Figure 6.1 The induction phenotype uses three basic pieces of information to detect and explain a biological change. By supporting both local and global interpretations, it optimizes a wide range of outcomes. Patterned after the way biology changes, it recognizes that detecting a change represents a problem-solving event wherein the cell becomes the solution.

6.1 Case Studies

The case studies follow the same updating procedures described in the previous chapter. In this chapter, hepatocytes respond to several inducers including barbiturates, Diazepam, vitamin B6, urease, thallium chloride, and chenodeoxycholic acid.

6.1.1 Case Study 1: Phenobarbital (Fed and Fasted Animals) – SG71

Source: Update applied to original data from Stetten M. R., Ghosh S. B. (1971) different properties of glucose-6-phosphatase and related enzymes in rough and smooth endoplasmic reticular membranes. Biochim Biophys Acta 233: 165-175. ER membranes: Herzfeld et al., (1973);HFG73.

Topic: Biochemical responses of hepatocytes to phenobarbital treatment.

Update: Apply corrections, expand data, report results in adaptability and rules layers.

Dataset: Assays for inorganic pyrophosphatase and glucose-6 phosphatase.

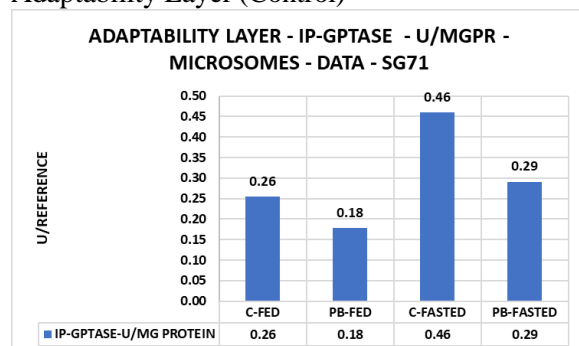
Stetten and Ghosh (1971; SG71) compared the effects of feeding and fasting on phenobarbital (PB) induced changes in two microsomal enzymes: (1) inorganic pyrophosphatase (IP-GPTASE) and (2) glucose-6-phosphatase (G6PASE). Experimental animals received intraperitoneal injections of sodium phenobarbital (80 mg/kg/5 days) with results related to two references (U/mg protein and U/g).

When preparing microsomes or estimating ER membrane surface areas, we fast the animals because it depletes the hepatocytic stores of glycogen. Apparently, this leads to better microsomal recoveries (perhaps by minimizes membrane clumping) and improves stereological estimates of ER surface areas *in situ* (glycogen particles can obscure SER membranes in electron micrographs).

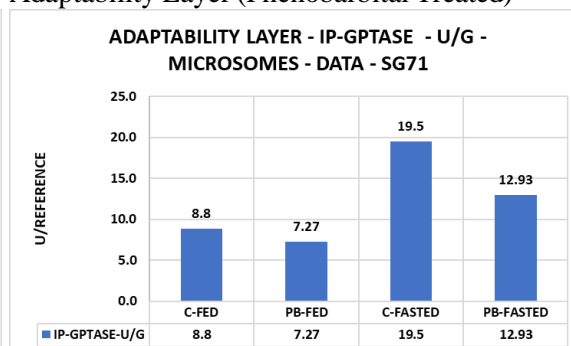
The update compares results related to a mg of protein and to a gram of liver. We want to know the extent to which our choice of data reference affects the results (Figures 6.2 and 6.3).

IP-GPTASE

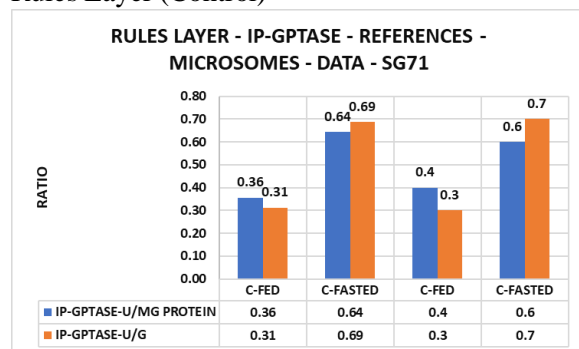
Adaptability Layer (Control)



Adaptability Layer (Phenobarbital Treated)



Rules Layer (Control)



Rules Layer (Phenobarbital Treated)

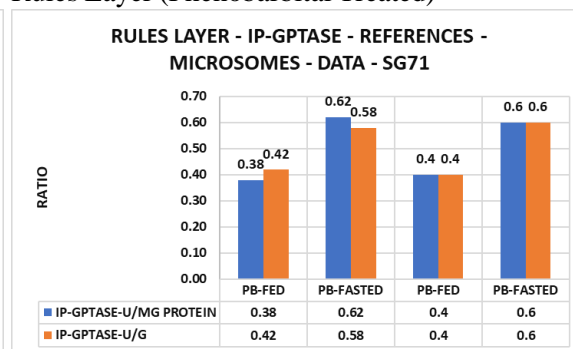
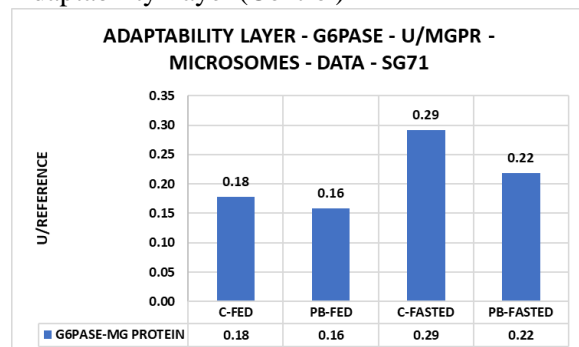


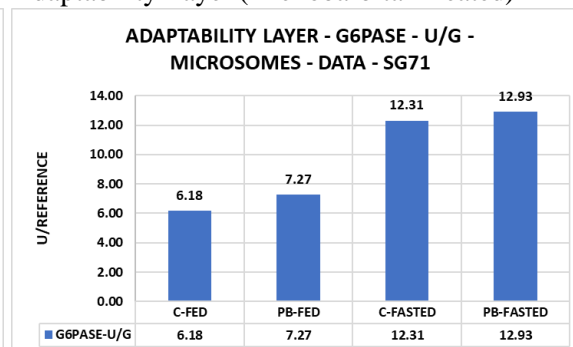
Figure 6.2 Changes in IP-GPTASE activity detected with mg protein and gram as references. In the adaptability layer, the fasted animals displayed larger amounts of enzyme activities than those of the fed group – in both control and phenobarbital (PB) settings. The rules layer, which included ratios of data pairs (data and data rounded), the fed and fasted animals displayed similar ratios when treated with PB (0.4:0.6) but not for the controls. Once again, the different data references produced different results. Since ratios were calculated across animals (fed vs fasted), the cell packing problem became an issue (a risky assumption).

G6PASE

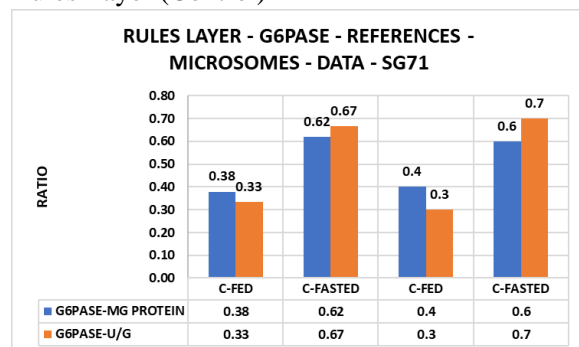
Adaptability Layer (Control)



Adaptability Layer (Phenobarbital Treated)



Rules Layer (Control)



Rules Layer (Phenobarbital Treated)

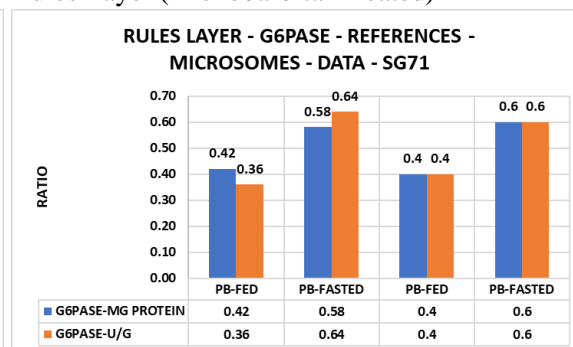


Figure 6.3 In the adaptability layer, the fasted animals displayed larger amounts of enzyme activities than the fed animals for both control and phenobarbital (PB) treated. The pattern seen in for IP-GPTASE for the U/G reference (C-FED, C-FASTED) duplicated that of the G6PASE.

Question 1: Why did the different data references (mg protein vs g liver) give different results (0.4:0.6 vs 0.3:0.7) for the controls but not for the phenobarbital treated animals (0.4:0.6 vs 0.4:0.6)? We can't answer the question because we don't have (1) the liver weights, (2) the enzyme densities, (3) the total

liver values, and (4) the microsomal recoveries [recovery = $U(\text{microsomes}/U(\text{homogenate}))$]. In other words, we don't have enough information to answer the question.

Question 2: Figure 6.4 separates the data by reference (U/mg protein and U/g). The mg protein gave the same set of results (ratios) for both enzymes. The gram of liver reference produced variable results in that the controls (fed and fasted) of both enzymes displayed a different ratio (3:7).

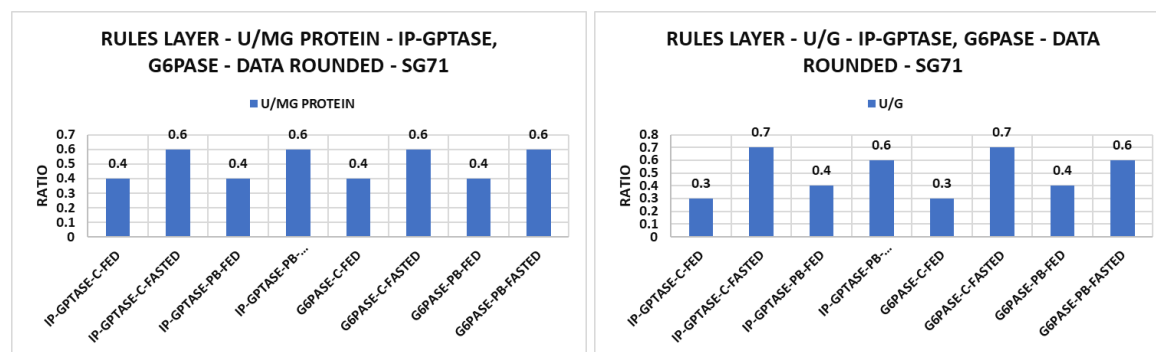


Figure 6.4 The two data references produced different results that the current dataset cannot explain.

Question 3: What else should we know? Calculating ratios with data taken from two different experimental animals introduced uncertainty. For example, hepatocytes treated with phenobarbital for five days became larger and fewer of them could fit into a gram of liver.

Comment: When an experimental question puts many variables in play, they become potential distractors: (1) control vs PB, fed vs fasted, (2) unstable data references (changes in the protein content of cells and in the number of cells per gram), and (3) the variability of microsomal fractions (percentage of total ER membranes recovered). In effect, seemingly simple questions call for complex answers.

6.1.2 Case Study 2: Barbiturates (Microsomal Enzymes) – VVAJ74

Source: Update applied to original data from Valerino D. M., Vesell E. S., Aurori K. C., Johnson A. O. (1974) Effects of various barbiturates on hepatic microsomal enzymes. A comparative study. *Drug Metab Dispos* 2:448-457.

Topic: Biochemical responses of hepatocytes to barbiturates.

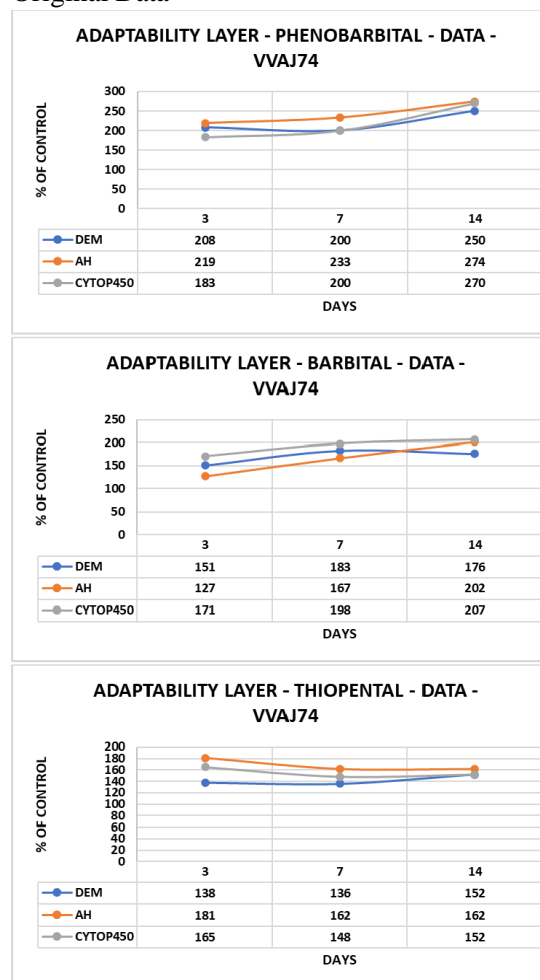
Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, and analyze patterns.

Dataset: n-demethylase (DEM), aniline hydroxylase (AH), and cytochrome p-450 (CYTOP450).

Valerino et al., (1974; VVAJ74) reported the effects of barbiturates on microsomal (ER) membranes. The update used pairs of enzymes to detect unique responses of hepatocytes to specific drugs in the rules layer. Duplicate patterns of change occurred only between phenobarbital and barbitol.

The liver enzymes used in the study included n-demethylase (DEM), aniline hydroxylase (AH), and cytochrome p-450 (CYTOP450). The experimental animals received daily doses of a drug as follows: phenobarbital (100 mg/kg/day), barbitol (150 mg/kg/day), and thiopental (100 mg/kg/day). Figure 6.5 summarizes the original and expanded data.

Original Data



Expanded Data

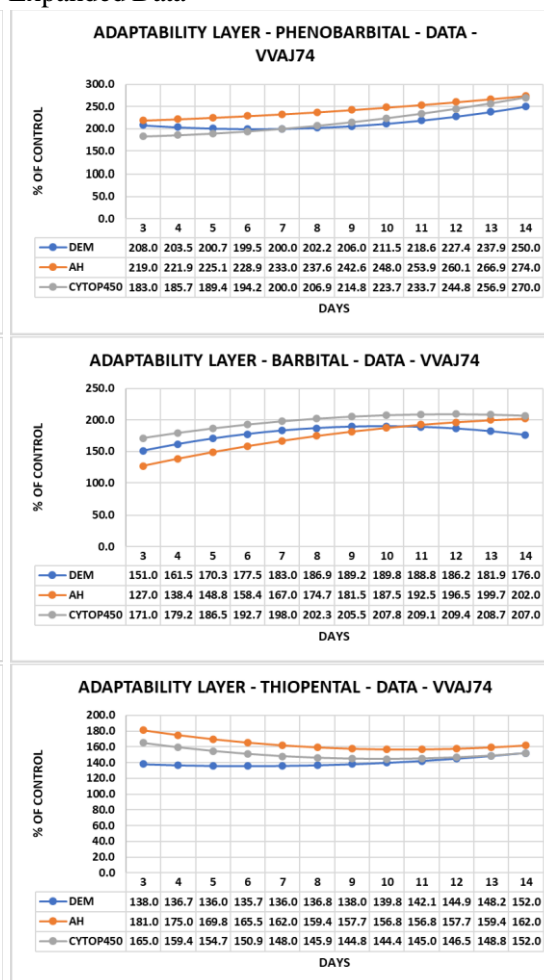


Figure 6.5 The responses of microsomal enzymes to different drugs displayed different patterns of change over 14 days.

Since the biochemical activities were related to a mg of protein, normalization allowed us to compare the changes in enzyme activities – without knowing if or how the expected changes in cell and liver volumes produced by the drugs influenced the results (Figure 6.6). We can, however, use the normalized ratio data in the rules layer to look for duplicate patterns in the responses of the enzymes to the drugs – without interference from the cell packing problem because the enzyme data came from the same livers.

Normalized Data (NORM1)

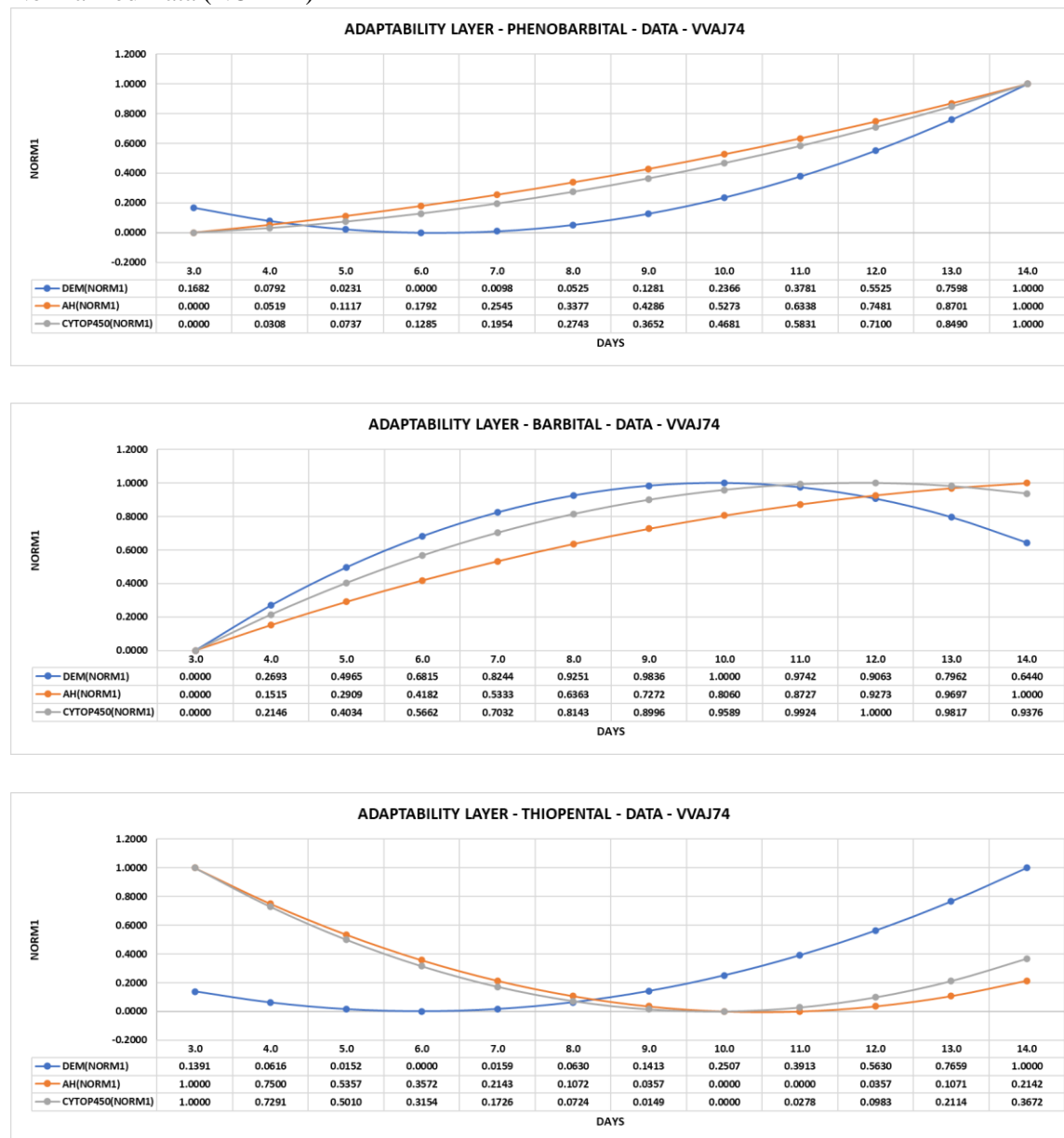
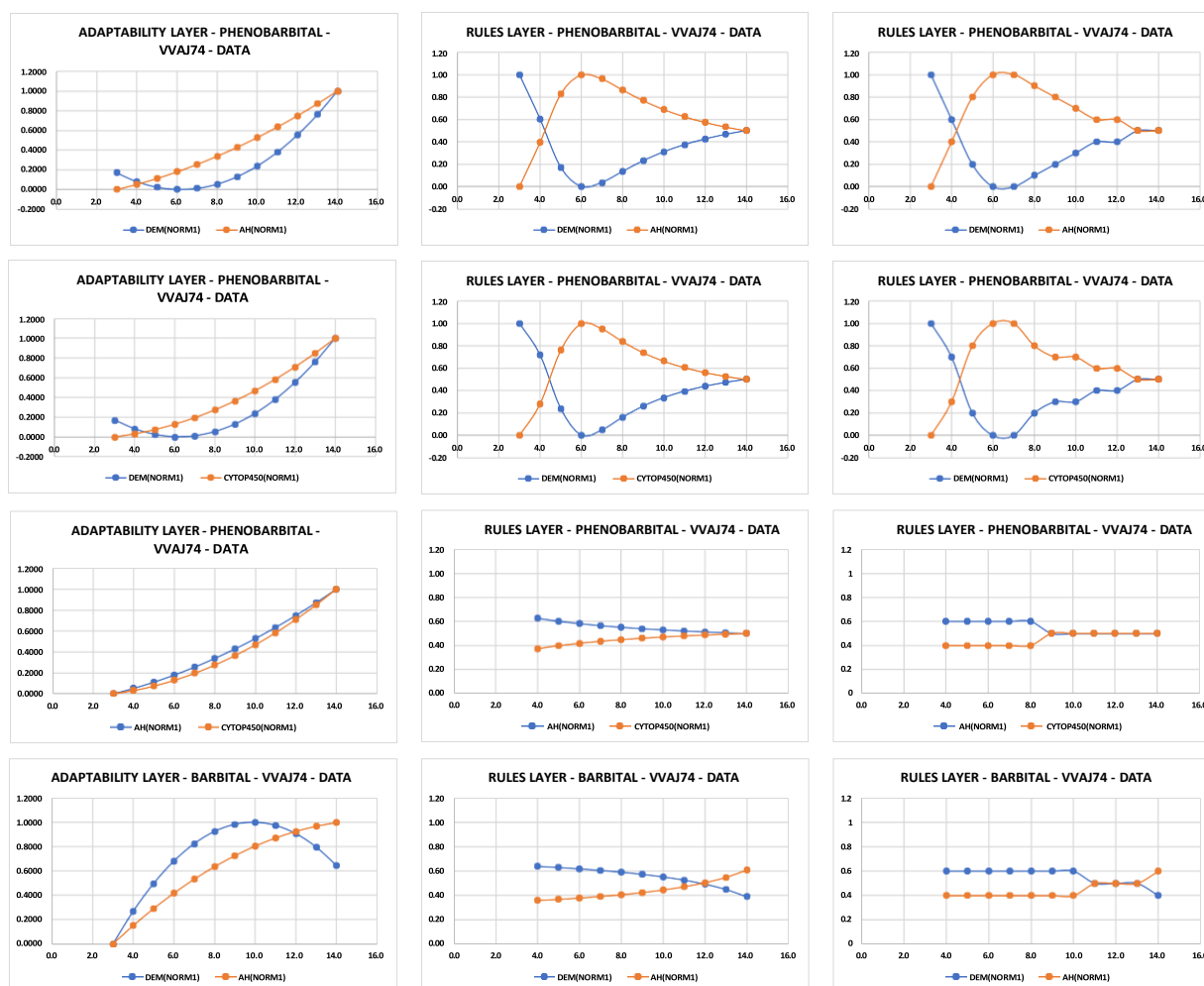


Figure 6.6 The normalized data show the responses of the hepatocytes to the three drugs. Each drug provided a unique set of curves, which means that the hepatocytes produced unique recipes for each drug.

Patterns (Enzymes): Figure 6.7 displays patterns produced in the rules layer by forming pairs of enzymes from the panel of barbiturates, which show that hepatocytes typically generate responses unique to a given drug. In one case, two related drugs (phenobarbital and barbital) generated duplicate curves involving n-demethylase (DEM), aniline hydroxylase (AH), and cytochrome p-450 (CYTOP450). Since some of the enzyme data pairs used to form ratios in the rules layer displayed duplicates, they identified subgroups sharing similar outcomes (Figure 6.8). Forward engineering consisted of recombining data pair ratios from the rules layer (data rounded) to reconstruct phenotypes, whereas reverse engineering reversed the process by taking the phenotype apart to identify the location of each duplicated data pair in a subgroup.

Rules Layer – (NORM1)



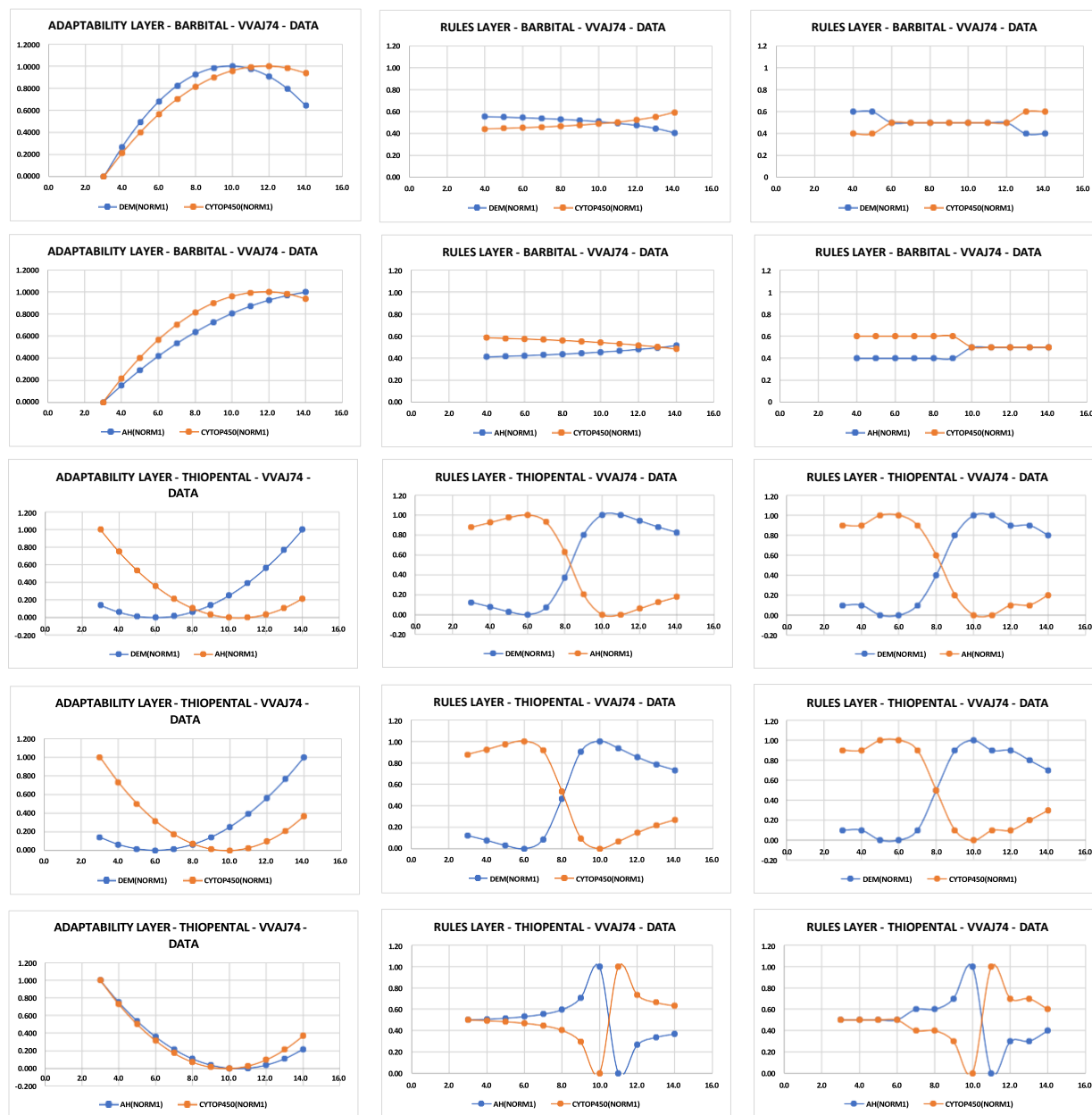
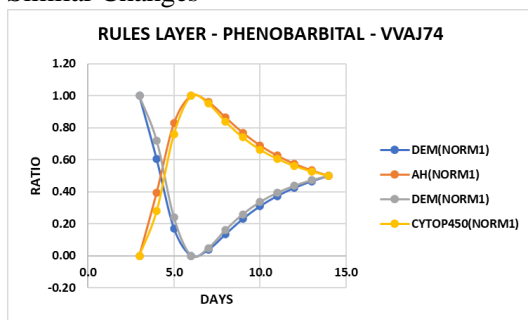


Figure 6.7 By plotting the interactions of the enzyme data pairs, patterns appeared in the rules layer.

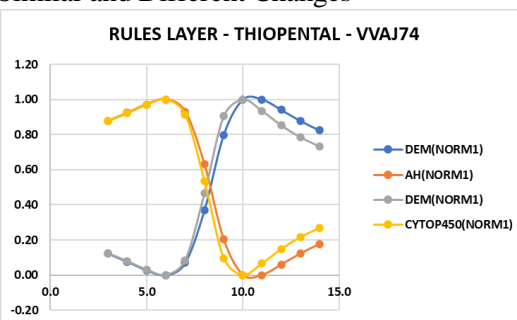
Since some of the enzyme data pairs used to form ratios in the rules layer displayed duplicates, they identified subgroups sharing similar outcomes (Figure 6.8). Forward engineering consisted of recombining data pair ratios from the rules layer (data rounded) to reconstruct phenotypes, whereas reverse engineering reversed the process to identify subgroups using duplicated data pair ratios.

Overlapping Patterns (Method 1): The overlapping patterns identified the times and locations when different pairs of enzymes responded similarly when exposed to different drugs. Figure 6.8 makes the point that results can be similar or different depending on the drug and when and where we look.

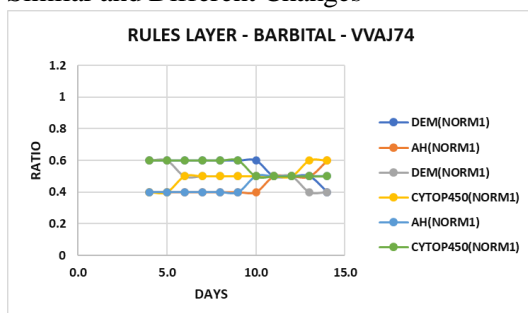
Similar Changes



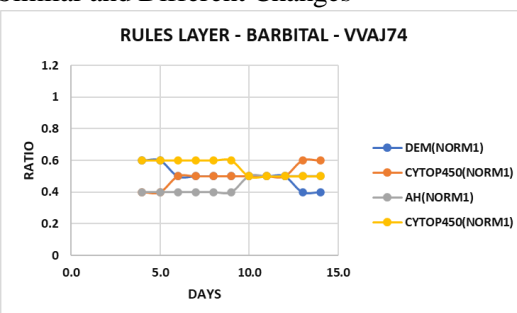
Similar and Different Changes



Similar and Different Changes



Similar and Different Changes



Similar and Different Changes

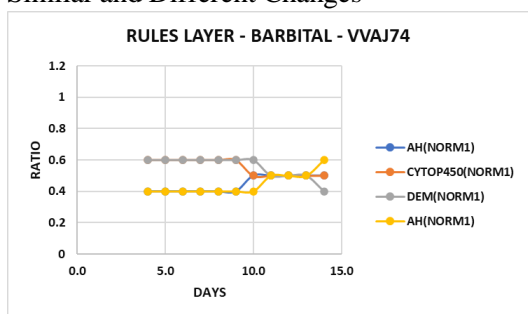


Figure 6.8 Usually duplicate data pair ratios occurred for responses to the same drug, with a single exception (see Figure 6.9).

Figure 6.9 shows that the same pairs of enzymes exposed to two similar drugs displayed the same solution at days 10 to 14 (as defined by at least three days of similar and consecutive ratios).

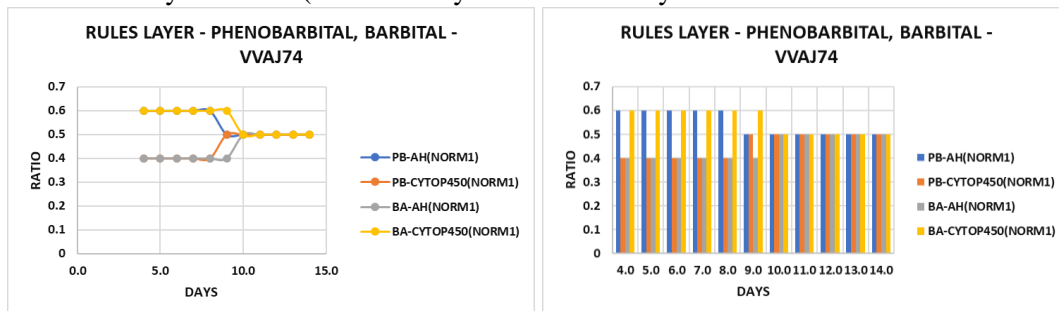


Figure 6.9 Duplicate patterns (data pair ratios) occurred when the same enzymes responded to different drugs.

Overlapping Patterns (Method 2): Alternatively, we can combine all the changes of the enzyme data pairs to form a phenotype (forward engineer) and then take it apart (reverse engineer) to look for similar

responses produced by the different drugs. This allows us to identify subgroups of data pairs contributing to the same solution.

Figure 6.10 includes a partially reconstructed phenotype with what may include the beginning of two solutions at day 14 (0.5:0.5, 0.4:0.6).

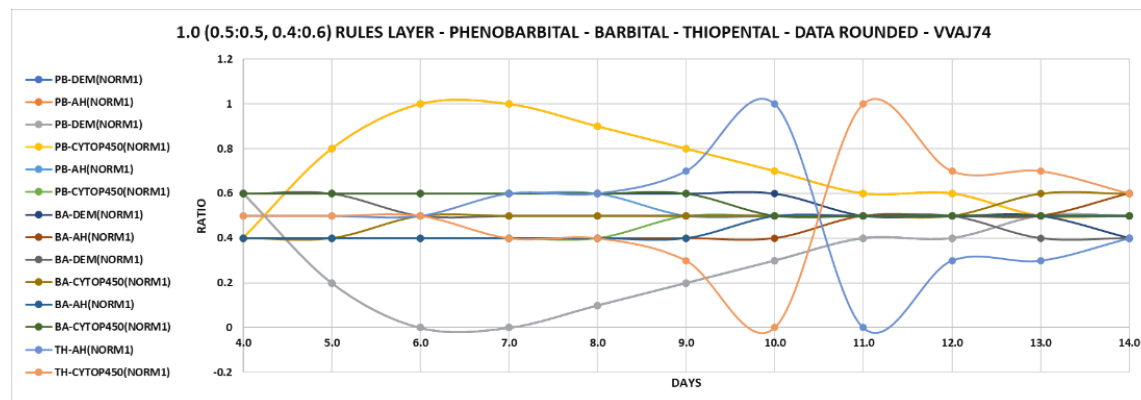


Figure 6.10 The combined responses of the hepatocytes to the drugs form a drug-response phenotype. Unfolding the phenotype into subgroups provides insights into the way the responses progressed over time.

The reverse engineering procedure separated the multiple drug phenotype into 0.5:0.5 and 0.4:0.6 subgroups according to the ratios at day 14 (Figure 6.11). The 0.5:0.5 subgroup detected similar solutions for two drugs - PB and BA; the 0.4:0.6 subgroup detected similar solutions for a different pair of pair of drugs – BA and TH.

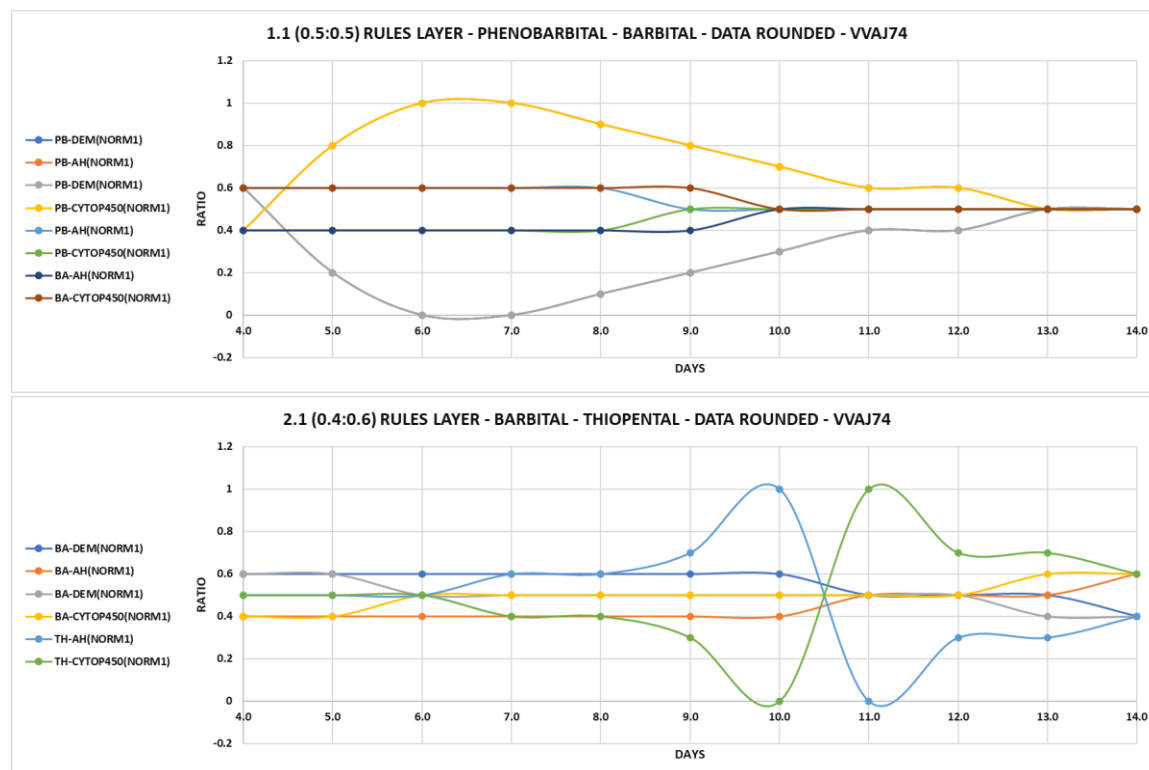


Figure 6.11 Two drugs may have found the same solution at day 14 using different strategies.

By continuing to separate the 0.5:0.5 subgroup of Figure 6.11 into just two data pairs (Figure 6.12), we find that the same pair of enzymes displayed almost the identical pattern of change when treated with two different drugs (PB and BA). In effect, the hepatocytes reproduced a result occurring in two different groups of animals exposed to different drugs. This identifies an experimental setting that might lead to an explanation of how and why this pair of enzymes shifted from one pattern of change (0.4:0.6) to another (0.5:0.5) at day 9. Specifically, what happened between days 8 and 10? Did gene expression change? Did the recipes of the enzyme densities change? Aging, for example, occurs at a time when long-established rules begin to collapse. Figuring out how cells shift from one set of rules to another becomes basic to understanding how cells change.

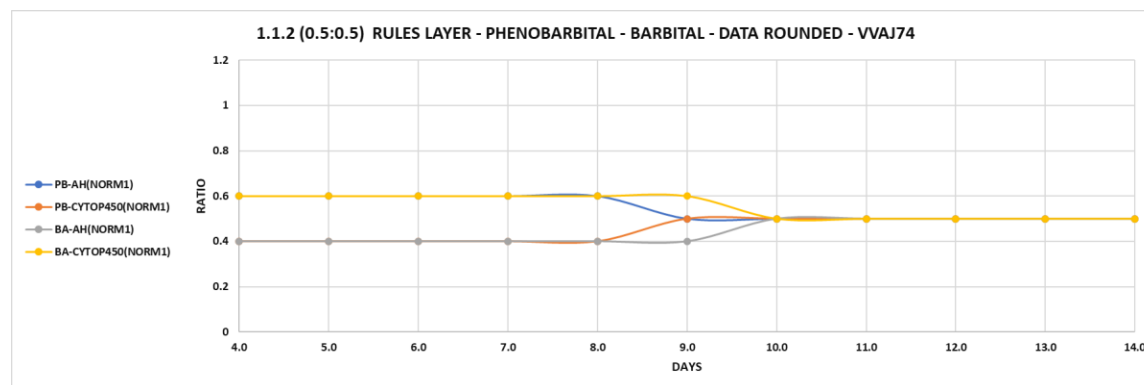


Figure 6.12 Two drugs found the same solution at day 10 using almost identical strategies.

Summary: By generating data pairs and using them to reconstruct mixed phenotypes, hepatocytes exposed to various drugs responded similarly and differently by adjusting their design rules. The timing of these adjustments points directly to a rule changing event of unknown origin that might have been triggered locally in the cytoplasm or upstream from the genome. Once again, reverse engineering a change leads to new information, ideas, and questions that need specific data to answer.

6.1.3 Case Study 3: Diazepam (Patients) – JKO74

Source: Update applied to original data from Jezequel M., Koch M., Orlandi F. (1974) A morphometric study of the endoplasmic reticulum in human hepatocytes: Correlation between morphological and biochemical data in subjects under treatment with certain drugs. Gut 15, 737-747.

Topic: Morphological responses of hepatocytes to diazepam.

Update: Apply corrections, expand data, report results in adaptability and rules layers, and analyze patterns.

Dataset: ER surface areas (RER, SER).

When addressing the central challenge of designing reproducible experiments, a better understanding of sampling theory as it relates to detecting biological changes benefits outcomes. Although we know that reproducibility becomes accessible experimentally by adopting biology's approach to change, how might we use the data of the rules layer to update our notion of representative sampling? Since the study by Jezequel et al., (1974; JKO74) includes individual patient data, we can explore empirically an answer to the question.

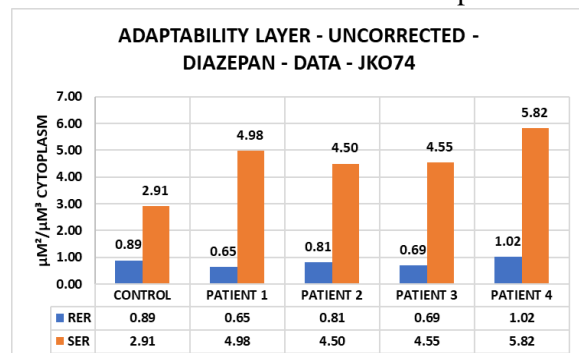
In the first study described in this paper, the investigators administered diazepam to four patients. When viewed in the adaptability layer, the changes of the RER and SER in response to the drug showed the expected individual variability. But the same data viewed in the rules layer displayed a constant ratio (RER:SER (local) = 8:2). The advantage of such a result is that it predicts the expected global response of the population as well (RER:SER (global) = 8:2). If a deep-rooted connection exists between experimental reproducibility and representative sampling, then encountering or ensuring reproducibility at the level of individuals would define a homogeneous population. If true, then enforcing reproducibility at the level of individuals – prior to calculating mean values – should result in greater success when attempting to demonstrate reproducibility at the population level. In effect, testing for reproducibility locally might improve experimental outcomes.

Think of it this way. Instead of starting with a population of similar individuals and if everyone has an equal chance of inclusion in the sample (definition of representative sampling applied to the adaptability layer), extending the representative sampling test to the rules layer would assure that we were dealing with a homogeneous population. Why, for example, would we want to study and compare heterogeneous groups displaying greater variation?

But why haven't we explored this approach to reproducibility earlier? Because our data wasn't up to the task. If we switch back to the original dataset uncorrected for section thickness and compression, then we get a conflicting result (Figure 6.13). The surface areas of the RER and SER become quite different, the ratio values change and lose their reproducibility. In short, in the absence of corrections for section thickness and compression, estimates for the membrane surface areas would have detected a heterogeneous population of patients when in fact they were homogeneous.

Original Data

Uncorrected for section thickness/compression



Corrected for section thickness/compression

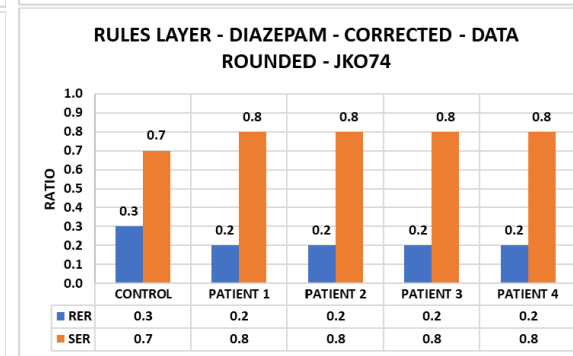
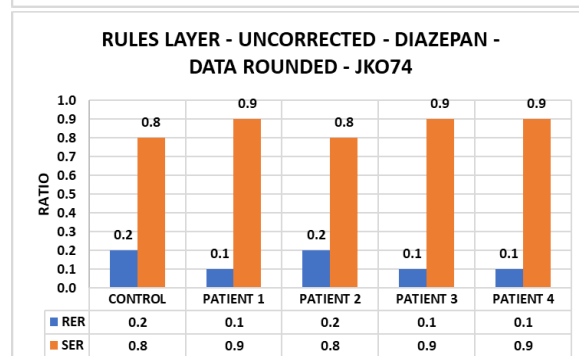
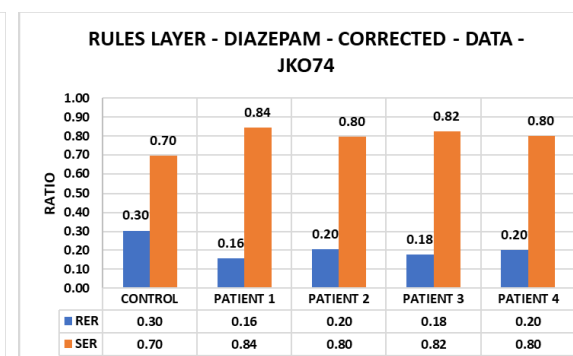
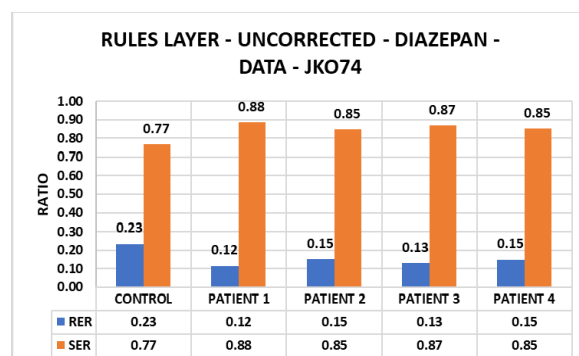
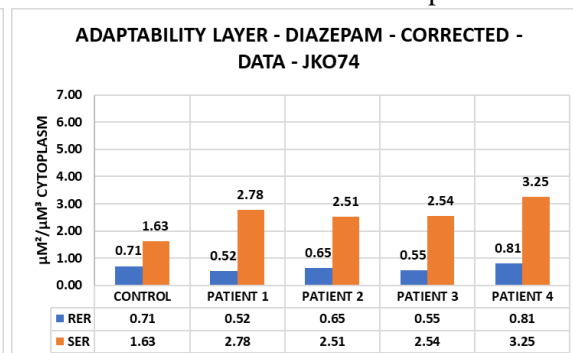


Figure 6.13 Correcting stereological estimates for section thickness and compression markedly improves the quality of the results. Uncorrected data identified a heterogeneous group of patients, whereas corrected data found homogeneous sampling. Identifying reproducibility at the outset eliminated distractions regularly imposed by heterogeneous populations. The point? Biased data produce biased results, which become more difficult to reproduce.

Summary: The uncorrected data found a heterogeneous group of patients whereas the corrected data discovered a homogeneous group. In effect, the rules layer allows us to use reproducibility at the level of individuals to demonstrate homogeneous (representative) sampling. Would representative sampling also gives us a mean value with a smaller standard deviation? Possibly. The implication? Were we to publish raw data (individual values), then the biology literature would become a renewable resource and we would improve our chances of answering such questions. For example, after correcting the individual ER estimates for section thickness and compression, we could have rerun the statistics to look for significant differences (p value plus effect size). *But how would we show a significant difference between a control and experimental phenotype?*

6.1.4 Case Study 4: Phenobarbital (RNA Synthesis) – HSR83

Source: Update applied to original data from Hardwick J.P., Schwalm F., Richardson, A. (1983) The effect of phenobarbital on the transcriptional activity of liver. *Biochem J* 210: 599-606.

Topic: Biochemical response to phenobarbital treatment.

Update: Report results in adaptability layer and analyze patterns.

Dataset: RNA.

Phenobarbital induces the synthesis of RNA, which appears first in the nucleus and then in the cytoplasm (Hardwick et al., 1983; HSR83). Of interest in the Figure 6.14 is the linear order (expressed as $R^2 = 0.999$) by which the production and transfer of mRNA occurs. Note, however, that the slopes of the two curves suggest a slower appearance of the radioisotope (labeled mRNA) in the cytoplasm. In any case, this is the second time we see RNA change linearly with an $R^2 \approx 1$ (Wanson et al., 1975), which suggests that such a pathway might serve as a temporal and quantitative link between gene expression and phenotypes.

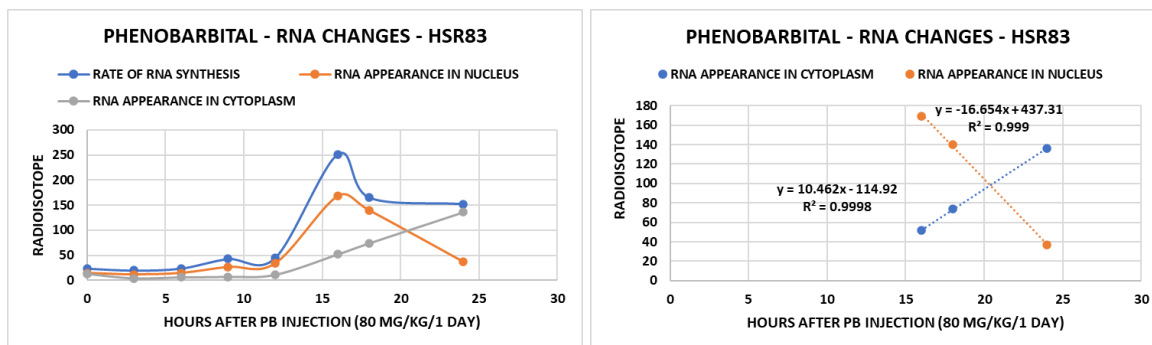
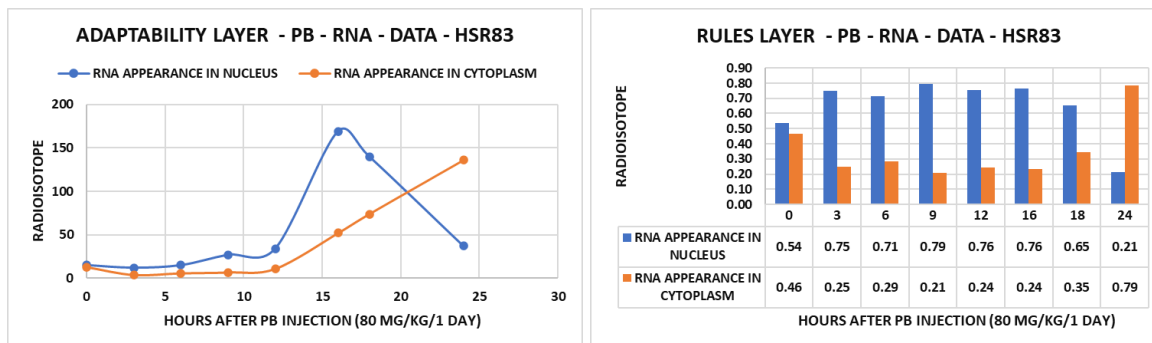


Figure 6.14 The timing of production and delivery of RNA from the nucleus to the cytoplasm might be short enough to account for many of the changes occurring in the phenotypes but perhaps not all. Curiously, hepatocytes have a history of responding to critical events with linear curves.

Notice in the rules layer below (Figure 6.15) that it took a day for the RNA produced in the nucleus to reach the cytoplasm. Synthesizing enzyme proteins from mRNA, assembling effective membrane-enzyme recipes, and distributing them to optimal locations in the cell also takes time.



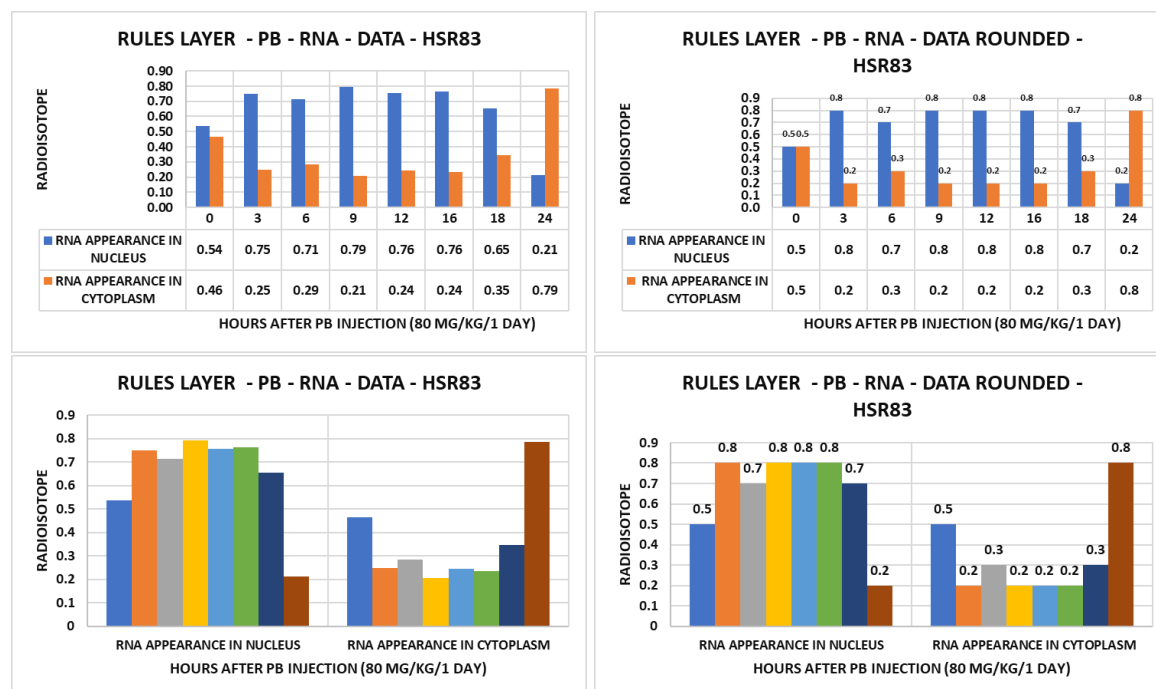


Figure 6.15 Moving RNA from the nucleus to the cytoplasm occurs as a highly ordered event. The patterns suggest an internal symmetry with an axis of rotation at 12 hours but a lateral switch in color (location) at 24 hours. Using the same ratio at the start and end of the exchange might suggest a quantitative coding link between cause and effect. We've seen this pattern of variables switching positions at days 3 (start) and 24 (end) before.

Comment: A cell supports two interconnected control mechanisms, one identified by changes occurring in the nucleus (DNA, RNA) and the other from the cytoplasm (enzymes, membranes, organelles). Understanding the temporal relationships of one to the other during changes becomes central to working out the patterns of the cell's information processing algorithms.

6.1.5 Case Study 5: Phenobarbital (Injured Livers) – NFHK94

Source: Update applied to original data from Noguchi M., Fujitsuka T., Honda K., Kawai Y. (1994) Effects of phenobarbital on drug metabolizing enzyme activities and other biochemical parameters in rats with DL-ethionine-induced liver injury. *J Toxicol Sci* 19:203-212. ER membranes: SWH69[Stäubli et al., (1969)].

Topic: Biochemical responses of hepatocytes to phenobarbital treatment.

Update: Apply corrections, report results in adaptability and rules layers, normalize data, and analyze patterns.

Dataset: Aminopyrine demethylase (DEM) and aniline 4-hydroxylase (ANH).

In this study, Noguchi et al., (1994; NFHK94), compared the activity of two microsomal enzymes [aminopyrine demethylase (DEM) and aniline 4-hydroxylase (ANH)] in control livers to those injured by exposure to DL-ethionine (ET). Both control and experimental animals received a single dose (oral; 100 mg/kgbw) of phenobarbital (PB).

The original data (Figure 6.16) compared the weights of the livers, microsomal protein/gram liver (largely ER), and enzyme activities (DEM, ANH). When normalized and viewed in the rules layer at 32 hours, the pattern of change for the two enzymes was similar.

Original Data

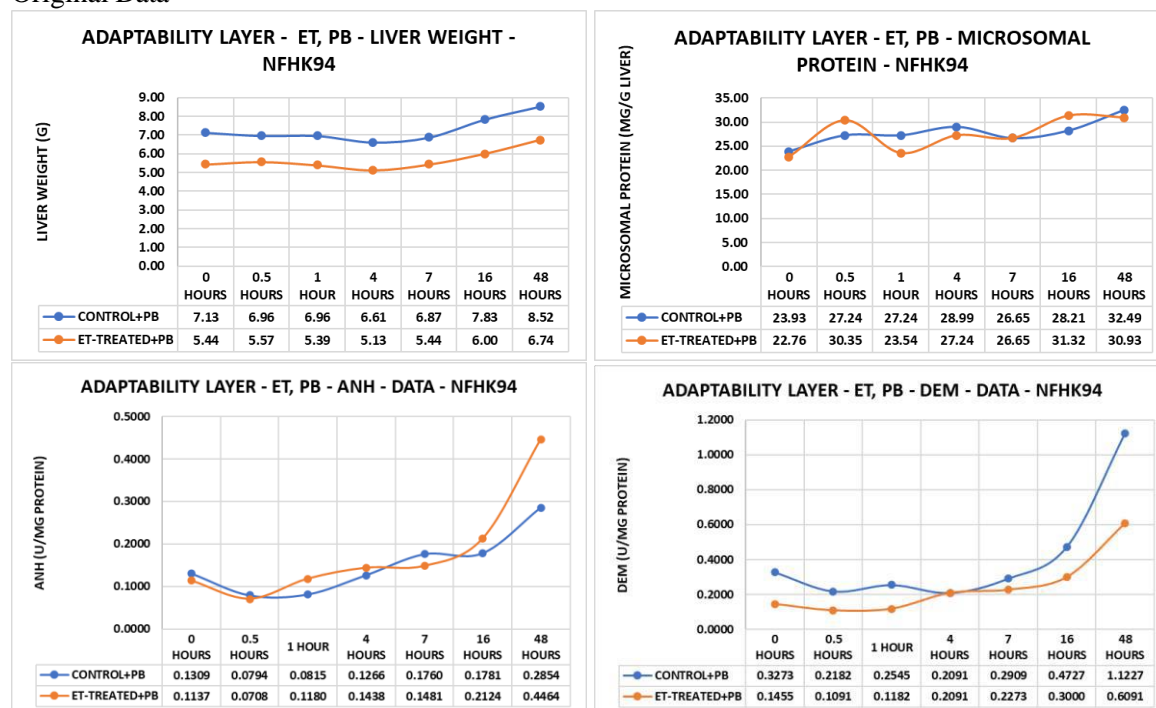


Figure 6.16 When first exposed to phenobarbital, the liver weight appeared to decrease slightly as the hepatocytes recycled old parts and generated new ones. At first, exposure to DL-ethionine muted the response of DEM to PB.

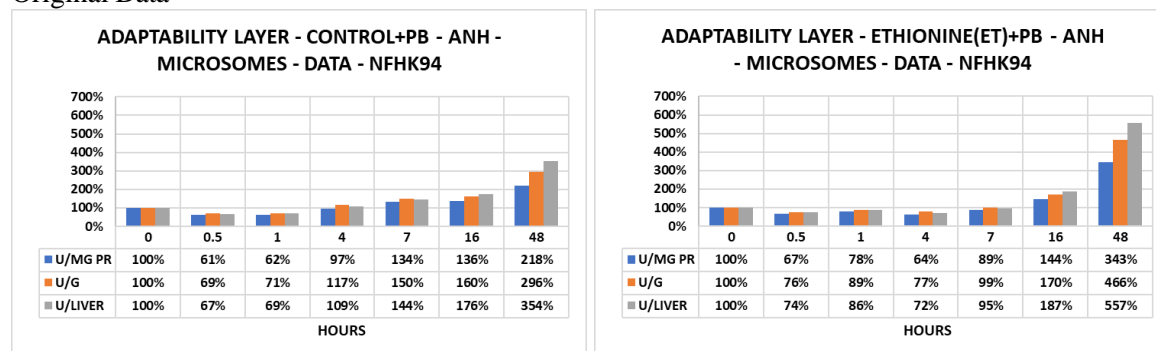
When given a problem to solve, solutions come from both the hepatocytes and the liver. By changing its phenotype, the hepatocytes change the capacity of the liver, which ultimately solves the problem. Expressing results relative to a mg protein or gram of liver, however, dilutes the authenticity of a result

because the data references become variables, the protein content and volume of the average cell change, as does the number of cells filling a gram of liver. Fortunately, there's a simple fix. Relate the results to the liver. The liver reference offers yet another advantage. When the number of cells per liver remains essentially constant, relative changes per liver equals relative changes per average cell.

Figure 6.17 summarizes the biochemical changes as a percentage of the control value (100%). Why were the results so different at forty-eight hours? The mg protein and the gram of liver references include a known set of variable errors that became readily apparent when updating published data. By exchanging accuracy and precision for a significant difference, one quietly assumes that the experimental errors associated with the mg protein and gram of liver references do not have an important effect on detecting or validating a “biological change.” Such assumptions - made within the widely supported framework of reductionism – have left published data without a rule-based mechanism to verify and reproduce results.

The single, most unrewarding outcome of likening a significant difference to a biological change is that the experiment abruptly ends. Moreover, our ability to reproduce, predict, explain, understand, and verify the results in terms of the underlaying first principles ends as well. The promise that we can recover from the criticisms of meta-analyses by simply passing p tests and effect sizes (Ioannidis et al., 2015) clearly demonstrates a disconnect between the basic principles of a significant difference and those of a biological change.

Original Data



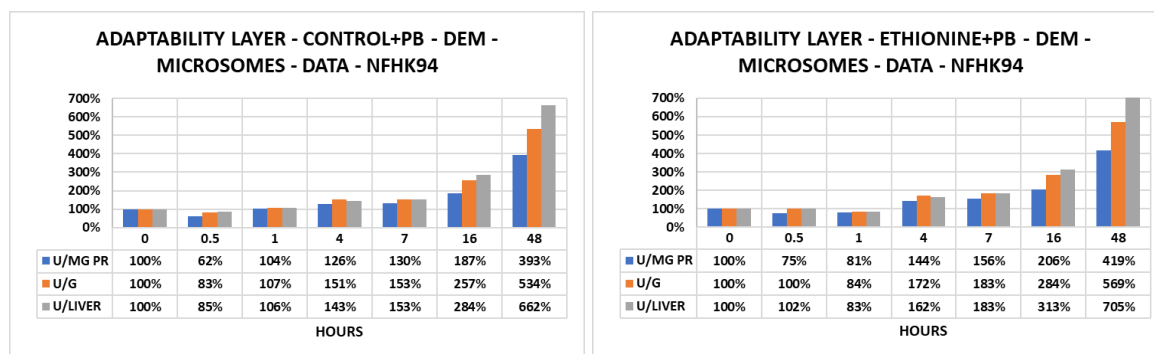


Figure 6.17 Notice how the data reference had a direct bearing on the amount of change detected. The liver detected the largest changes, whereas the mg protein the smallest. This pattern appears repeatedly throughout the case studies.

By normalizing the data, we can form data pairs and look for patterns in the rules layer (Figures 6.18 and 6.19).

Normalized Data (NORM1)

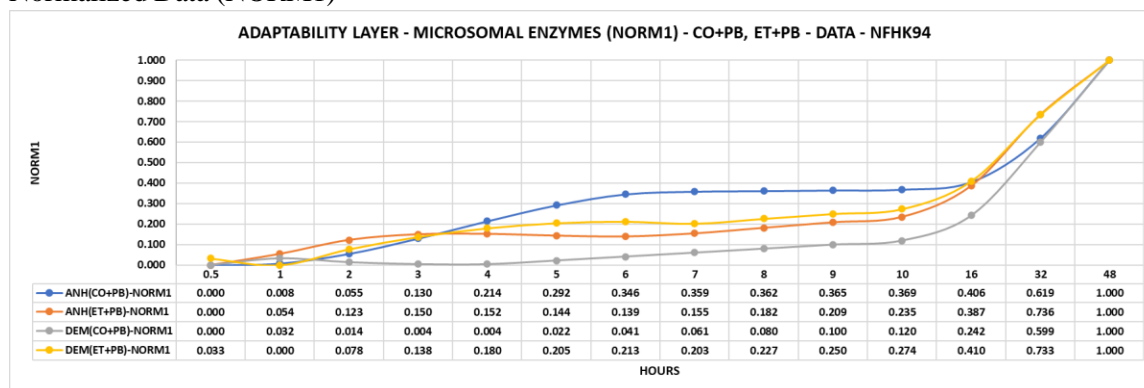
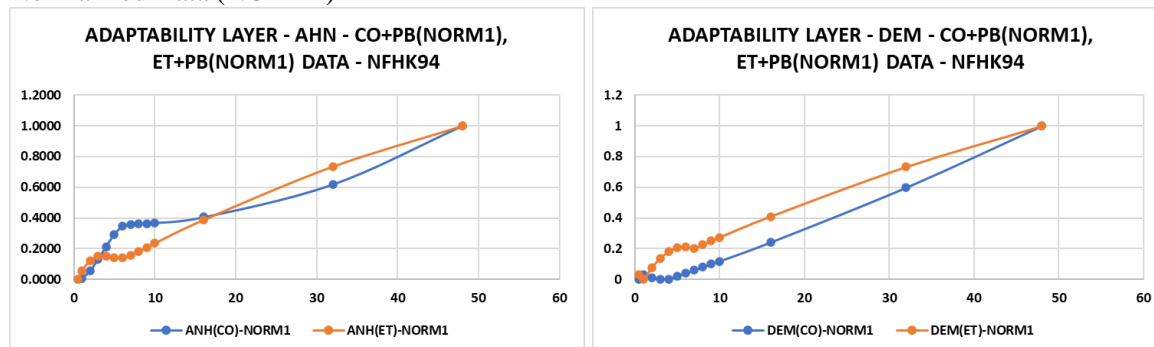


Figure 6.18 Both enzymes (ANH and DEM) responded similarly to the PB and ET exposure.

In Figures 6.19 and 6.20, a solution to the problem (ethionine+PB) for aniline 4-hydroxylase (ANH) seems to have occurred at 16 hours (0.5:0.5), whereas the one for aminopyrine n-demethylase (DEM) remained a work in progress at 48 hours. However, without the enzyme densities, we don't know how the hepatocytes found the solutions by altering their enzyme-membrane recipes.

Normalized Data (NORM1)



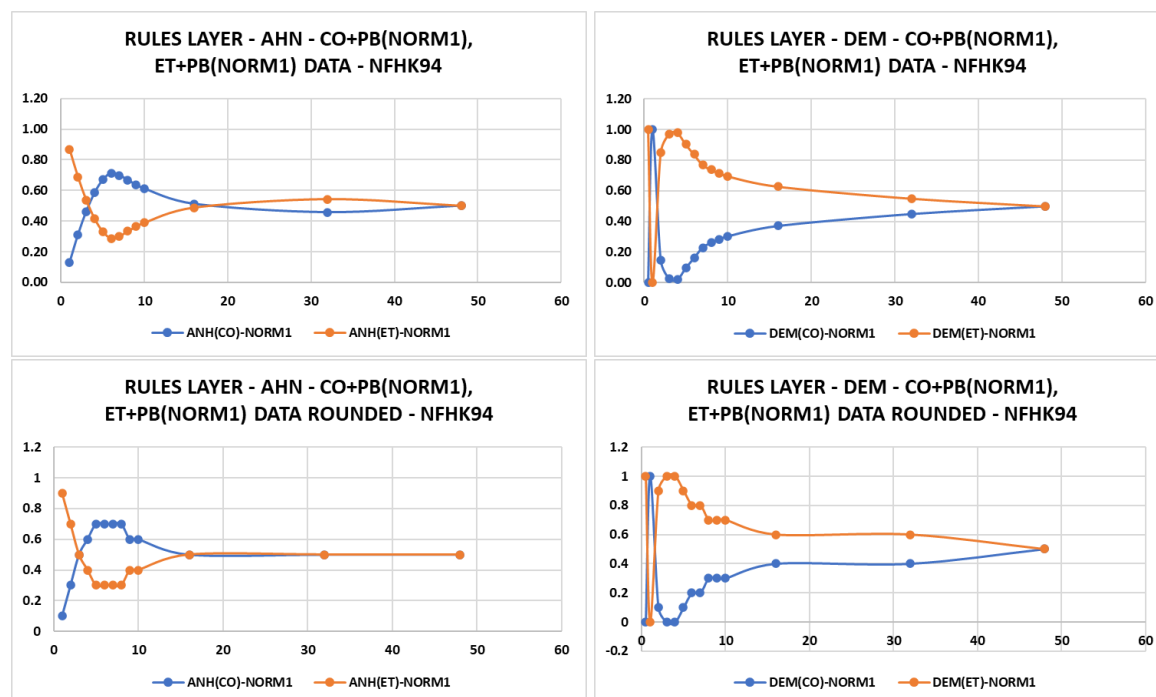


Figure 6.19 The rules defining the relationship between the control and experimental data differed for the two enzymes. Recall, however, that the data compared came from different groups of animals that displayed differences in liver volumes. Such differences suggest that the average size of the hepatocytes may have changed, which would have altered the results.

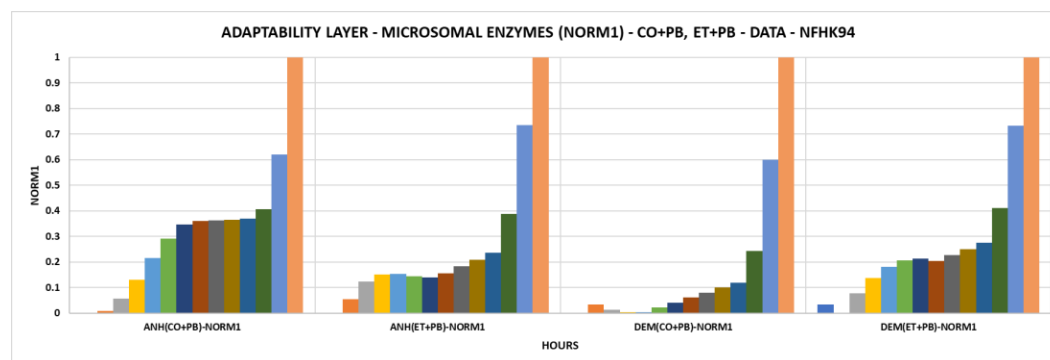


Figure 6.20 By plotting the enzymatic changes as histograms, we can detect similar expression patterns (days 16+) in the adaptability layer for both ANH and DEM following exposure to (ET+PB)-NORM1.

Next, we can calculate ratios for the ANH-DEM data pair in the rules layer (Figure 6.21). Notice that the two enzymes appeared to solve the problem together after only nine hours in that they continued to change (to increase the liver capacity) at the same rate (0.5:0.5). The shared 0.5:0.5 ratio might be explained by (1) coordinated gene expression, (2) the hepatocyte deciding to produce both enzymes at the same rate, or (3) some combination of the two events. The point? Without morphological data, detecting and interpreting biochemical data often becomes a challenging task because of the assumptions in play.

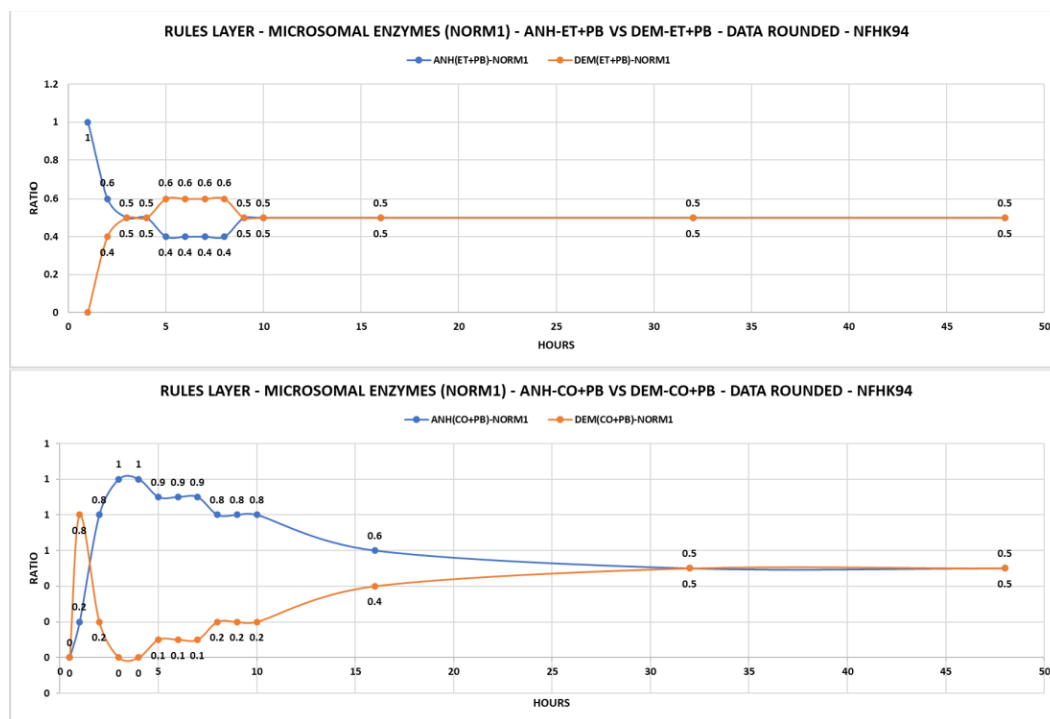


Figure 6.21 ANH(ET+PB) and DEM(ET+PB) found the same solution to the problem and continued to produce new enzymes at the same rate. DEM often serves as a seed (initiator) enzyme for the 0.5:0.5 solution. The controls, however, were working on just the phenobarbital part of the problem. When exposed to just the phenobarbital, the control enzymes [ANH(CO+PB), DEM(CO+PB)] took longer to find a solution to the toxicity problem, which began to appear thirty-two hours after exposure to the drug.

To test the data for equal rates of change, we can plot a linear regression (Figure 6.22). The regression equation indicated that the activity of demethylase increased roughly the same as that of aniline 4-hydroxylase (the slope was close to 1.0 (0.9436)). The point? Ratios supply predictable and reproducible results.

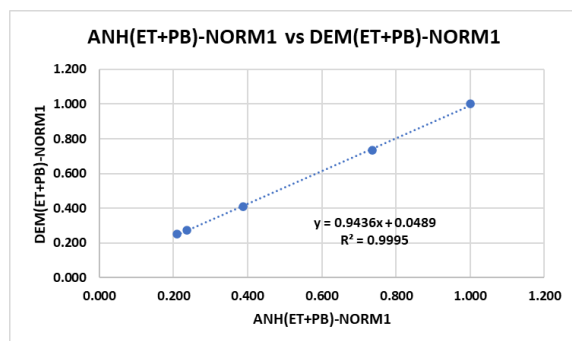


Figure 6.22 A linear curve with an angle at (or near) 45° and a supportive R^2 suggest the possibility of a one-to-one relationship.

Comment: Without the ER surface areas, enzyme densities, and microsomal recoveries we can't explain how the hepatocytes solved the problem presented by the experiment.

6.1.6 Case Study 6: Phenobarbital (Glucose-6-Phosphatase) – OE66

Source: Update applied to original data from Orrenius S., Ericsson J. L. E. (1966) On the relationship of liver glucose-6-phosphatase to the proliferation of endoplasmic reticulum in phenobarbital induction. J Cell Biol 31: 243-236. ER membranes and liver weights: (SWH69).

Topic: Response of hepatocytic enzymes to phenobarbital (90 mg/kg/day/5 days).

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, calculate enzyme densities [(ED = ED(NORM2))], analyze patterns, and report biological solutions (recipes).

Dataset: Activities of demethylase (DEM), glucose-6-phosphatase (G6PASE), and ATPase (ATPASE); ER surface areas and liver weights (SWH69).

Orrenius and Ericsson (1966; OE66) wanted to know why treatment with phenobarbital increased the activity of demethylase (DEM) – a drug-metabolizing enzyme – but decreased the activity of glucose-6-phosphatase (G6PASE) at a time when the ER surface area was rapidly increasing. Note that both enzymes serve as marker enzymes of the ER and become concentrated in the microsomal fraction (P).

Since we now know that interpreting cellular changes requires both morphological and biochemical data, we'll start by explaining what happened by working through the steps of the update. Recall that cells represent phenotypes consisting of related parts distributed into groups. For hepatocytes, demethylase activity belongs to a drug-metabolizing group and glucose-6-phosphatase to a carbohydrate metabolism group. Arranging enzymes into functional groups promotes order and allows cells to triage resources, as the need arises.

When cells change, several biochemical and morphological events occur simultaneously. In this study, phenobarbital (PB) induced the production of demethylase and ER membranes by the hepatocytes. This led to increases in average cell volumes, which meant that fewer of them could fit into a gram (or cm³) of liver. Compared to the control, data coming from a gram of the PB-treated livers came from fewer and fewer hepatocytes as the cells continued to respond to the PB treatment. Fewer cells per gram underestimated the true enzyme activity. Since the demethylase activity increased faster than what the gram of liver lost to the decrease in cell number, the increases persisted. Glucose-6-phosphatase, which is not a PB inducible enzyme, showed a loss of activity because it was coming from fewer and fewer hepatocytes. Since the liver weights were absent in the original publication, we'll borrow an expanded dataset from Stäubli et al., (1969; SWH69). However, the two studies used slightly different doses of PB (OE66: 90mg PB/kg body weight/day; SWH69: 100mg PB/kg body weight/day. This introduced a slight error.

Starting with the original data, we'll look at the same changes related to different references. Since we're trying to copy the way biology changes, knowing what data to use and how to detect a change experimentally became the basic questions. In short, we're trying to answer the following.

- Why do different data references produce different results?
- When detecting and explaining a biological change, why do we need two solutions?
- How do changes in the relationships of structure to function define a new phenotype?
- How do phenotypes support forward and reverse-engineering?
- How do enzymes change relative to other enzymes?
- What do we gain by normalizing experimental data?

Figure 6.23 shows the same enzyme data related to three different references, each one detecting a different response to phenobarbital. We already know that the first two – mg protein (MGPR) and gram of liver (G) – were becoming unstable references because the hepatocytes were increasing their average volumes as they made the transition from a control phenotype to one capable of removing the drug.

Original Data (Log Plot)

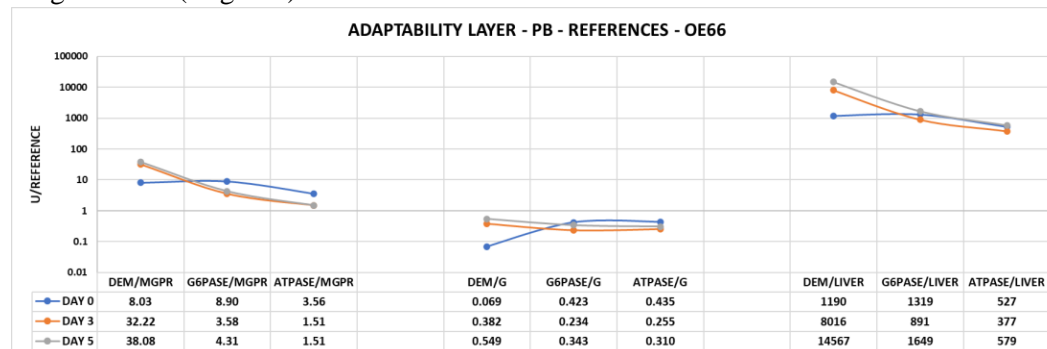
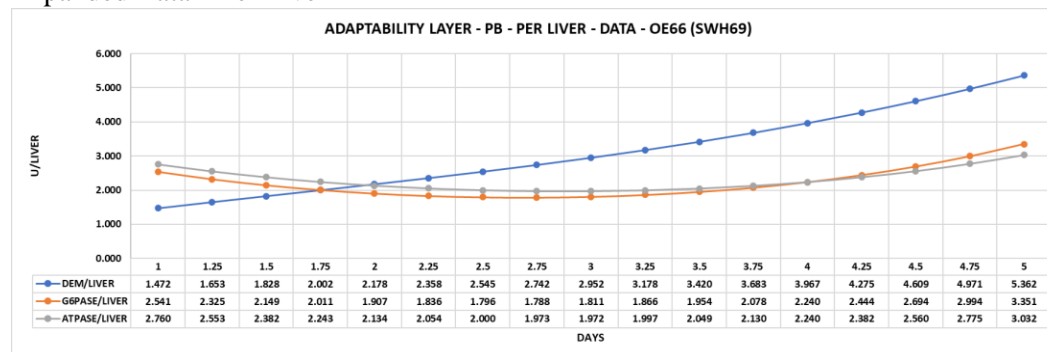


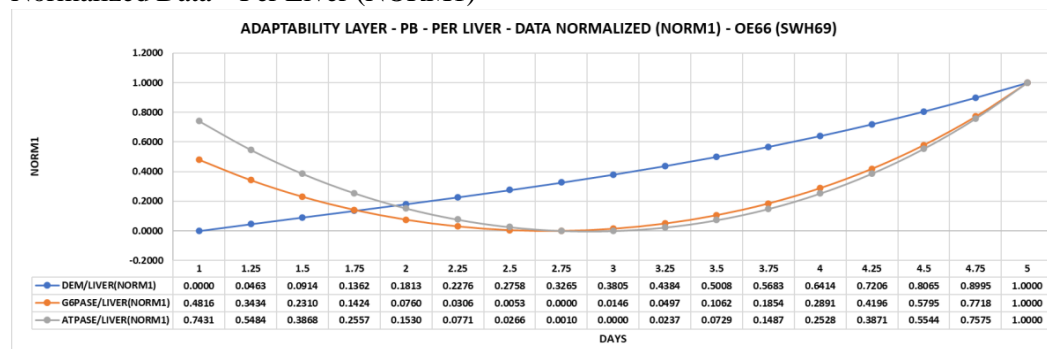
Figure 6.23 The same data related to different data references produced different results. The mg protein and gram references suffer the problem of estimates coming from a constantly changing number of cells (the cell packing problem) whereas the liver reference captures all the data from all the cells all the time. In the absence of evidence to the contrary, we can assume that the total number of hepatocytes in the liver remained essentially constant.

Expanding a dataset creates enough data points (3 \rightarrow 17) to allow normalization. We begin by calculating results related to the liver, gram of liver, and mg protein. While each data reference produced a different result in the adaptability layer (Figure 6.24), the liver and gram of liver gave the same ratios in the rules layer (multiplying parts related to a gram of liver by the weight of the liver changes the amounts of the parts but not the ratios).

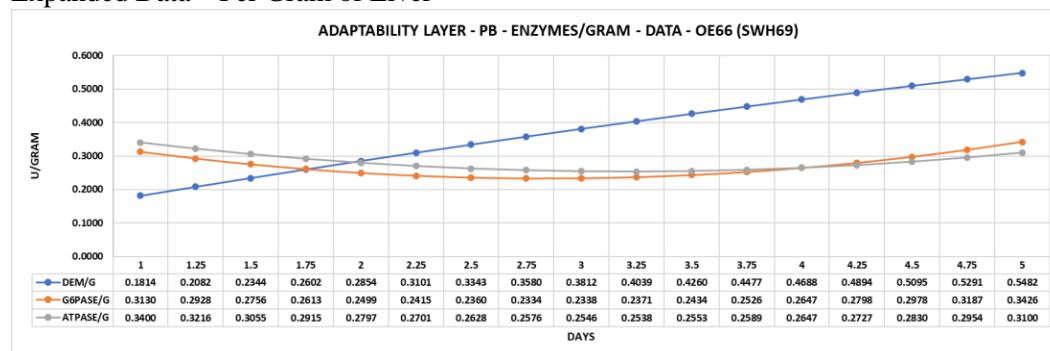
Expanded Data – Per Liver



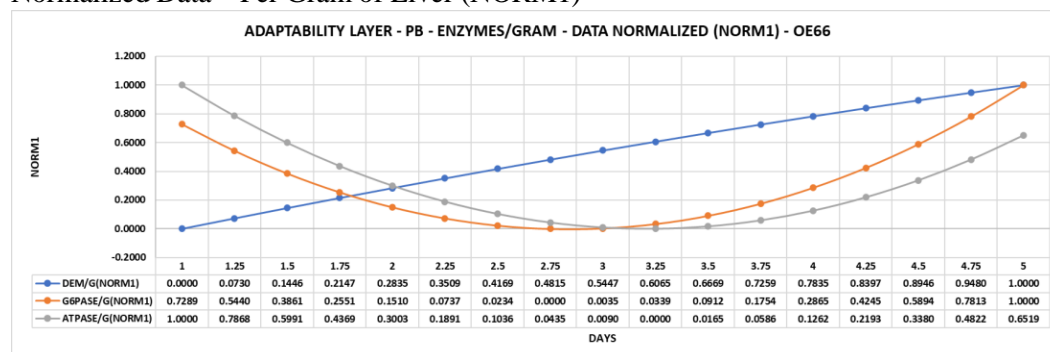
Normalized Data – Per Liver (NORM1)



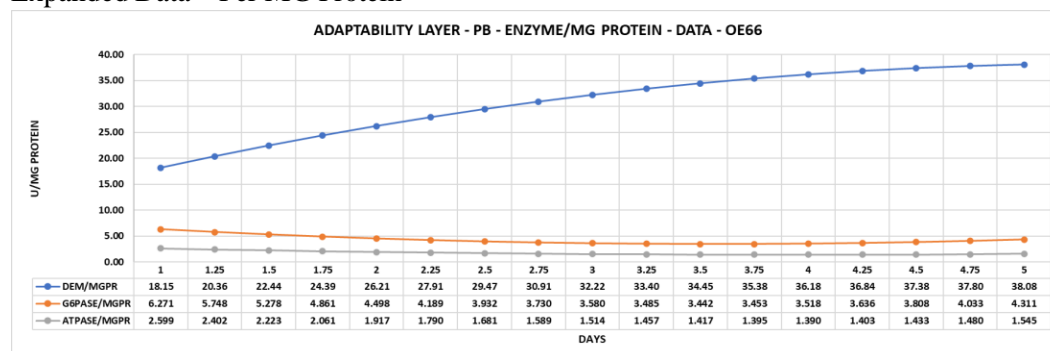
Expanded Data – Per Gram of Liver



Normalized Data – Per Gram of Liver (NORM1)



Expanded Data – Per MG Protein



Normalized Data - MG Protein (NORM1)

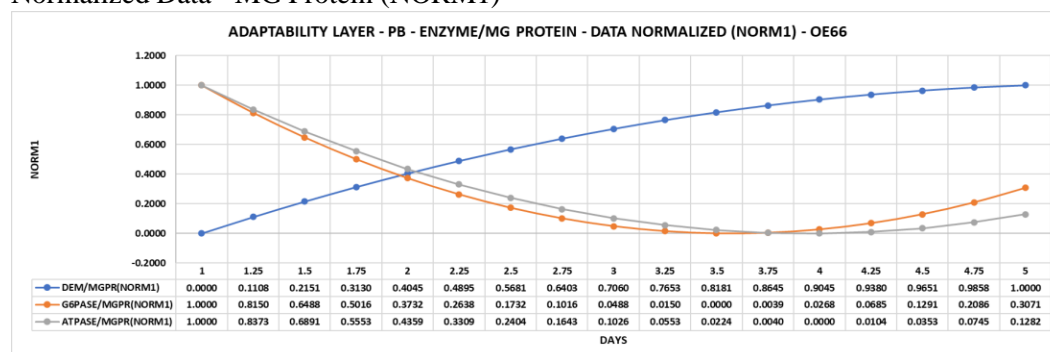
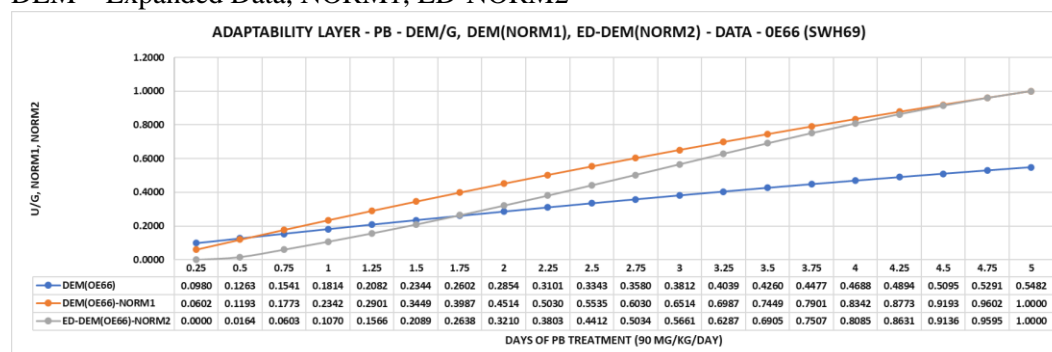


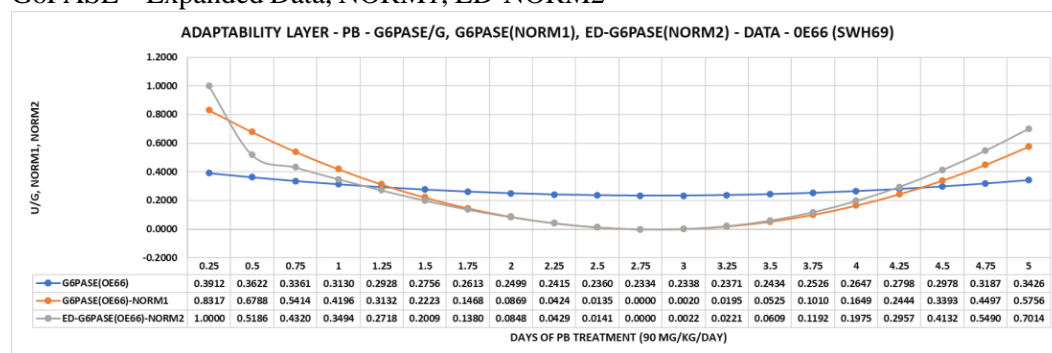
Figure 6.24 Different references produced different results.

Figure 6.25 summarizes the results for each enzyme (original expanded data, NORM1, and ED-NORM2).

DEM – Expanded Data, NORM1, ED-NORM2



G6PASE – Expanded Data, NORM1, ED-NORM2



ATPASE – Expanded Data, NORM1, ED-NORM2

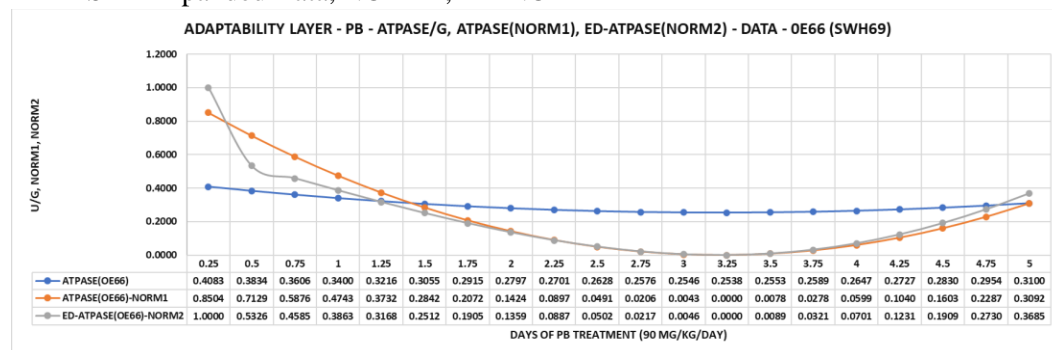


Figure 6.25 The update expanded, normalized (NORM1), and expressed the results as normalized enzyme densities (ED-NORM2). All the enzyme density calculations used the same data reference (U/G).

Figure 6.26 plots the results as percentages, wherein the three enzyme densities identify their relative contributions to the enzyme-membrane recipe. Notice that the hepatocytes originally assigned priority to the synthesis of the drug-metabolizing enzyme (DEM), but that the other enzymes (e.g., G6PASE as a housekeeping enzyme) caught up a few days later. The point? Generating a new phenotype to solve the phenobarbital problem also included solving other problems as well. However, after five days of phenobarbital treatment, all three enzymes contributed equally to the recipe (33%:33%:33%).

Moreover, the figure provides answers to the questions originally raised by the authors. The relative proportions of the enzymes attached to the ER changed as the ER membranes increased in surface area. While the total amount of DEM activity remained elevated, the relative proportion of the three ER

(microsomal) enzymes became similar after five days of treatment with phenobarbital. In short, the enzyme-membrane recipe supplied the answer for the investigators and the solution for the hepatocytes. On PB day five, it appeared that the hepatocytes knew how much of each enzyme to produce to detoxify the phenobarbital and to meet its other responsibilities.

Enzyme recipe (%)

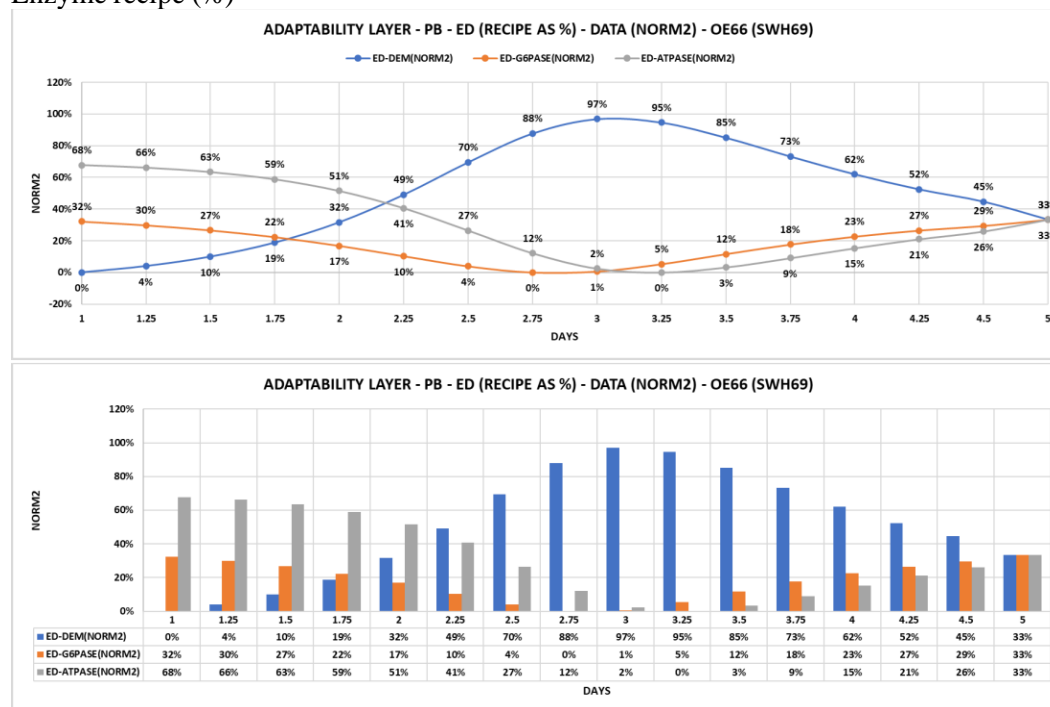
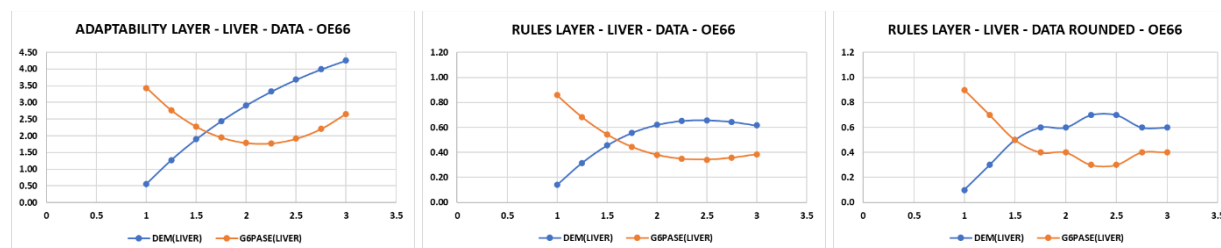


Figure 6.26 The enzyme recipes of the ER membranes appear in each column - expressed as percentages. Notice how heavily weighted they become for the drug-metabolizing enzyme (DEM) compared to these of housekeeping enzymes (G6PASE, ATPASE). It appears that adaptability includes an element of triage, which posits that the hepatocytes optimized all the parts and connections as they constructed the new recipe for the phenobarbital phenotype. The figure makes several points: (1) all parts become connected, (2) change is complex because it consists of many changes, (3) the cell becomes the solutions, as (4) told by the recipes.

Let's finish by working through the rules (Figures 6.27, 6.28). Starting with the original data related to the liver, we can compare changes in the data pairs ratios of enzymes to look for repeating patterns in the rules layer.



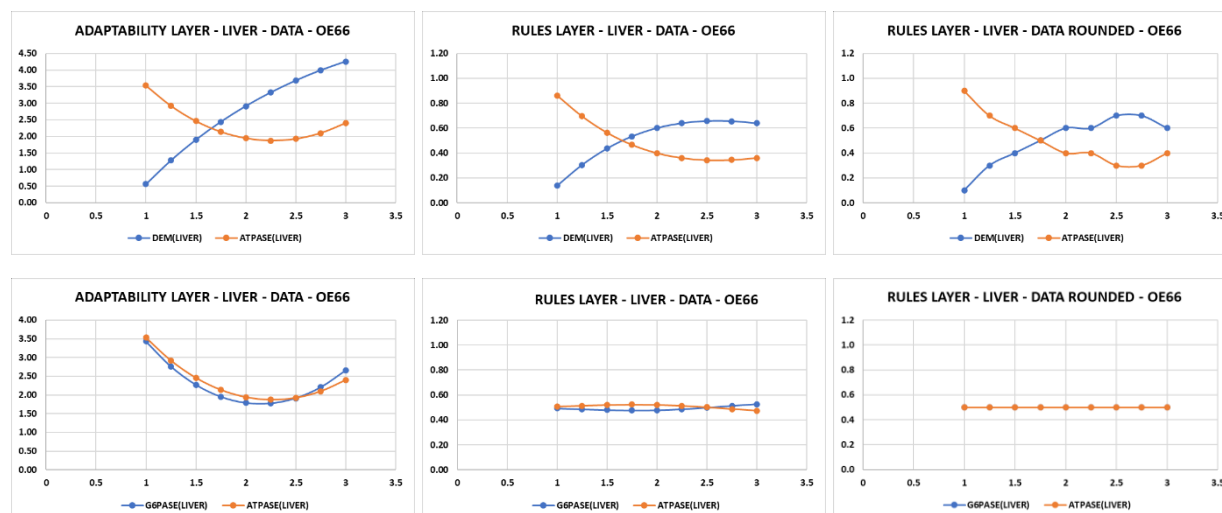


Figure 6.27 The data rounded plot shows that between PB days 1 to 3 G6PASE and ATPASE changed at the same rate (0.5:0.5).

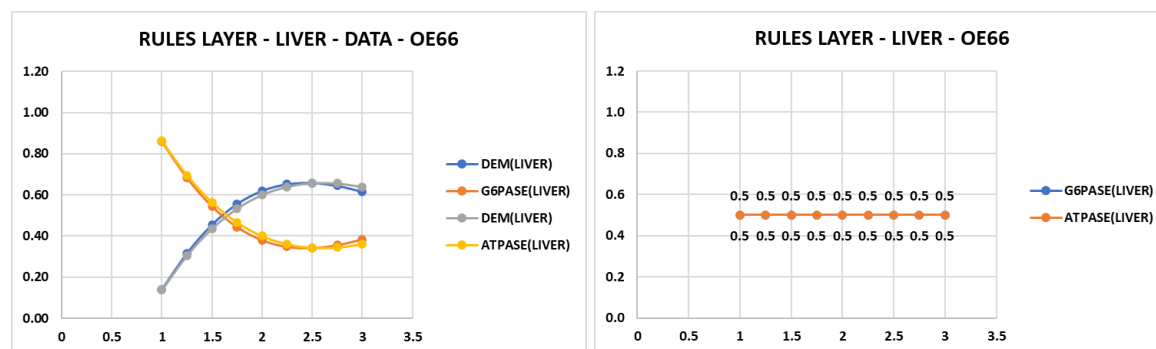


Figure 6.28 When superimposed, the DEM:G6PASE and DEM:ATPASE data pairs showed a good fit. In turn, plotting the ratios of G6PASE against ATPASE produced the expected straight line (0.5:0.5) telling us they changed together at the same rate.

By including PB day 5, we can see how our choice of data reference leads to different results in the rules layer (Figure 6.29). The ratios for the mg protein reference were 0.5:0.5, but 0.4:0.6 for the two liver references. Such a difference becomes important when using ratio data to test for reproducibility and representative sampling or when aggregating data to generate global datasets and phenotypes.

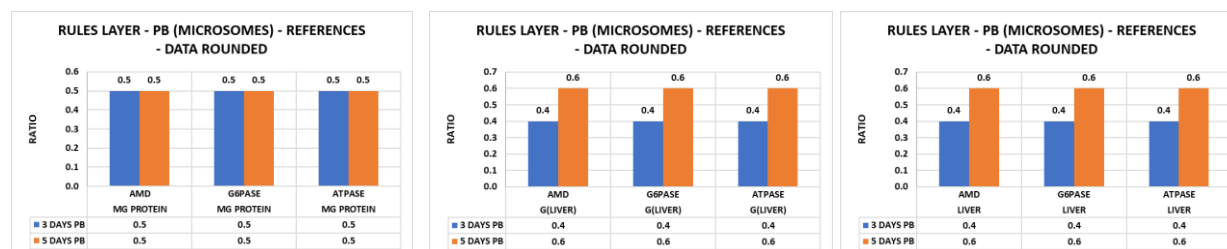


Figure 6.29 Different references can produce different results in the rules layer. The ratios of the gram of liver and the liver always remained the same because differ only by a constant – the weight of the liver. Notice that both the liver and the gram of liver generated the same ratios even though the number of hepatocytes in a gram of liver may have been different on PB days 3 and 5.

Summary: The hepatocytes solved the drug problem by progressively replacing their “control” phenotype with one that could metabolize and eliminate the drug phenobarbital. They accomplished this task by increasing the surface area of the endoplasmic reticulum and changing the enzyme-membrane recipes. It took the hepatocytes a day or so to figure out the right recipe, which the cells then mass-produced until the drug metabolizing capacity of the liver matched the severity of the threat. At the same time, it managed two housekeeping enzymes (G6PASE, ATPASE), which belonged to a different metabolic group.

6.1.7 Case Study 7: Vitamin B6 Deficiency (Hepatocytic Organelles) – RSPR80

Source: Update applied to original data from Riede, U. N., Sandritter, W., Pietzsch A., Rohrbach R. (1980) Reaction patterns of cell organelles in vitamin B6 deficiency: Ultrastructural-morphometric analysis of the liver parenchymal cell. Pathology - Research and Practice 170: 376-387.

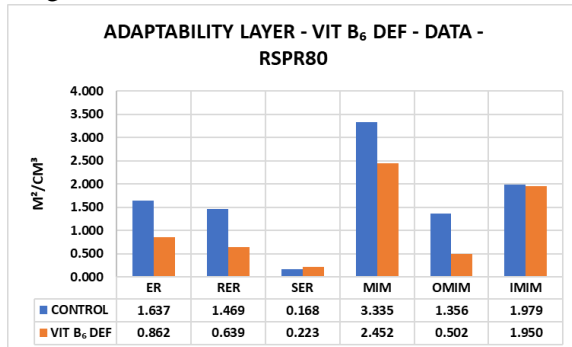
Topic: Morphological changes in response to vitamin deficiency.

Update: Apply corrections, expand data, report results in adaptability and rules layers, analyze patterns.

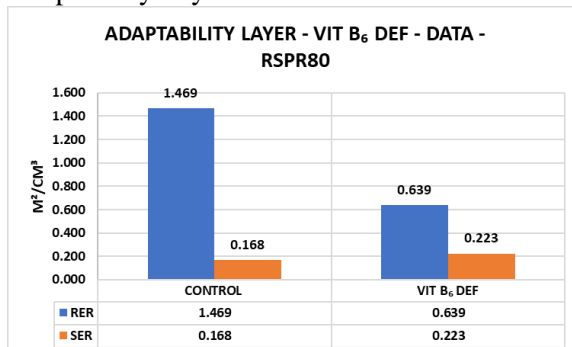
Dataset: ER (RER, SER) and mitochondrial (OMIM, IMIM) membranes related to the cytoplasm of hepatocytes.

Riede et al., (1980; RSPR80) looked at the effect of vitamin B₆ deficiency on the surface areas of the ER (RER:SER) and mitochondrial (OMIM:IMIM) membranes. The updated data in Figures 6.30 and 6.31 showed differences in the control and experimental ratios (RER vs SER, OMIM vs IMIM), but not when combined (ER:MIM).

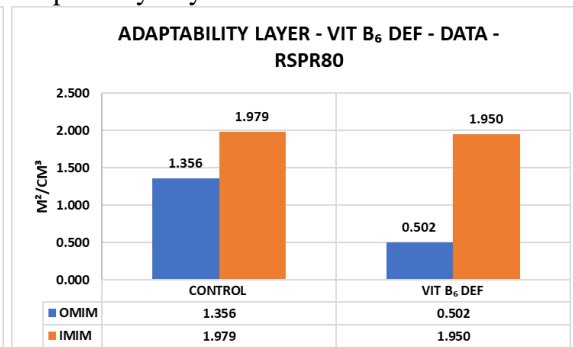
Original Data



Adaptability Layer – ER



Adaptability Layer - MIM



Rules layer – ER

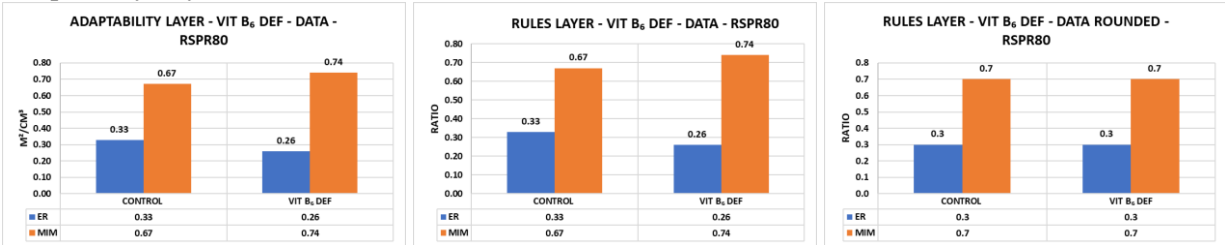


Rules layer – MIM



Figure 6.30 The vitamin deficiency decreased the RER but increased the IMIM. Without the enzyme densities of the ER and mitochondrial marker enzymes, one can show that the membrane surface areas changed but not explain what the hepatocytes did to solve the vitamin deficiency problem. Figure 6.31 summarizes the changes in membranes: ER (RER, SER) and MIM (OMIM, IMIM).

Adaptability Layer – ER:MIM



Rules Layer – ORGANELLES

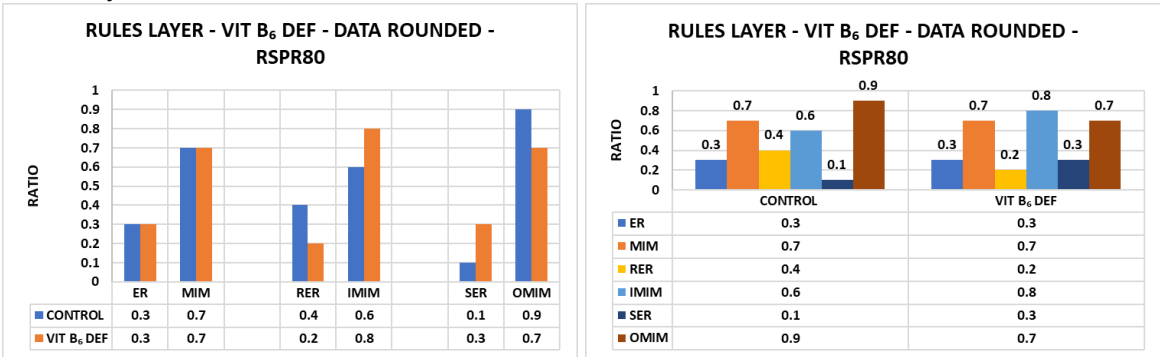


Figure 6.31 The histograms suggest that a priority exists for maintaining the same relative amounts of ER and mitochondrial membranes but not for their subcompartments. Since the RER decreased when the IMIM increased, it suggested that the enzymes had become more concentrated on the ER membranes or that the energy producing effectiveness of the IMIM decreased. Without enzyme densities for both the ER and mitochondrial membranes, one can report the results but not explain them.

Unanswered Questions: Did the organelles compensate for the losses in membrane surface areas by increasing the concentrations of the membrane bound enzymes? How, for example, did the enzyme densities and enzyme recipes change? To what extent did the hepatocytes succeed or fail in reaching their goal of solving the problems created by the vitamin B6 deficiency? The point? By shifting focus from detecting a change statistically to explaining what the hepatocytes did in response to the vitamin deficiency, one discovers the advantage of applying a first principles approach when detecting biological changes.

6.1.8 Case Study 8: Urease (Hyperammonemia) – ORG84

Source: Update applied to original data from O'Connor J. E., Renau-Piqueras J., Grisol, R. A. S. (1984) The effects of urease-induced hyperammonemia in mouse liver. Ultrastructural, stereologic and biochemical study. Virchos Arch B Cell Pathol 46:187-197.

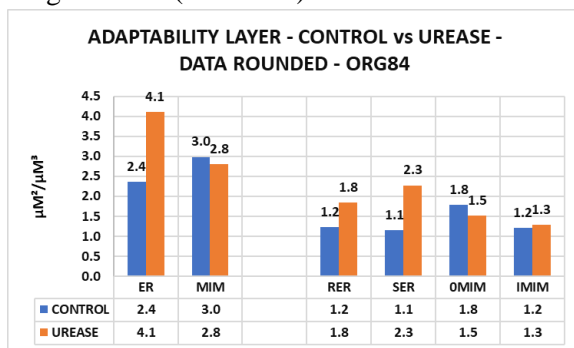
Topic: Hepatocytic injury response: morphological changes in ER and mitochondrial membranes exposed to urease.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, and analyze patterns.

Dataset: ER(RER, SER) and MIM(OMIM, IMIM).

O'Connor et al., (1984; ORG84) used urease injections to induce hyperammonemia, which brought about changes to the hepatocytic organelles. In response to the urease treatment, the ER and mitochondrial membranes switched from a control to a urease phenotype displaying a symmetrical pattern (Figures 6.32 and 6.33).

Original Data (Corrected)



Rules

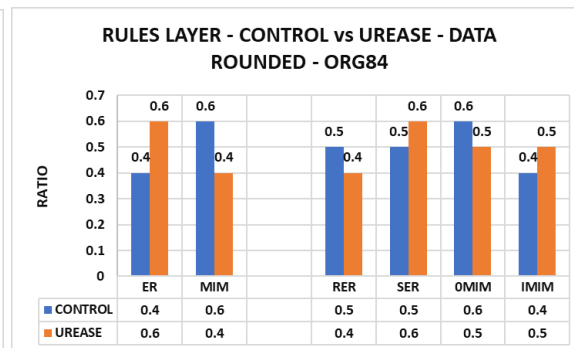


Figure 6.32 Data shown the adaptability layer suggested that changes occurred in ER, RER, and SER in response to urease. Notice that the patterns in the rules layer detected an underlying symmetry of the changes – RER paired with the IMIM and SER with the OMIM. This suggests a rule-based linkage between pairs of membrane compartments wherein the rules involved the inexplicable (but reoccurring) left-right switch.

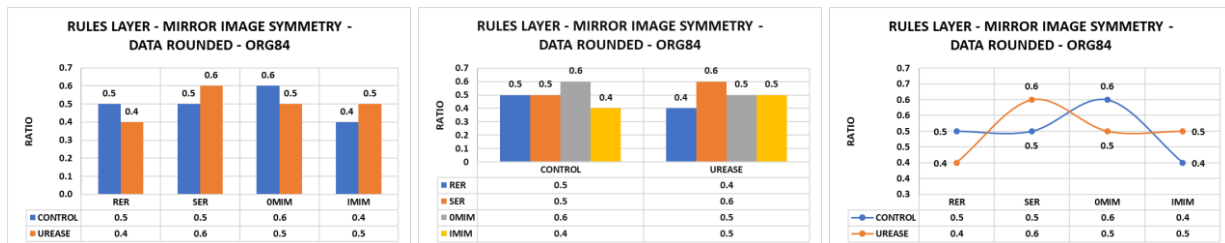


Figure 6.33 Symmetrical patterns often encountered during changes may have something to do with the efficiency of changing to a new phenotype and then returning the original phenotype by reading the same instructions backwards. Since connections exist between ER and mitochondrial membranes, a possible mechanism for the observed changes might be as simple as exchanging membranes between the mirrored compartments and then repurposing or replacing them. The algorithm for a change includes three inputs (function, structure, and function/structure). Since cells prefer some ratios over others, and if optimizing outcomes is the driving force, then updating published data will eventually reveal the optimizing algorithms.

Summary: The original data related to a μm^3 of hepatocyte cytoplasm in the control and experimental data points may not have come from the same number of cells because we don't know if the cells changed their sizes in response to the urease treatment. Although urease changed the organelle membranes and ratios, without enzyme data, enzyme densities, and total liver values, we don't know if or how the hepatocytes solved the problem. However, by applying corrections to the original data for section thickness and compression, we now have a new insight into the way cells might use data pair ratios and symmetry to forward and reverse changes.

6.1.9 Case Study 9: Thallium Chloride (Injury Model) – WF86

Source: Update applied to original data from Woods J. S., Fowler B. A. (1986) Alteration of hepatocellular structure and function by thallium chloride: ultrastructural, morphometric, and biochemical studies. Toxicology and Applied Pharmacology 83: 218-229.

Topic: Hepatocytic responses to injury by thallium chloride.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, calculate enzyme densities [ED(NORM2)], analyze patterns, and report biological solutions (recipes).

Dataset: Enzymes [Monoamine oxidase (MAO), ferrochelataze (FERASE), aminolevulinic acid (ALA), NADPH cytochrome c (P-450) reductase (NADPHCCR), aniline hydroxylase (ANHY), aminopyrine demethylase (DEM), ALA synthetase (ALA SYN), MONOAMINE OXIDASE (MAO), malate dehydrogenase (MADH), cytochrome P-450 (CYTOP450)] and hepatocytic membranes (ER and MIM).

Woods and Fowler (1986; WF86) reported on the dose dependent toxicity of a trace metal (thallium chloride) on hepatocytes using morphology and biochemistry. The authors related their data to mixed references, wherein the biochemistry used a mg of protein and the morphology a cm³ of hepatocyte cytoplasm.

In response to thallium chloride, several things changed. Enzyme activities associated with the ER decreased, but the surface area of the ER increased. MAO activity increased, but the surface area of the OMIM remained essentially unchanged. Both the activity of FERASE and the surface area of the IMIM increased. Enzyme densities decreased as the dose of thallium chloride increased. When normalized, the enzyme data told the story differently. In the rules layer, the membranes ratios remained nearly constant, whereas the enzymes increased and or decreased. While the enzyme densities generated a thallium chloride phenotype, no liver weights meant no liver capacities. Table 6.1 lists the enzymes with suggested locations, Figures 6.34 and 6.35 include the original, expanded, and normalized data.

Table 6.1 Enzymes with likely organelle location.

Enzyme	Organelle
Monoamine oxidase (MAO)	OMIM
Ferrochelataze (FERASE)	MI
Aminolevulinic acid (ALA)	MI
ALA synthetase (ALA SYN)	MI
Malate dehydrogenase (MADH)	MI MATRIX
NADPH cytochrome c P-450 reductase (NADPHCCR)	ER & MICROSOMAL
Aniline hydroxylase (ANHY)	ER & MICROSOMAL
Aminopyrine demethylase (DEM)	ER & MICROSOMAL

Original Data (Biochemistry)

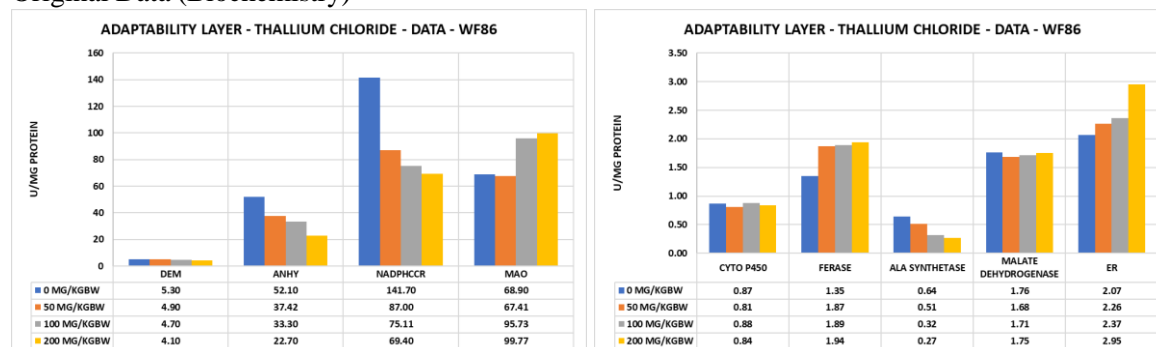
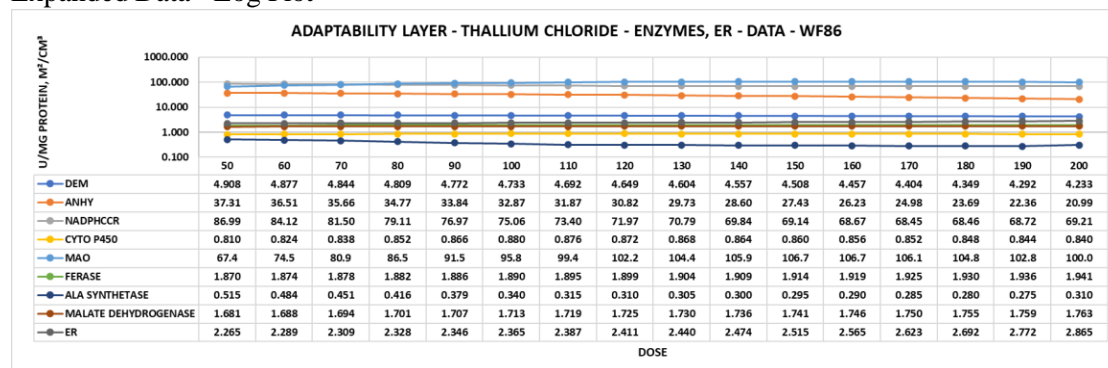


Figure 6.34 Original enzyme data uncorrected for changes in average cell volumes (the cell packing problem).

Expanded Data - Log Plot



Normalized Data (NORM1)

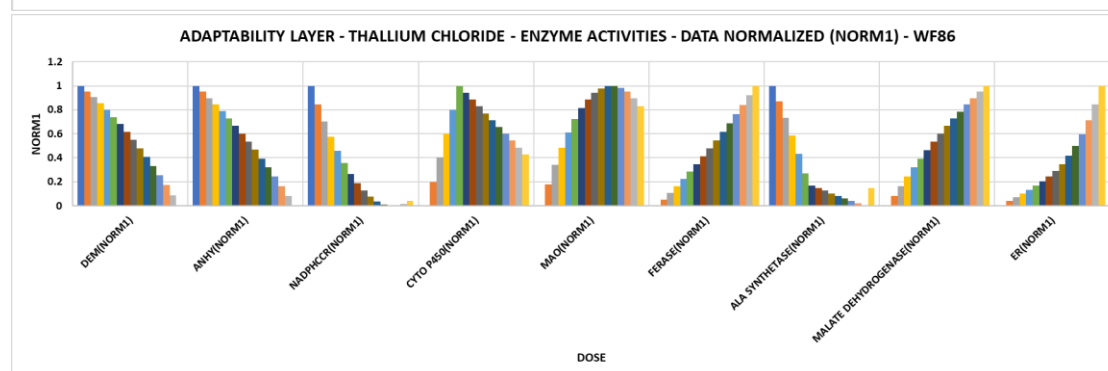
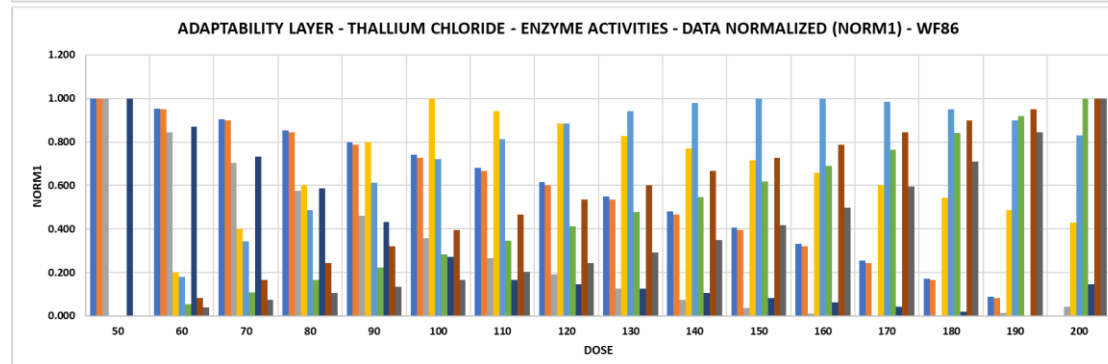
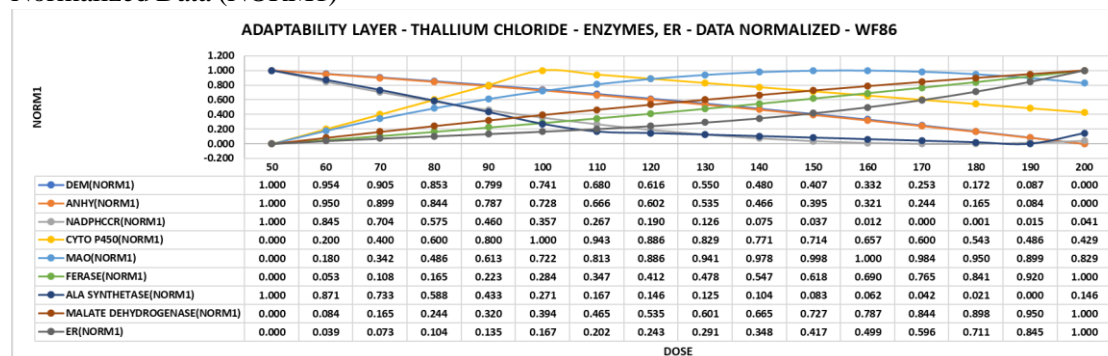
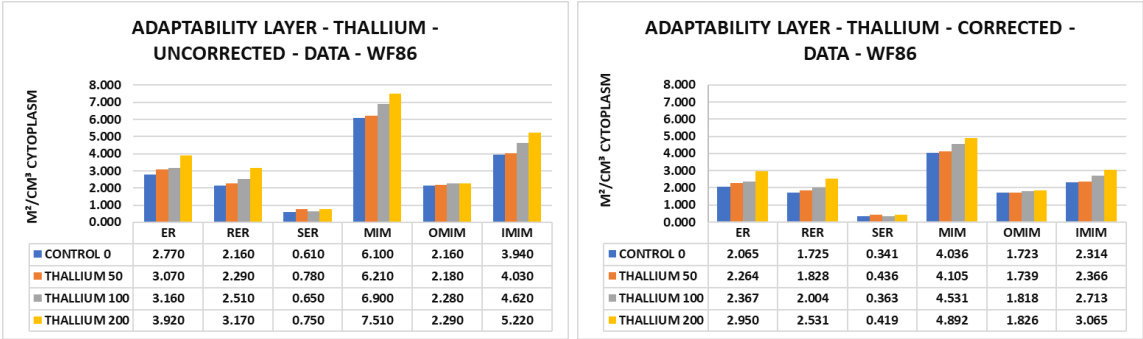


Figure 6.35 The normalized data simplified the task of identifying patterns in the data. In response to the increasing doses of thallium chloride, the hepatocytic enzymes often displayed near linear responses to the toxin. Moreover, pairs of enzymes showed similar responses. Once again, we see the triaging of enzyme activities wherein some enzyme activities increased while others decreased.

Notice in Figure 6.36 that changes in the membranes also appeared to be dose dependent, but the data pair ratios of the rules layer changed only once (for RER at 200 mg/kg, the highest dose of thallium chloride). Without the liver data we cannot rule out an error caused by the cell packing problem.

Adaptability Layer



Rules Layer

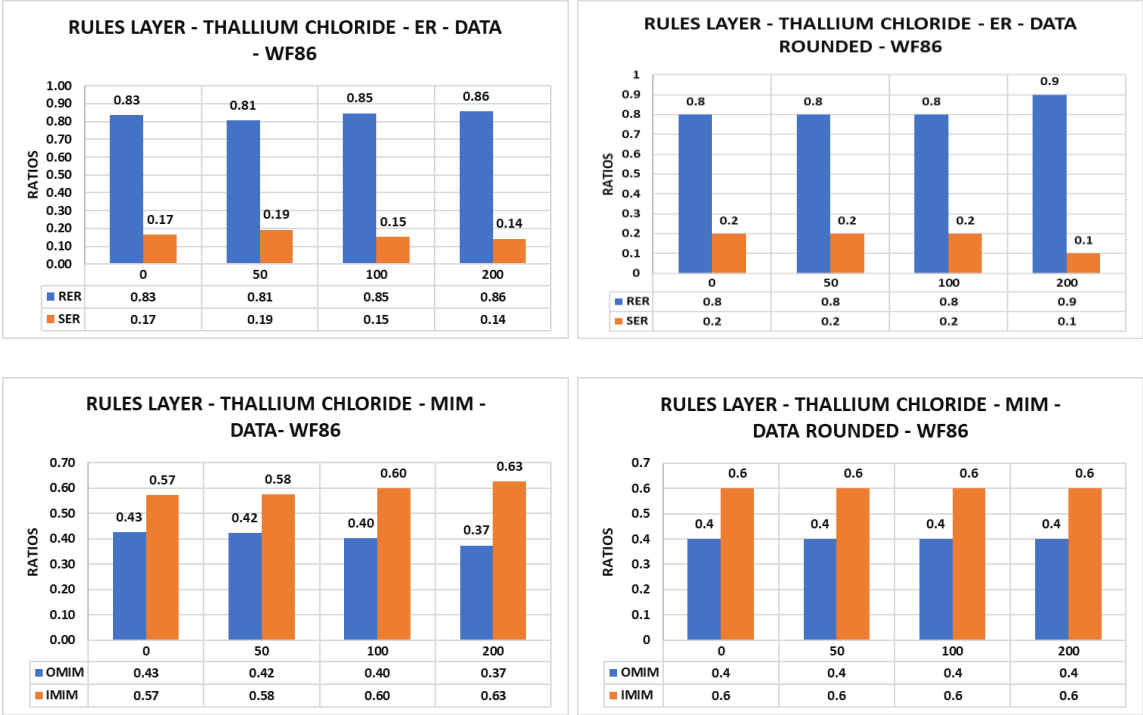


Figure 6.36 While most of the membrane compartments exposed to thallium chloride showed dose dependent responses, the increasing doses eventually changed RER:SER ratio but not that of the OMIM:IMIM.

Figure 6.37 shows that amounts can change while the rules remain constant (parallel).

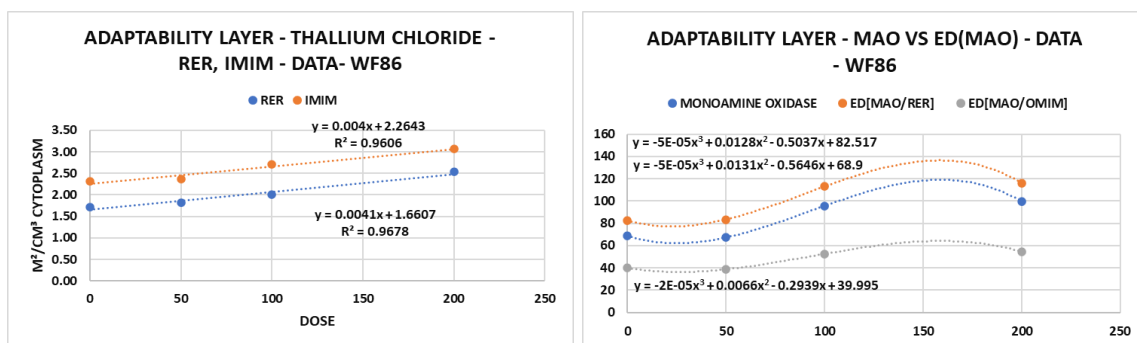
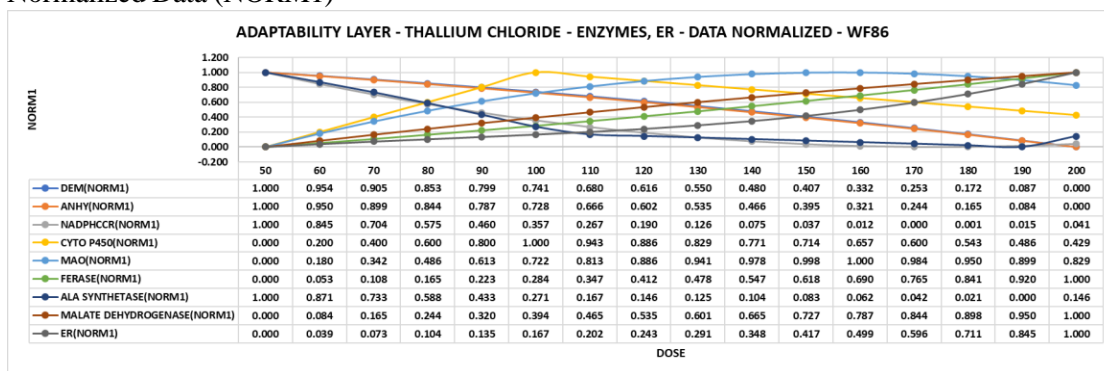


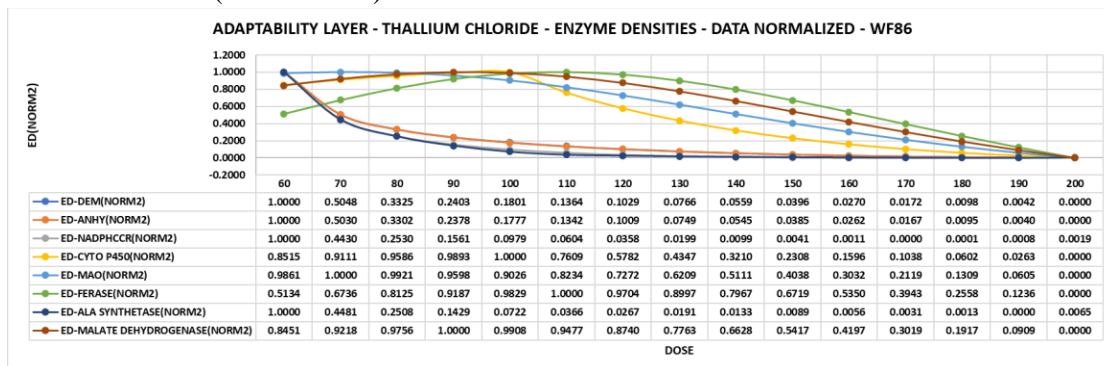
Figure 6.37 Changes in cell parts often display parallel patterns, which can be linear or polynomial. Monoamine oxidase (MAO) serves as a marker enzyme for the outer mitochondrial membrane (OMIM).

Enzyme Densities: Calculating enzyme densities from normalized data allows us to follow the changes in the hepatocytes as they worked out a solution to the problem caused by the exposure to thallium chloride. The overall downward trend in the amount of enzyme activity associated with the ER in response to increasing doses of thallium chloride underscores the toxicity of the trace metal. We're also adding an unknown measure of uncertainty by using two mixed and untested data pair references: (1) U/mg protein for the enzymes and (2) m^2/cm^3 of hepatocyte cytoplasm for the membranes. This means that we're assuming that both references produce change curves that run parallel. Given our earlier assessments of the mg protein reference, this puts the chances of the assumption being correct roughly within the range of 50% to 100%. Figure 6.38 includes the normalized (NORM1) and enzyme density (ED-NORM2) data.

Normalized Data (NORM1)



Normalized Data (ED-NORM2)



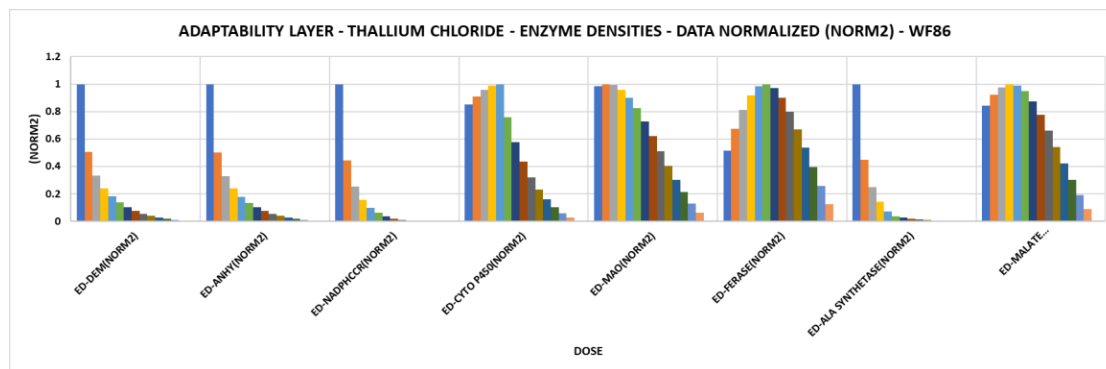


Figure 6.38 At a thallium chloride dose of 200, the curves representing the normalized ED-NORM2 data converged on zero (the smallest numbers). The histograms identify (1) substantial losses of membrane associated enzyme activity in response to increasing doses and (2) several different enzymes responding similarly. The damage appears selective.

Next, we can move the normalized enzyme densities (ED-NORM2) onto the rules layer, generate patterns by plotting the ratios taken two at a time, and look for duplicate patterns. Using the rounded data ratios of the rules layer (Figure 6.39), we can identify duplicate sets of curves. These plots illustrate the remarkable ability of hepatocytes to apply the same changes to different pairs of enzymes as the dose increased. This finding of a widespread duplication of data pair ratios undergoing changes identifies a basic property of a biological change.

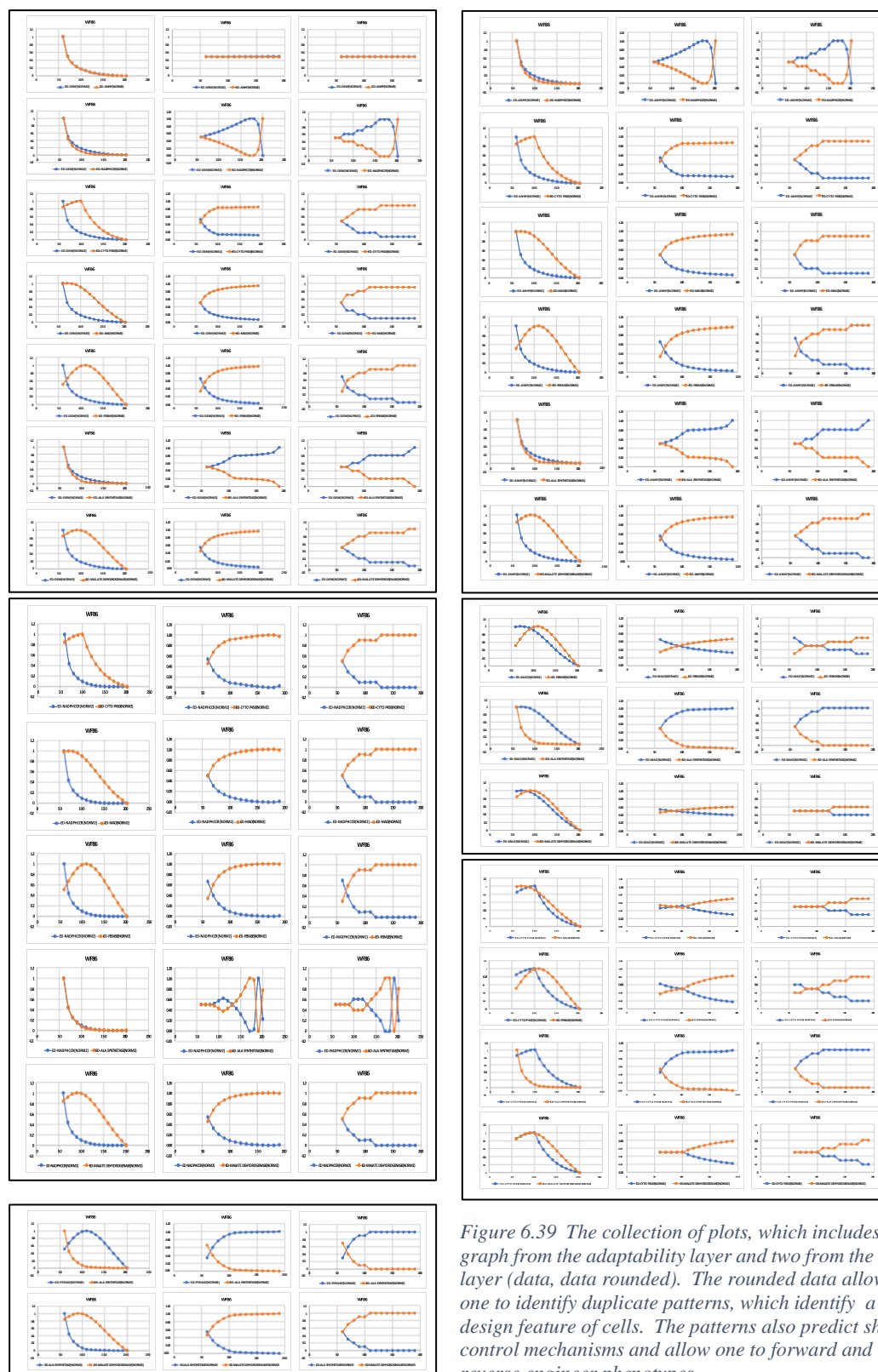


Figure 6.39 The collection of plots, which includes one graph from the adaptability layer and two from the rules layer (data, data rounded). The rounded data allows one to identify duplicate patterns, which identify a design feature of cells. The patterns also predict shared control mechanisms and allow one to forward and reverse engineer phenotypes.

By forming ratios of data pairs, we can see how cells apply rules to maintain order between parts. The first example shown below in Figure 6.40 identifies two ER enzymes (DEM, ANHY) related to a third (NADPHCCR). When one enzyme changes, the remaining enzymes change as expected (by rule).

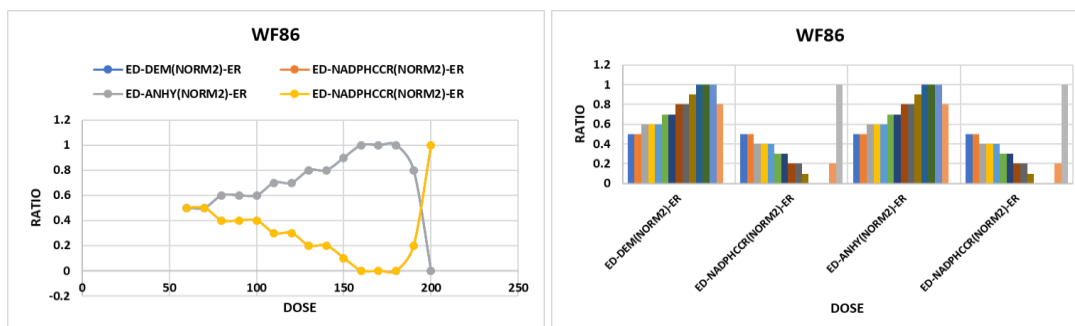


Figure 6.40 Two pairs of enzyme densities displayed the same changes. This means that a plot of ED-DEM(NORM2) to ED-ANHY(NORM2) would produce a straight line with a ratio of 0.5:0.5.

In the next example (Figure 6.41), we have the same two ER enzymes (DEM, ANHY) related to a third enzyme (MALATE DH) coming from mitochondria. As before, a change in one enzyme led to predictable changes in the remaining two. By allowing enzymes to connect by rule within similar and different organelles, cells use data pairs to keep their many parts in touch and on the same page.

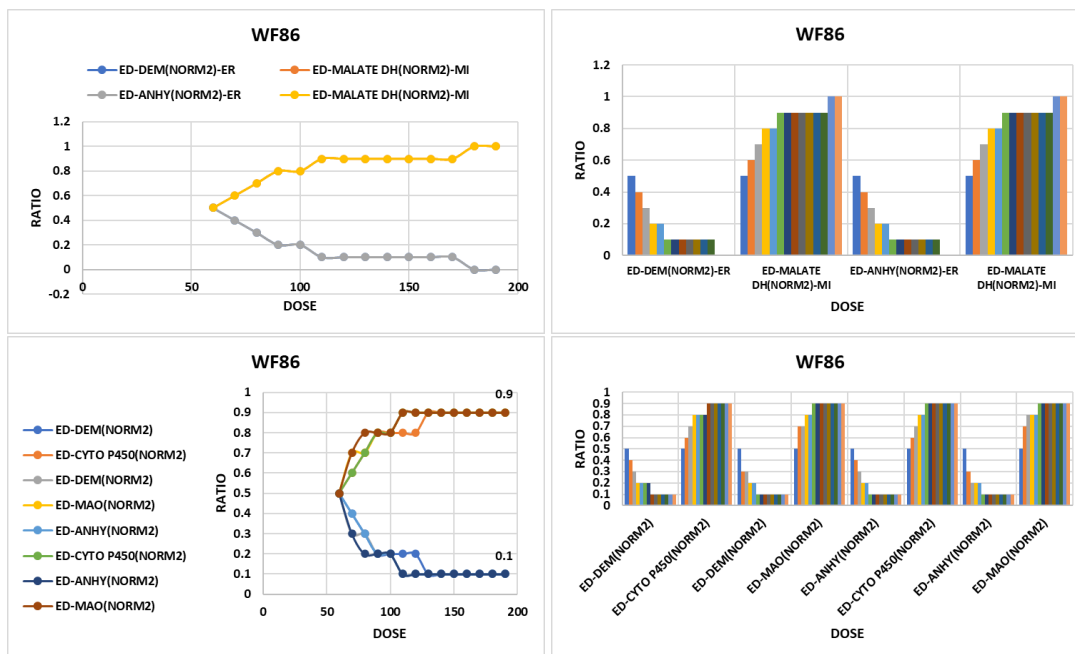
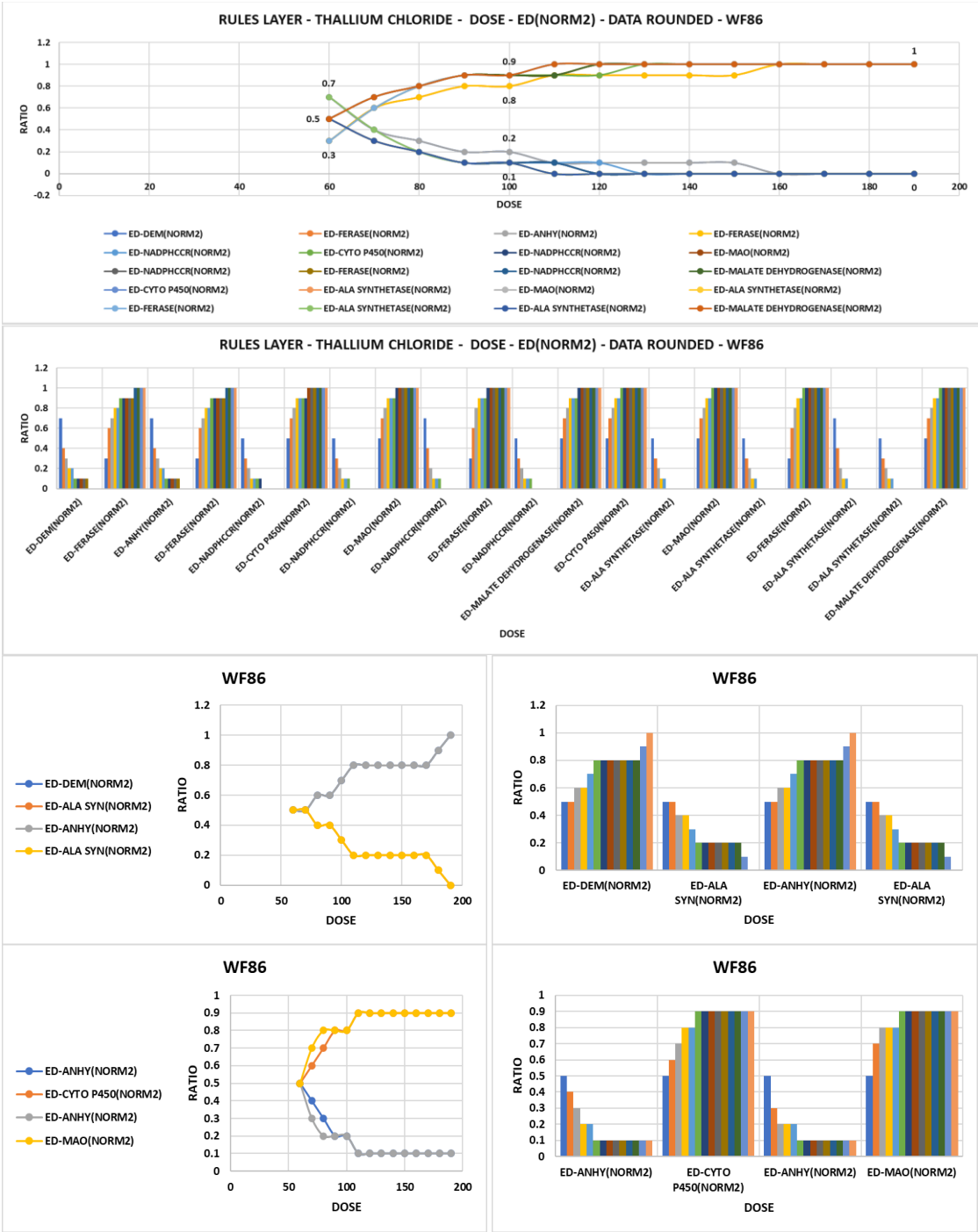
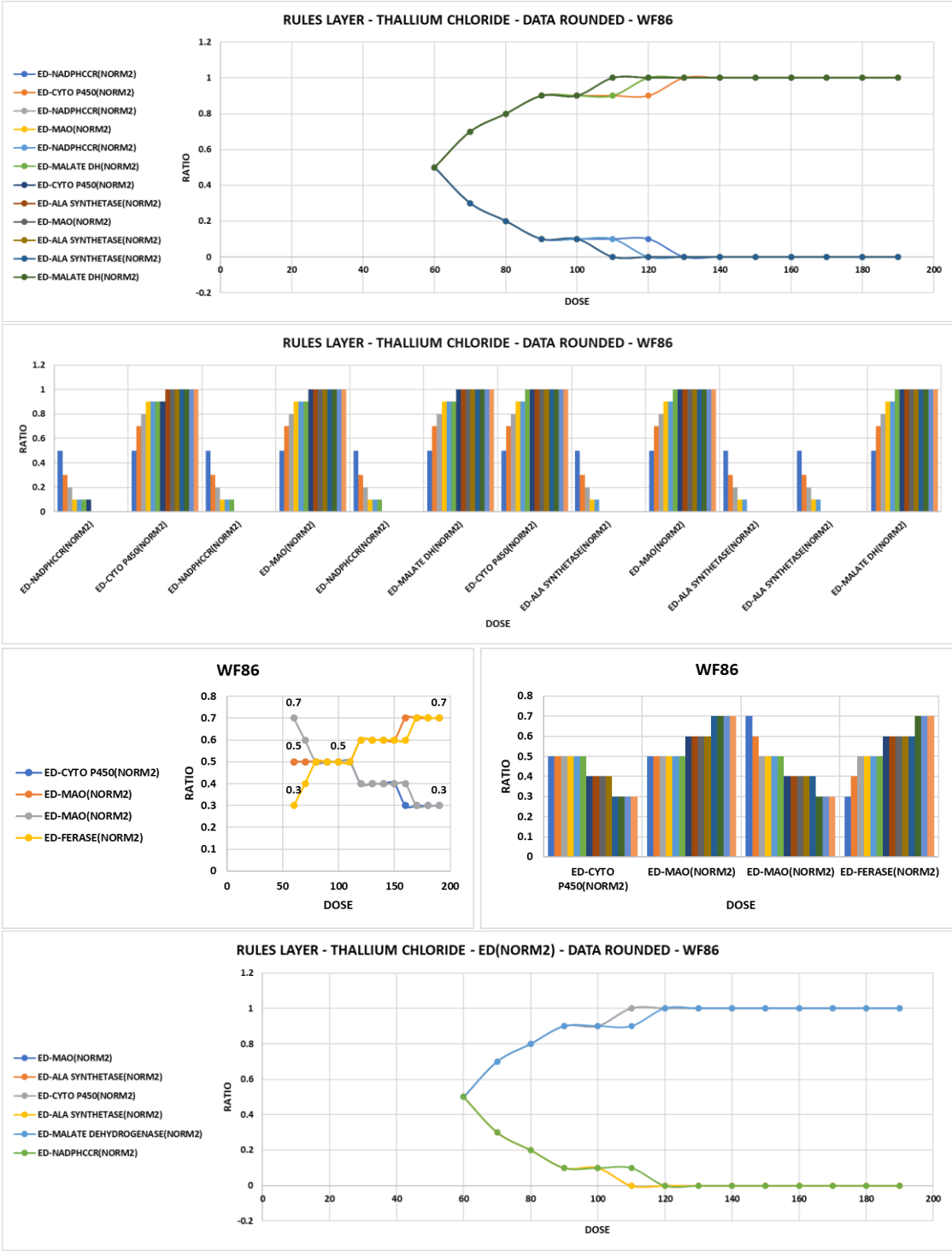


Figure 6.41 Duplications can occur within and across organelle compartments. For example, ER (DEM, ANHY) and MI (MALATE DH, MAO). Such quantitative crossovers between structure-function compartments suggests a strategy for linking information across multiple membranes and enzymes. Moreover, such crossover relationships appear repeatedly between subgroups when reverse engineering phenotypes. In fact, duplication plays a key role in the learning process basic to the mechanism of a biological change that cells apply to solve problems. This explains why change as a complexity involves many parts and connections, not just a single event coming from a single part. Cells start a change simply with parts and connections and then continue by optimizing many such combinations into new recipes that solve problems.

When we collect data pairs that share the same ratio repeatedly, we identify a subgroup and its solution. Basically, data pair ratios become the building blocks of biological complexity. In Figure 6.42, each plot represents either a subgroup or a subgroup of a subgroup.





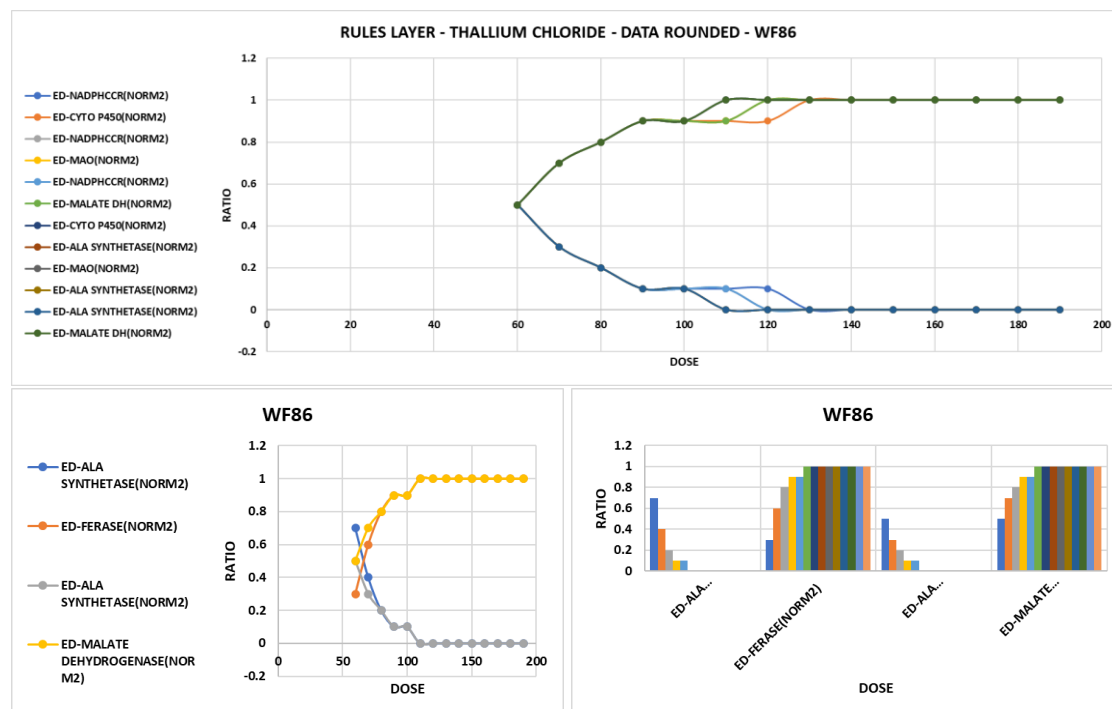
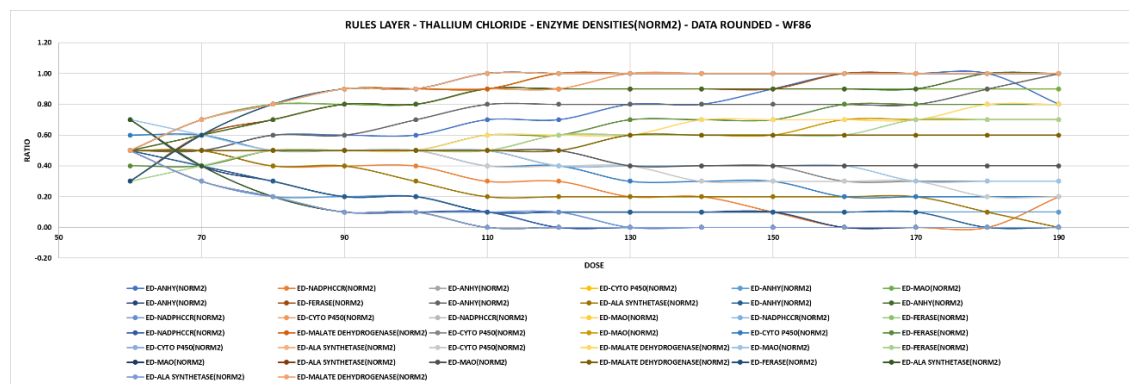


Figure 6.42 Duplications occur within and across enzyme densities. These quantitative crossovers between enzyme densities provide a way for the changes in one data pair to interact with data pairs in other subgroups. This increases complexity while at the same time keeps the data pairs connected within and across subgroups. For example, a pair of enzyme densities can optimize the relationship between two parts, but in turn each part can optimize its relationship with other parts. Duplicate data pairs display the exact properties needed to process a change as a massive fine-tuning event. The point? The duplications of enzyme densities appear to play a key role in the learning process that cells use to solve problems by changing.

Figure 6.43 summarizes the responses of the data pairs to the different concentrations of the trace element. Moving from one concentration of thallium chloride to another, the concentration of the various enzymes in the ER membranes change to make the accommodation. The summary plots explain when and how. The point to take from the figures is that some pairs of enzyme densities no longer change their ratios (persistent parallel horizontal lines suggest that a solution exists), whereas others continue to change (still looking for a solution).



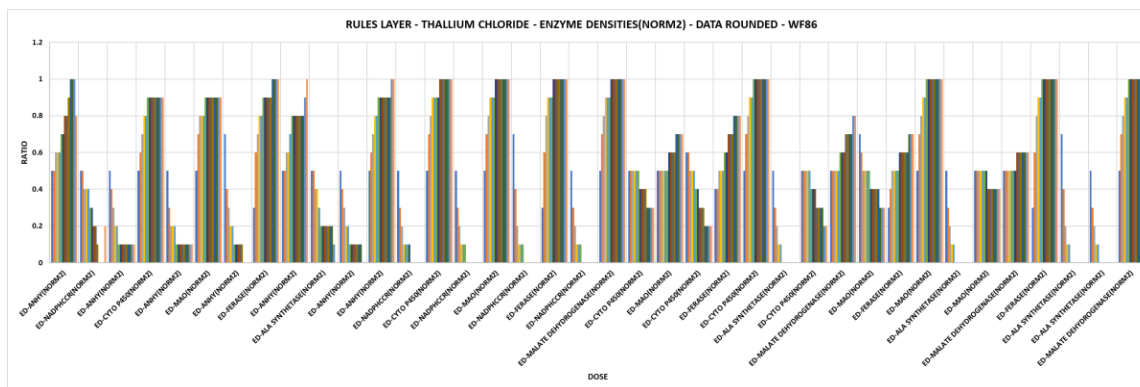


Figure 6.43 The plots summarize the changes occurring step-by-step as the hepatocytes respond to increasing concentrations of thallium chloride. Note the many duplicates.

By defining the solution (the recipe) as the relative amounts of the eight EDs, Figure 6.44 shows us how the recipes changed as the dose of thallium chloride increased (from 60 to 190). The 200 data point was lost to the normalization.

Enzyme-Membrane Recipes (Shown as Columns of Percentages)

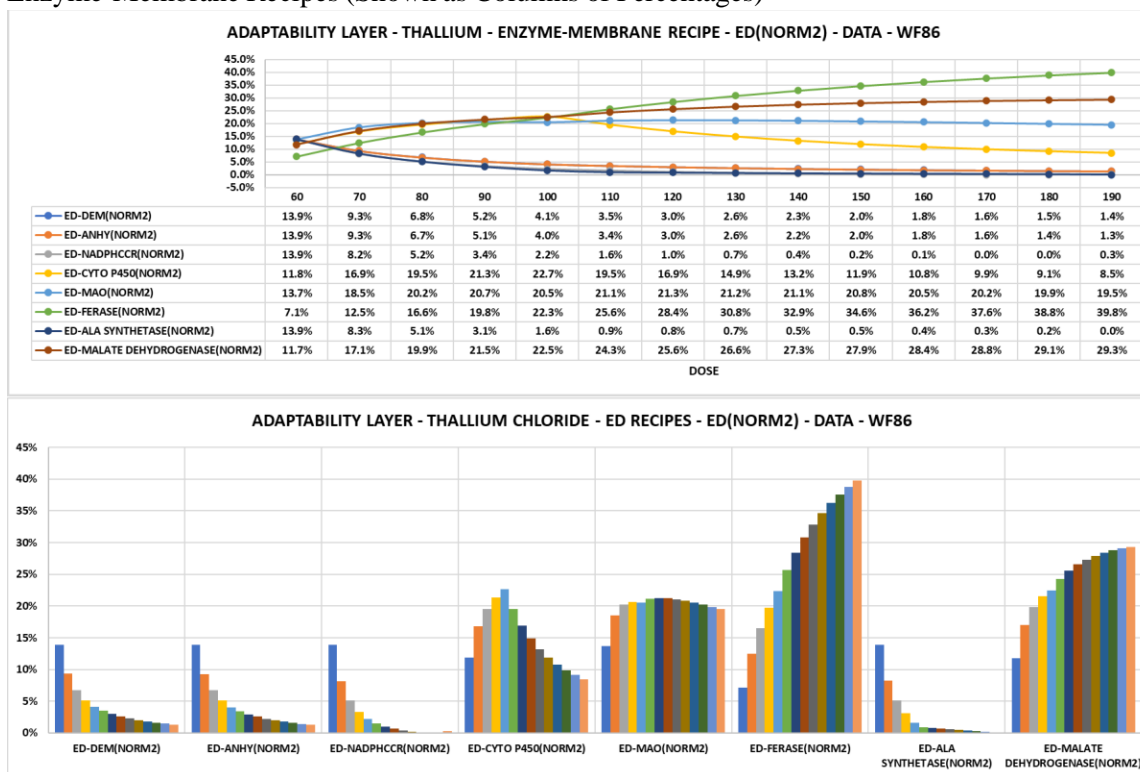


Figure 6.44 The plots record the changes in the enzyme membrane recipes as the dose of thallium chloride increased.

Summary: By forming data pairs, the update captured the complexity of a change from one phenotype to the next. The solution to the problem – as indicated by the recipes listed in Figure 6.44 – came largely from just three mitochondrial enzymes: MAO, FERASE, and MALATE DH.

6.1.10 Case Study 10: Dissolving Gallstones - Chenodeoxycholic Acid (CDCA) -KGLJO80

Source: Update applied to original data from Koch M. M., Giampieri M. P., Lorenzini I., Jezequel A. M., Orlandi F. (1980) Effect of chenodeoxycholic acid on liver structure and function in man: a stereological and biochemical study. Digestion 20:8-21.

Topic: Morphological responses of hepatocytes to chenodeoxycholic acid, a treatment for dissolving gallstones.

Update: Apply corrections, expand data, and report results in adaptability and rules layers.

Dataset: Surface areas of ER (RER, SER) and mitochondrial (MIM, OMIM, IMIM) membranes.

Reportedly, treating patients with chenodeoxycholic acid (CDCA) removes gall stones by dissolving them. Koch et al., (1980; KGLJO8) found no evidence of hepatotoxicity in nineteen patients treated with the drug (CDCA). Figure 6.45 summarizes the published data before and after the section corrections.

Original Data

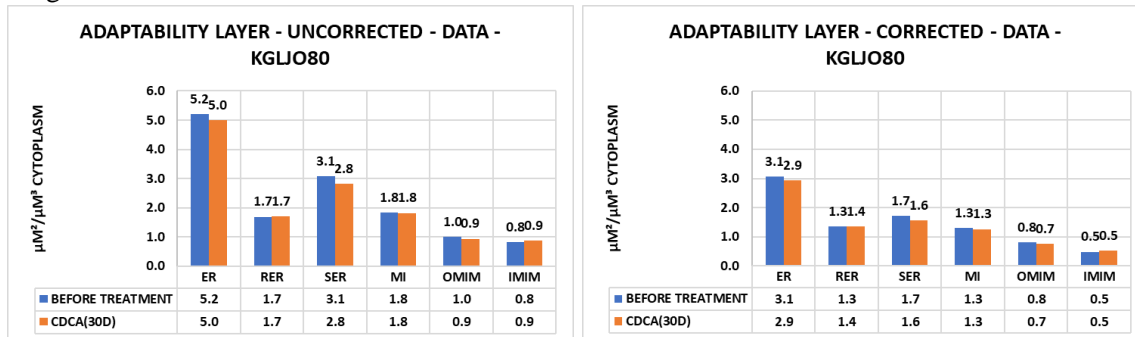


Figure 6.45 Substantial differences existed between the uncorrected and corrected values.

Short term experiment: Switching to the rules layer (Figure 6.46), the rounded data showed changes in the ratios of RER to SER (4:6 → 5:5), but not for the mitochondrial membranes (6:4 → 6:4). Be careful. Calculating ratios using different groups of animals assumes the absence of the cell packing problem.



Figure 6.46 The drug changed the rules for the RER to SER ratio but not for OMIM to IMIM ratio.

Long term experiment: The results from three patients included data reported before and after long-term treatment with CDCA. Notice in Figures 6.47 and 6.48 the shifts displayed by the histograms.

Original Data

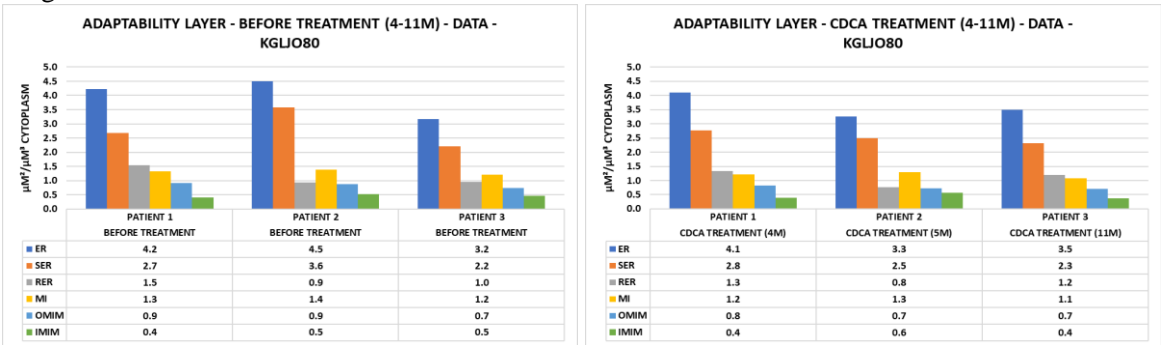


Figure 6.47 Before treatment, a greater similarity existed between patients 2 and 3, but with treatment the better match shifted to patients 1 and 3.

When checked in the rules layer (Figure 6.48), all three patients before treatment started out with slightly different rules for their ER and mitochondrial membranes. After exposure to CDCA, however, the hepatocytes of two patients (1 and 3) showed an identical rule-based response whereas patient 2 showed no change at all.

Rules Layer

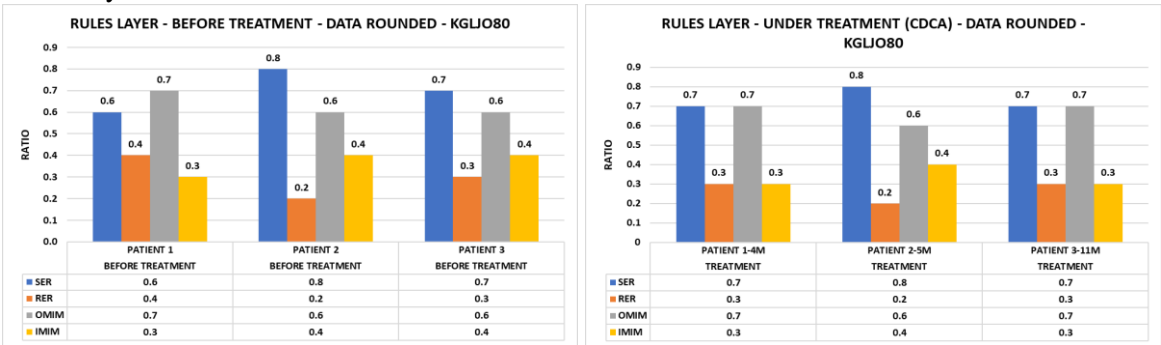
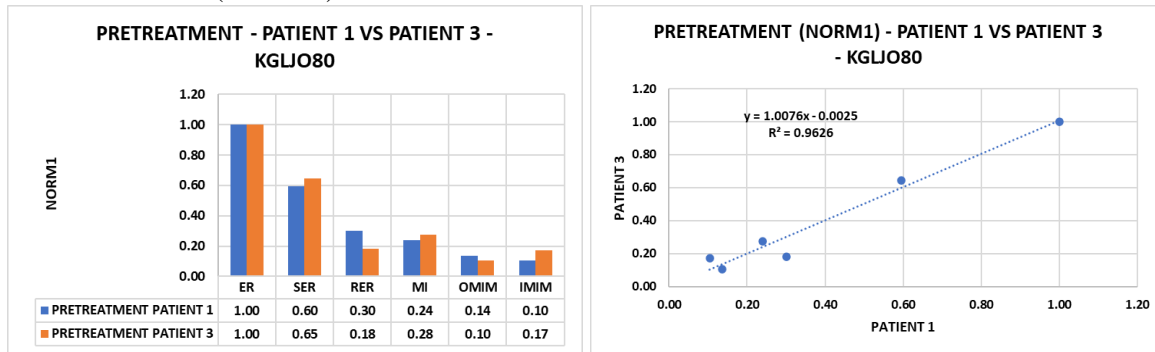


Figure 6.48 Two patients (1, 3) starting with completely different organelle ratios delivered identical results (ratios) when treated with the drug. In effect, the hepatocytes of patients one and three demonstrated empirically an ability to reproduce the others results. Moreover, the drug demonstrated an effectiveness of 67%. Knowing why patient 2 failed to respond becomes an interesting question. Individual patient data represents raw data.

The patterns in the rules layer suggested that the drug affected only two of the three patients. By normalizing and plotting the data of patients 1 and 3 pre and posttreatment, we can see that their hepatocytes managed to find nearly an identical solution to CDCA treatment (Figure 6.49).

Normalized Data (NORM1) – Pretreatment



Normalized Data (NORM1) - Treatment

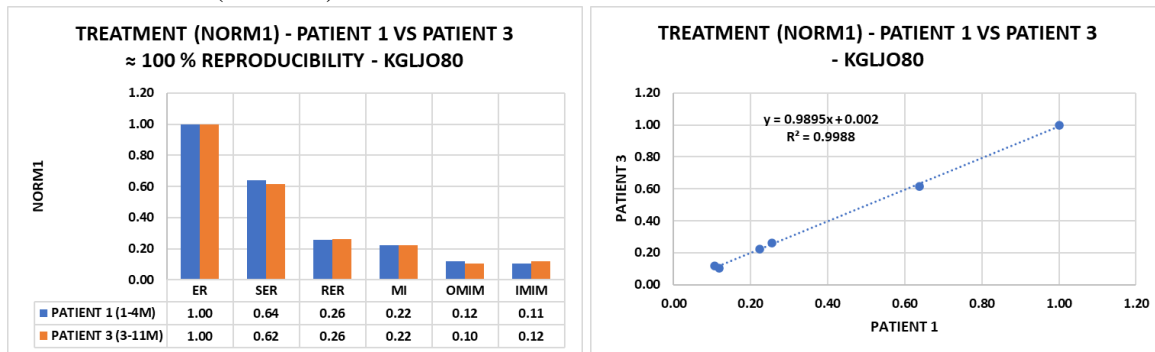


Figure 6.49 An $R^2 = 1$ represents an exact match. A slope of almost 1 (0.99) supports the conclusion of reproducibility.

Summary: This is what we don't know. Was the CDCA treatment successful clinically in dissolving the gallstones for all three patients? Since patient two showed no drug-induced changes, was the treatment ineffective? After treatment, did a CT scan of patient two still detect the presence of gallstones? Patients two and three?

For the hepatocytes of two patients (one and three) to find the same solution, they either applied the same internal algorithm or arrived at the same optimal solution. This triggers a basic question of the update. If hepatocytes can demonstrate reproducibility independently, why can't we do the same? The point? By applying the rules of hepatocytes, we just did.

Reminder: When comparing surface density data ($\mu\text{m}^2/\mu\text{m}^3$ cytoplasm), we trigger the cell packing problem, which can reduce the reliability of the result by roughly 50% (Bolender, 2019). The workaround consists of using ratios in the rules layer because they are not affected by changes in cell volume when both estimates come from the same animals (the same reference - μm^3 cytoplasm - cancels out).

6.1.11 Case Study 11: Phenobarbital (Glucose-6-Phosphatase) – MBH74

Source: Update applied to original data from Menard D., Berteloot A., Hugon J. S. (1974) Action of phenobarbital on the ultrastructure and the enzymatic activity of the mouse intestine and the mouse liver. *Histochemistry* 38: 241-252. ER membranes: Stäubli et al., (1969);SWH69.

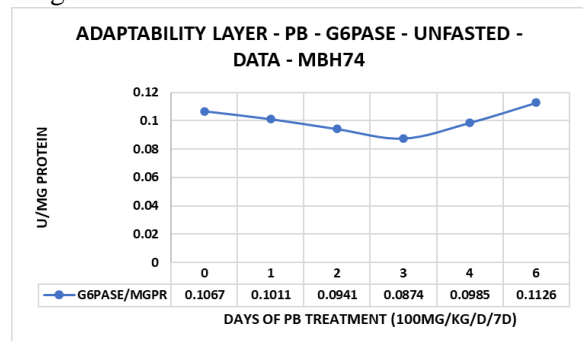
Topic: Biochemical changes in response to phenobarbital treatment.

Update: Apply corrections, expand data, report results in adaptability layer, normalize data, calculate enzyme densities [ED(NORM2)], and analyze patterns.

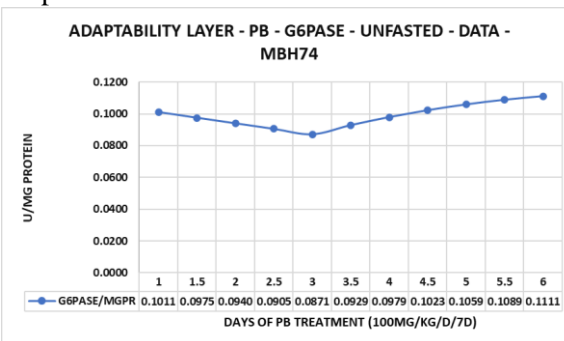
Dataset: Glucose-6-phosphatase (G6PASE) activities and membrane surface areas (ER).

Menard et al., (1974; MBH74) treated adult (5-month-old) Swiss ICR mice with phenobarbital (100mg/kg/day/7days) and assayed liver microsomes for glucose-6-phosphatase (G6PASE) activity. Although the animals were not fasted, they showed the expected response of this enzyme to phenobarbital (PB) treatment namely a decrease followed by an increase. Figure 6.50 shows the original, expanded, and normalized data.

Original Data



Expanded Data



Normalized Data (NORM1)

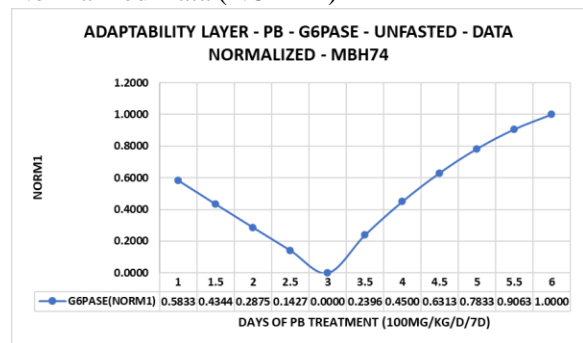


Figure 6.50 The original and normalized data show similar changes.

But how do we explain the initial decrease in G6PASE activity? Several factors were involved. When replacing one phenotype (control) with another (phenobarbital), the average volume of the hepatocytes change, organelles recycle, and synthetic priority goes to the drug-metabolizing enzymes. G6PASE is a carbohydrate metabolizing enzyme.

But what else might have been happening? After day three of PB treatment, the Figure 6.51 shows that the enzyme density of G6PASE ($ED_{G6PASE} = U_{G6PASE}/S_{ER}$) remained relatively constant during most of the first three days, but then increased thereafter. Note that the mashup for the enzyme density estimate

included normalized data coming from mice (G6PASE activity per mg of protein) and the ER surface area per gram of liver from rats (Stäubli et al., 1969). Moreover, the rats were fasted, the mice were not. We're assuming that changes related to a mg of protein paralleled those related to a gram of liver, which introduces an unknown but risk (not recommended except for primers teaching the basics).

ED-NORM2 = $[(U/G-NORM1)/(S/G-NORM1)] \rightarrow \text{NORMALIZED}$

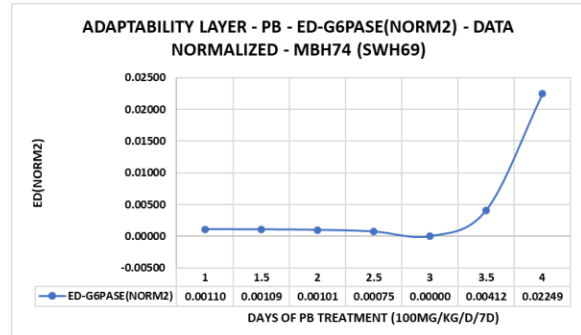


Figure 6.51 The enzyme density data showed little change during the first three days but afterward increased rapidly.

Notice in Figure 6.52 that the normalized results of the current study (MBH74) were like the pattern published earlier (Orrenius and Ericsson, 1966).

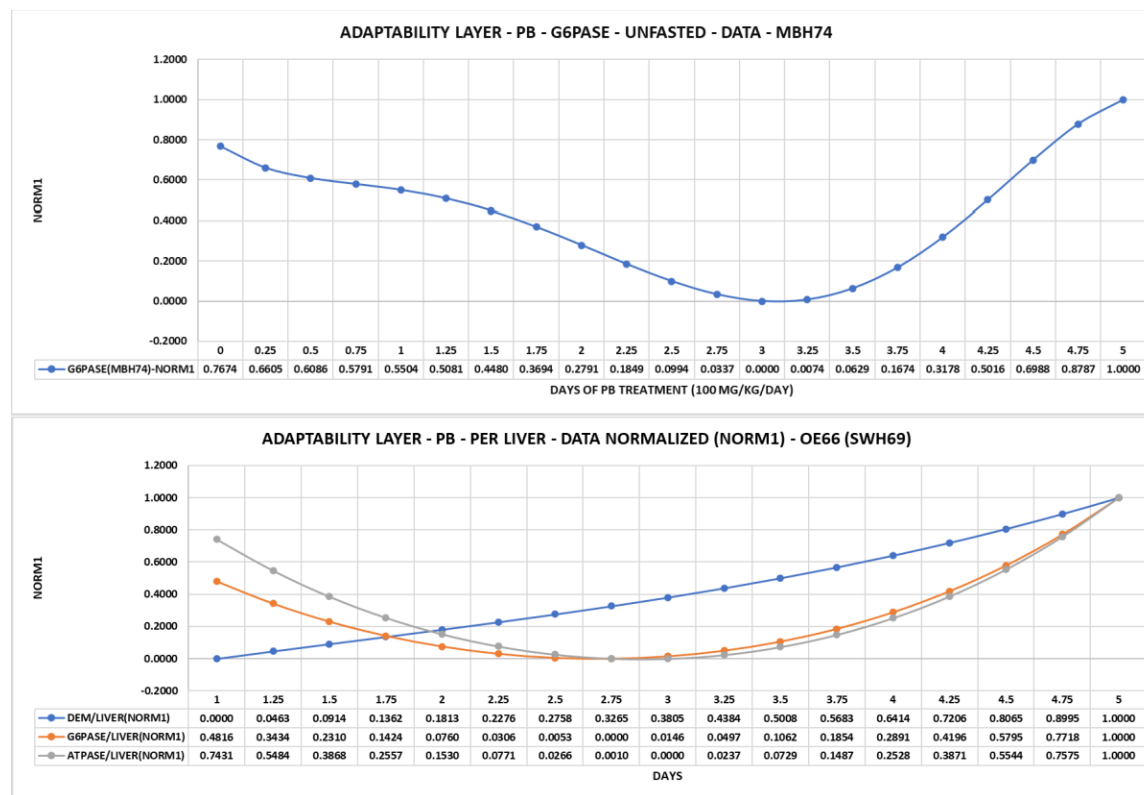


Figure 6.52 Normalized G6PASE activity coming from the microsomal fraction of phenobarbital treated animals (Original data adapted from Menard et al. (1974)- top panel and Orrenius and Ericsson (1966) – bottom panel. Recall that we used mixed data references (mg protein vs liver) for the MBH74 update.

Comment: Figure 6.52 – Different studies, different species, different data references, different nutritional states, different drug doses, yet roughly similar results (reproducibility).

6.1.12 Case Study 12: Phenobarbital (Drug-metabolizing Enzymes) – OE66A

Source: Update applied to original data from Orrenius S., Ericsson J. L. E. (1966A) Enzyme-membrane relationship in phenobarbital induction of synthesis of drug-metabolizing enzyme system and proliferation of endoplasmic membranes. J Cell Biol 28: 181-198. ER membranes: Stäubli et al., (1969); SWH69 & Bolender and Weibel (1973); BW73.

Topic: Morphological and biochemical responses of hepatocytes to phenobarbital.

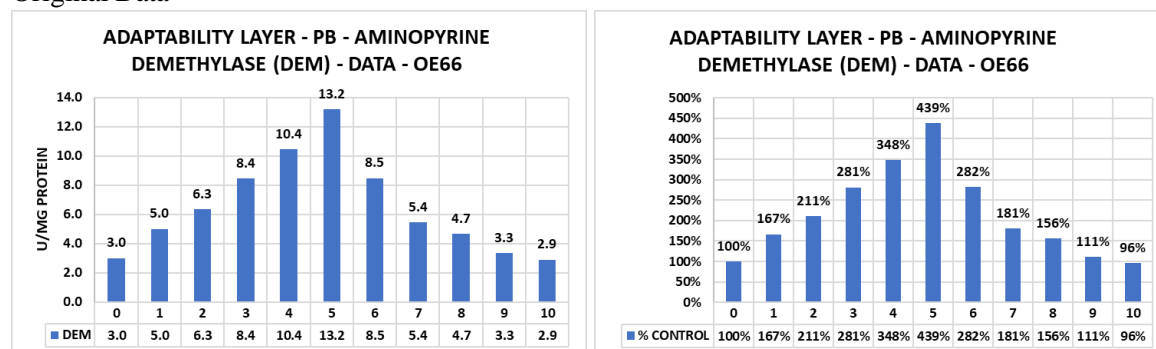
Update: Apply corrections, expand data, report results in adaptability layer, normalize data, calculate enzyme densities [(ED = [(U/G)/(S/G)]; ED(NORM2)], and analyze patterns.

Dataset: Aminopyrine demethylase (DEM) activities and ER surface areas.

In this study, Orrenius and Ericsson (1966; OE66A) studied the biochemical and morphological effects of phenobarbital (PB) on hepatocytes, wherein fasted, adult male rats received five daily injections (100mg/kg/day) of the drug. In response to PB, the drug-metabolizing enzyme aminopyrine demethylase (DEM) increased more than four-fold when related to a mg of protein. The update calculated enzyme densities using a mashup and normalized data. The PB induction part of the study used the ER data of Stäubli et al., (1969; SWH69) whereas the PB recovery portion used ER data from Bolender and Weibel (1973; BW73).

The original data (OE66) detected an increase in demethylase activity in response to the drug followed by a recovery to the control state upon its withdrawal. Normalized data allowed the update to calculate enzyme densities for both the induction and recovery phases of the experiment using mixed data references. Cautions apply because curves produced with data related to the mg protein reference do not always parallel those related to a gram of liver (see Orrenius S., Ericsson J. L. E. (1966) J Cell Biol 31: 243-236). Figure 6.53 includes the original and expanded data for aminopyrine demethylase (DEM).

Original Data



Expanded Data (Induction (PB (100 mg/kgbw/day/5 days) and Recovery (Days 5 to 9.5))

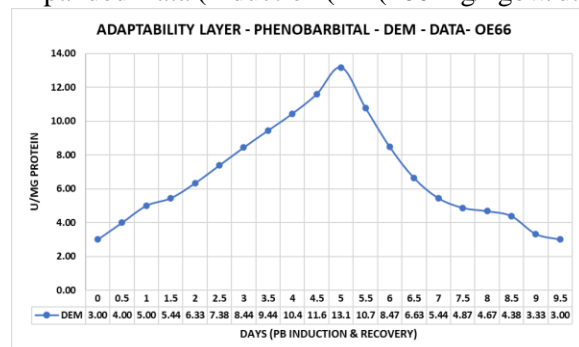


Figure 6.52 DEM activity increased in response to the PB treatment, which returned to normal after the drug was withdrawal.

PB Induction (Days 1 to 5 - 100mg/kg/day/5days): Recall that we're starting with mixed data references - DEM related to a mg of protein and ER membrane surface area related to a gram of liver. The enzyme density calculation will not work algebraically because the references fail to cancel out. We do know, however, that the two data references can detect roughly the same changes given the limitations noted earlier. The workaround consists of using normalized data to remove the references but not the unknown differences between the references.

The changes shown in Figure 6.54 appear inconsistent. Why, for example, did the ER surface area decrease when it's supposed to increase due to the induction of new ER membranes and constituent marker enzymes? By synthesizing ER membranes capable of metabolizing the phenobarbital, the cells became larger and fewer of them fit into a gram of liver. As the number of hepatocytes in a gram of liver decreased, surface area estimates (ER/g) came from fewer and fewer cells. Presumably, this produced the observed decrease. Since the liver didn't lose any hepatocytes, it eventually became larger and heavier. Multiplying membrane surface areas (ER/g) by the weight of the liver would have detected the expected increase.

Expanded Data

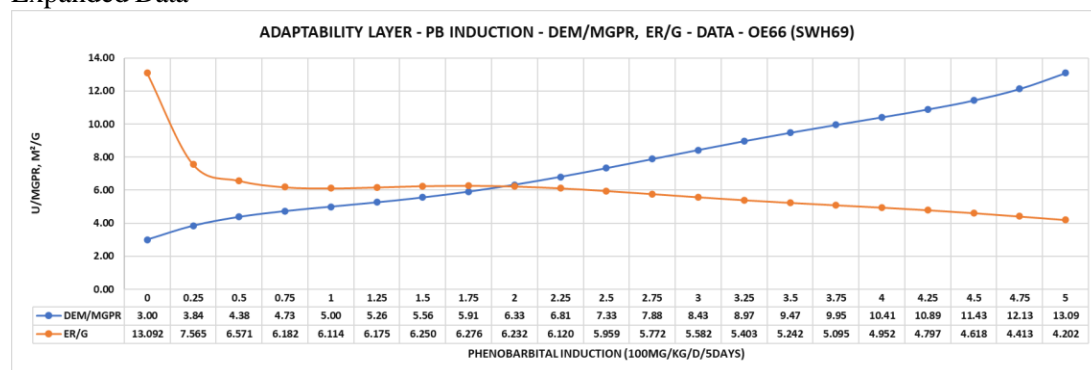


Figure 6.53 Note the presence of mixed data references (mg protein and gram of liver).

By normalizing the data (Figure 6.55), the units disappear, and we can calculate the enzyme densities (ED-NORM2) – acknowledging that we might have added an unknown error by including the mg protein as one of the mixed data references.

Normalized Data (NORM1), ED(NORM2)

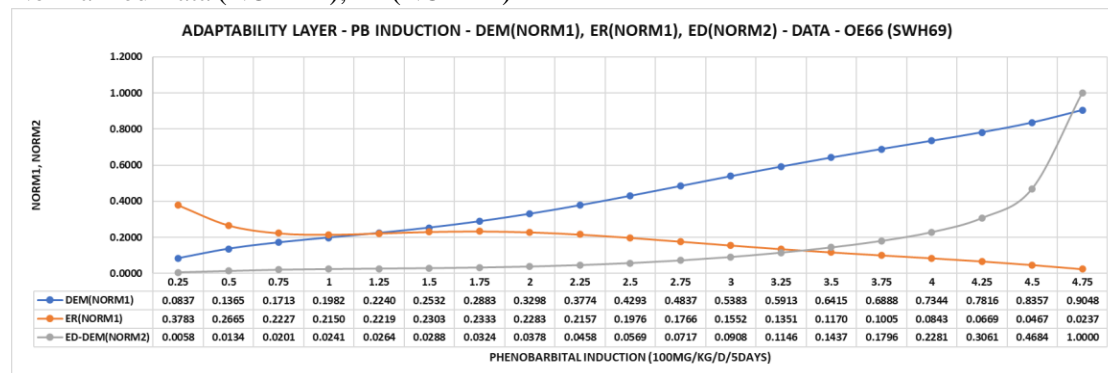


Figure 6.54 Note the steady increase in the enzyme density of demethylase, a drug-metabolizing enzyme.

Comment: The hepatocytes solve their part of the problem by delivering a membrane-enzyme recipe defined by enzyme densities that include parts presumably in the most beneficial amounts and proportions. By scaling the solution to the liver, the cells redefine the liver's capacity.

To explain the way a hepatocyte approaches a change as a problem-solving event, we can copy the way it turns changes in enzyme activities and membrane surface areas into the complex relationships of biochemistry to morphology that little by little redefines the properties of the cell. Since we know that biology changes according to rule-based ratios, we can use the parts expressed as ratios to put the cells back together, which amounts to forward engineer the phenotype with its solution intact. In turn, reverse engineering the cell phenotype sharpens our understanding of a change by detecting multiple subgroups of data pairs each focused on solving a specific part of the larger problem. In effect, a biological change consists of many basic changes that become complex because of the large numbers of parts and relationships in play.

Since the process of updating the literature involves moving data from one theory structure (reductionist) to another (complexity), the gap between what we need and what's available often becomes considerable. This requires workarounds that sometimes solve, but other times only mitigate problems. Although normalization allows us to avoid the mathematical problem of incompatible references when calculating enzyme densities, it nonetheless can add unknown errors coming from the data references. Mixing the mg protein reference with a gram of liver or a cm³ of hepatocyte cytoplasm produces the greatest uncertainty because it appears that we can expect the corresponding curves to run in parallel for only part of an experiment. The point? For the biology literature to become a renewal resource, we need to rethink our current policy of not publishing raw data.

6.1.13 Case Study 13: Phenobarbital (Hepatocytic Enzymes and Membranes) – SHW69

Source: Stäubli, W., Hess, R., Weibel, E.R. (1969) Correlated morphometric and biochemical studies on the liver cell. II Effects of phenobarbital on rat hepatocytes. J Cell Biol. 42:92-112.

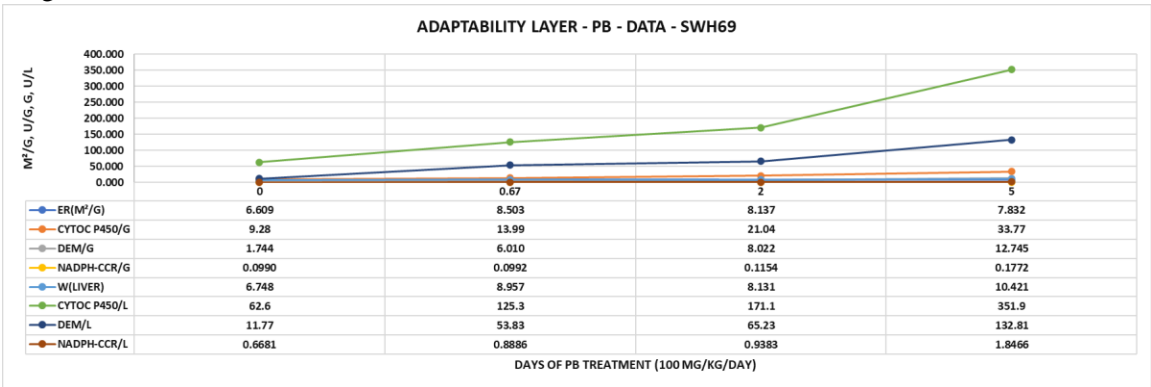
Topic: Morphological and biochemical responses of the liver to phenobarbital.

Update: Apply corrections, expand data, include data from adaptability and rules layers, normalize data, calculate enzyme densities (ED, ED(NORM2), and analyze patterns.

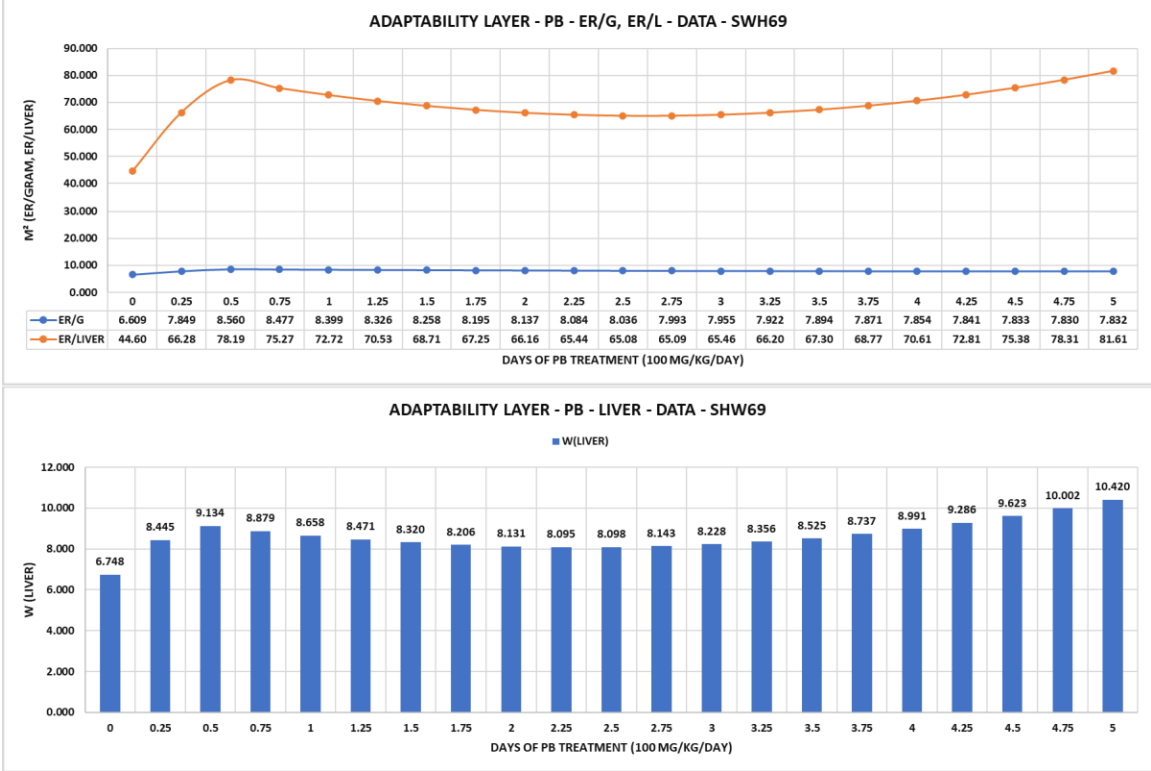
Dataset: Aminopyrine demethylase (DEM), cytochrome P450, NADPHCCR activities, and ER surface areas.

Stäubli et al., (1969; SHW69) used morphology and biochemistry to detect changes in hepatocytes exposed to phenobarbital (PB) for five days (100mg/kg/day/5 days). The update applied section corrections, recalculated the results, normalized the data, calculated ratios and enzyme densities, and reported recipes. Figure 6.55 starts with the basic data.

Original Data



Expanded Data



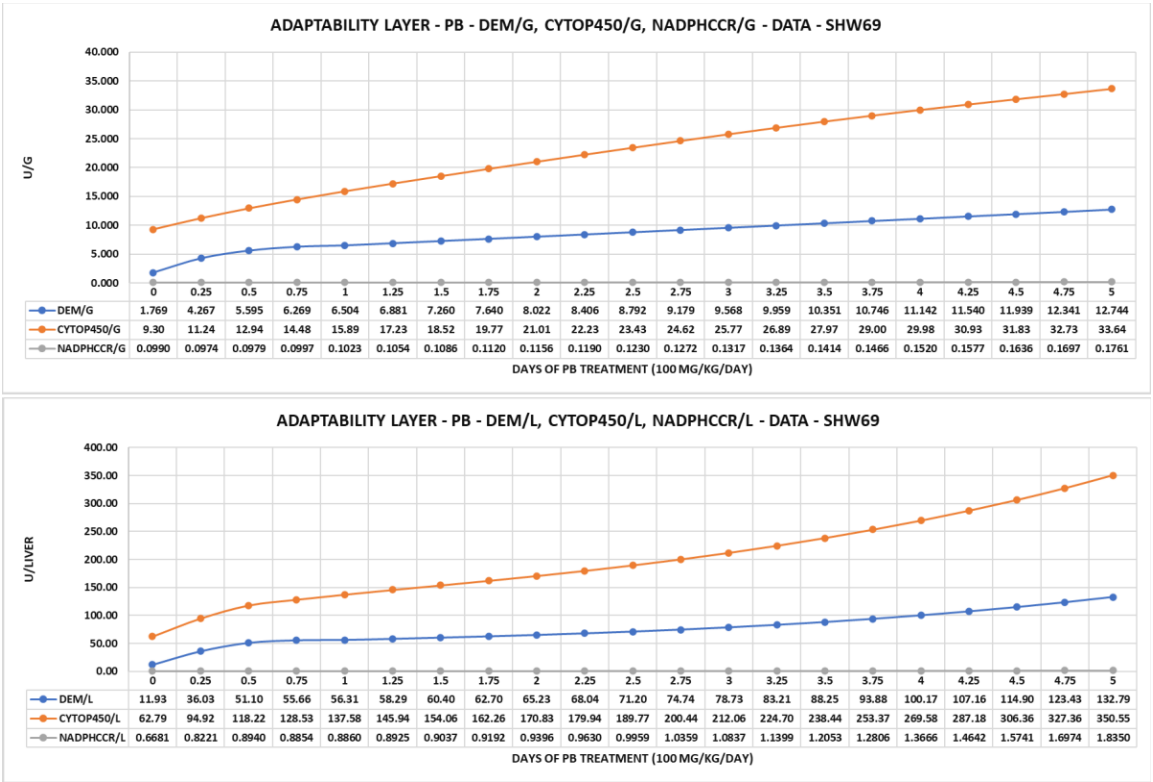


Figure 6.55 Original and expanded data expressed relative to the gram and liver references.

Figure 6.56 used the expanded data to calculate local changes in enzyme densities [ED = (U/G)/(S/G)].

Enzyme Density [ED = (U/G)/(S/G)]

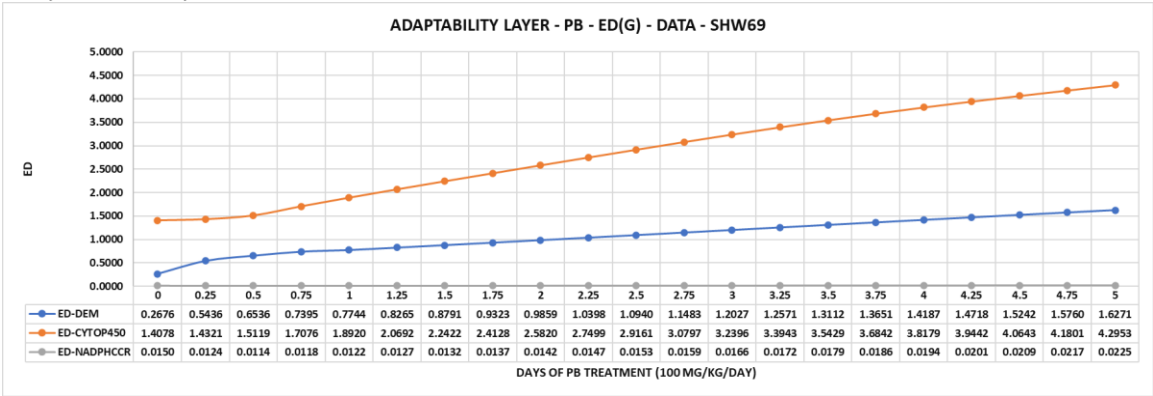


Figure 6.56 Enzyme densities showed a wide range in values, which explains why the doubling of the values for ED-NADPHCCR did not appear in the graph. [Note that when expressed in the rules layer (data rounded), all three enzymes ED-DEM, ED-CYTOP450, and ED-NADPHCCR appeared to change in parallel. However, the smaller values for ED-NADPHCCR activity became overwhelmed by the larger values of the other enzymes. Normalizing the local enzyme densities fixed the data range problem.]

Normalizing the changes related to different references (gram of liver and the liver), simplified comparisons (Figure 6.57).

Data (NORM1)

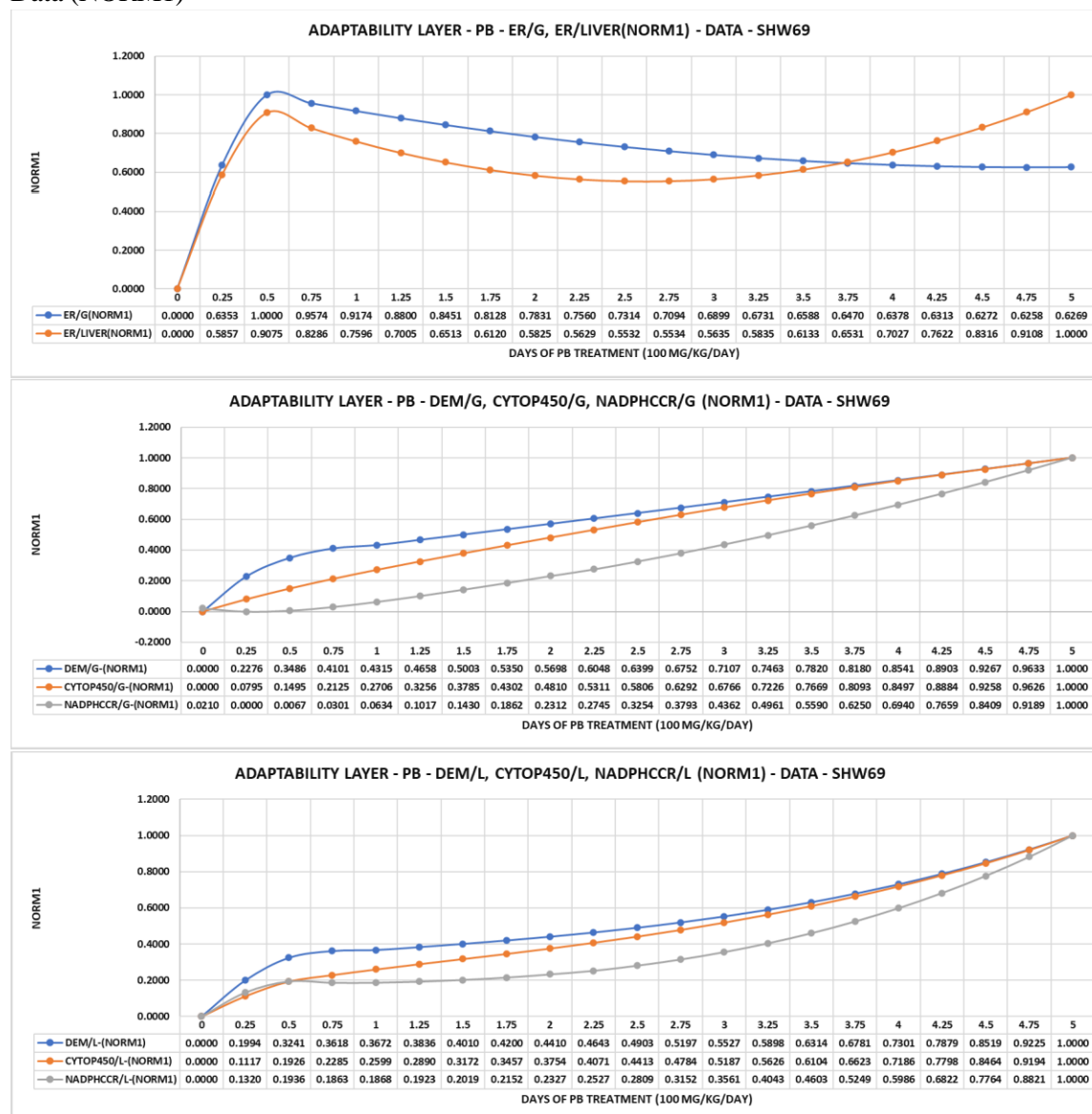


Figure 6.57 After normalizing the data, one can distinguish between changes related to a changing number of hepatocytes per gram of liver and to a fixed number of hepatocytes per liver.

Calculating Data Pair Ratios from the Normalized Data: By normalizing the enzyme densities shown in Figure 6.56, we can generate data pair ratios in the rules layer and compare patterns of change (Figure 6.58). Notice that the three drug-metabolizing enzymes eventually shared the same solution to the phenobarbital problem (0.5:0.5). By sharing the same solution (0.5:0.5), the three enzyme densities become part of the same drug-metabolizing subgroup (0.5:0.5).

Data Pair Ratios - (ED-NORM1)

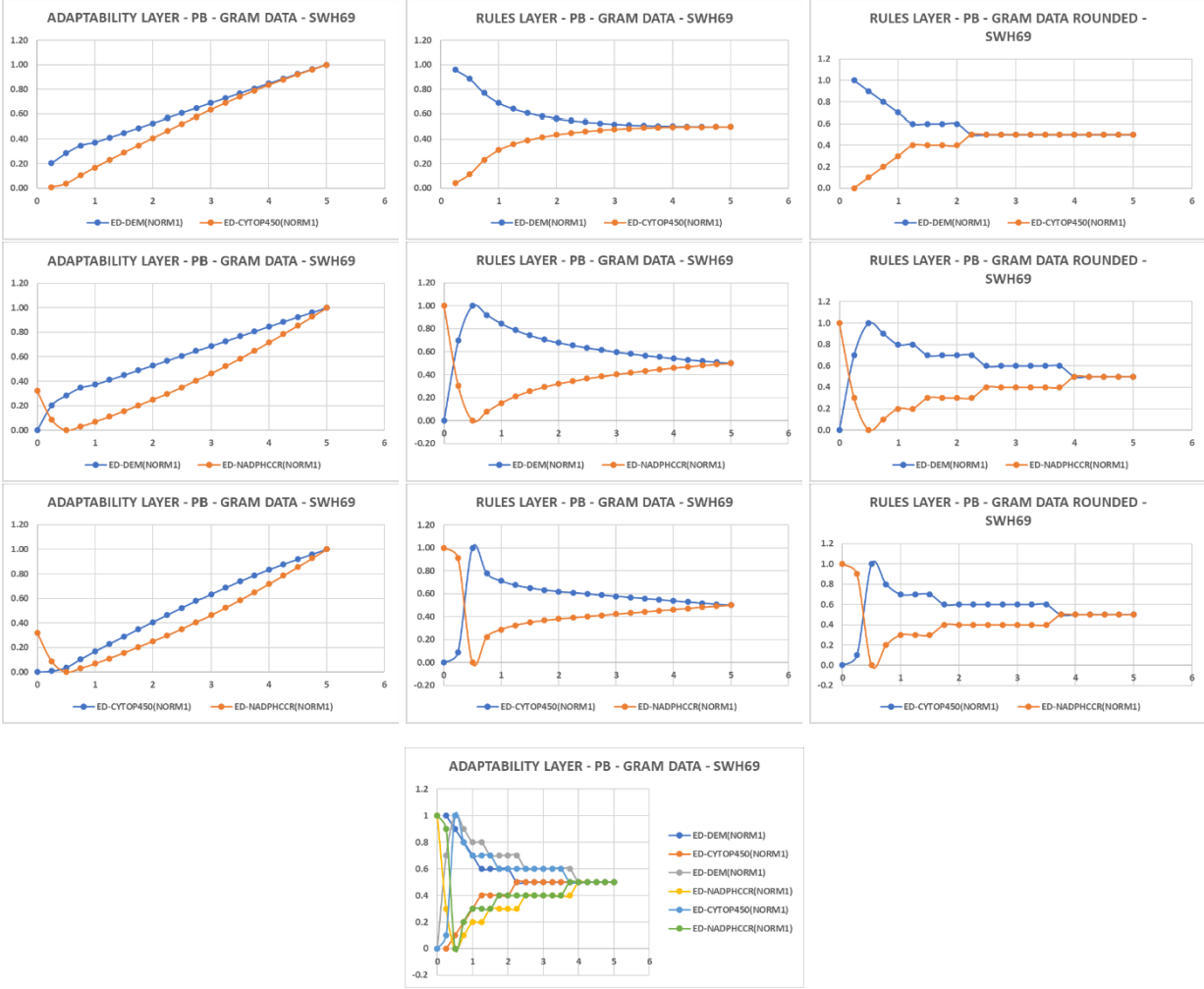
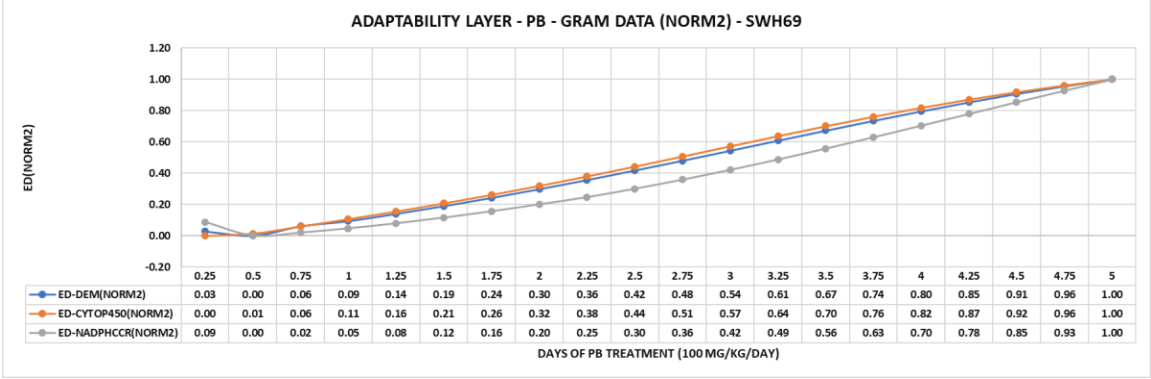


Figure 6.58 All three drug-metabolizing enzymes eventually belonged to the same subgroup (0.5:0.5). By superimposing the plots of the three data pairs, we can see when and where each data pair found its solution (0.5:0.5).

Calculating global enzyme densities (ED-NORM2) and data pair ratios from the normalized data of Figure 6.57 produced the patterns shown in Figure 6.59.

Enzyme Density – ED(NORM2)



ED Rules (NORM2)

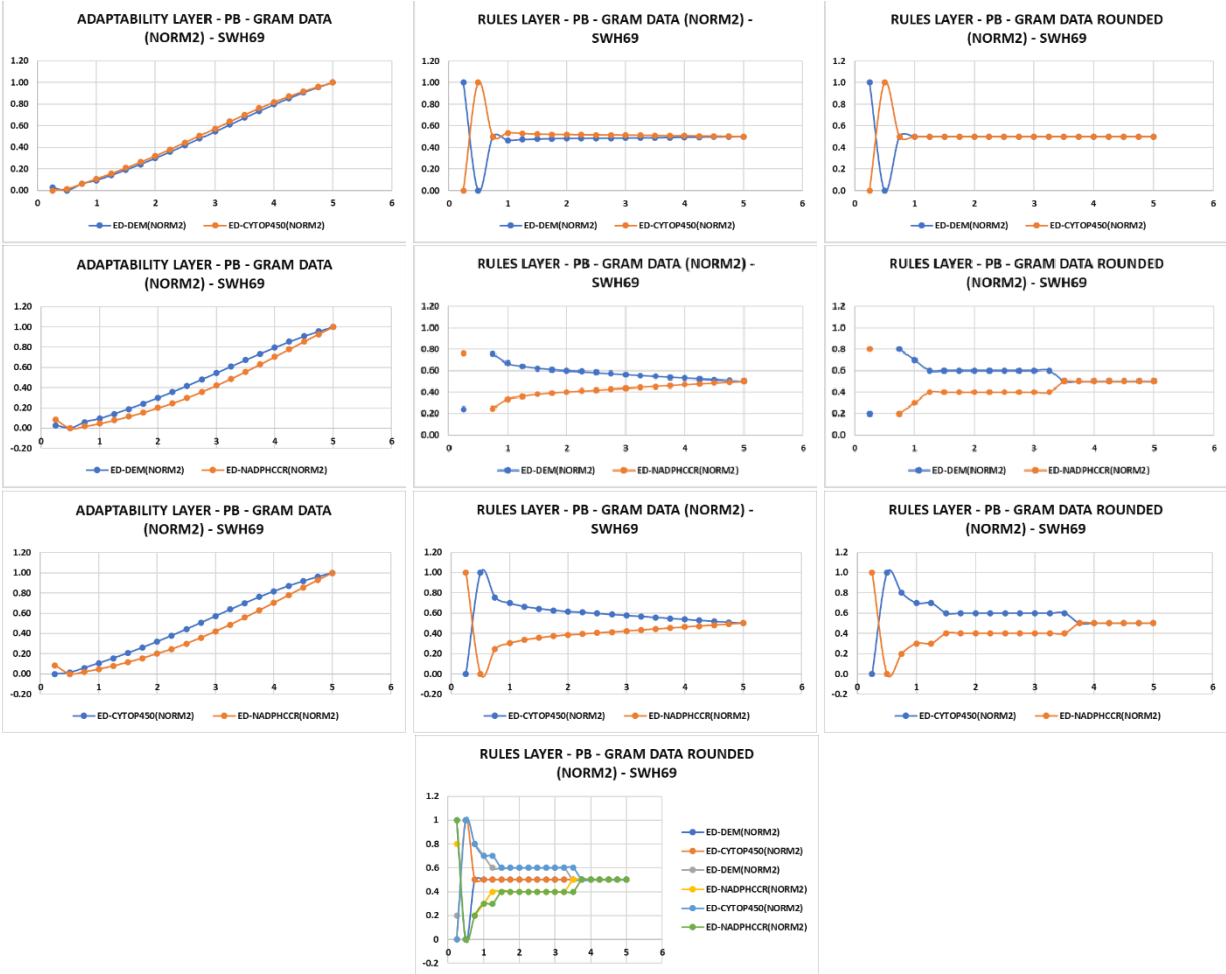
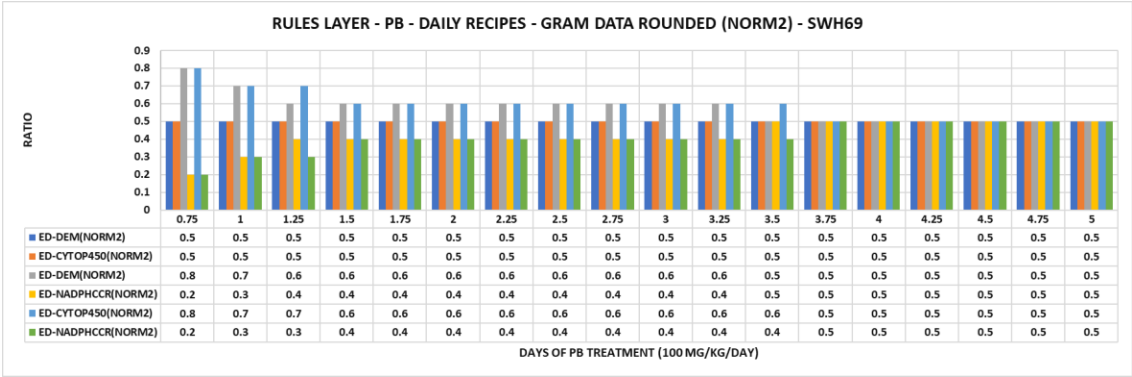


Figure 6.59 One data pair ratio (DEM and CYTOP450) found the solution first followed by the remaining data pairs of the subgroup. A point of some interest is that hepatocytes provided empirical evidence that they use reproducibility in the rules layer to tune complex relationships of structure to function. If arriving at a solution involves optimizing the relationship of one enzyme density to another, then the staggered solutions suggest that optimizing outcomes involve a learning process.

The enzyme density recipes became the solution to the PB problem, which in turn provide the emergent properties of the adaptation. Figure 6.60 chronicles the progressive changes to the recipes (the PB solution).

ED Recipes (NORM2)



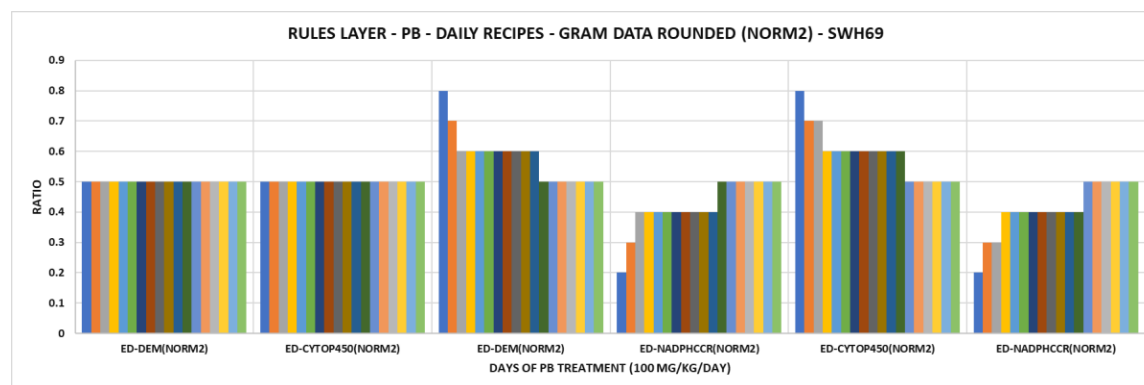


Figure 6.60 The histograms summarize changes occurring in the relationships of structure to function (enzyme densities) for a given enzyme density and for changes occurring between pairs of enzyme densities. In effect, biology creates complexity by embedding ratios within ratios and by forming ratios between data pairs within and across the subgroups of phenotypes. By copying hepatocytes, one can use patterns as measure of reproducibility.

Notice in Figure 6.61 that the original local (ED) and normalized global (ED-NORM2) enzyme densities produced reasonably parallel curves.

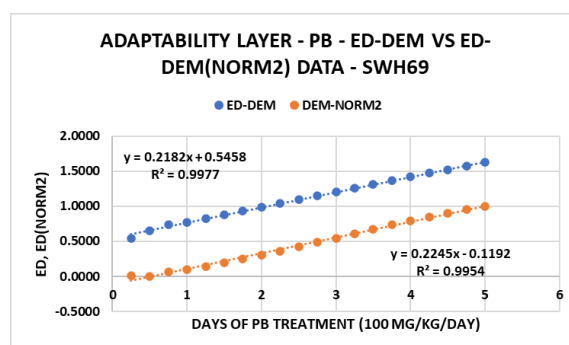


Figure 6.61 By plotting the original enzyme densities (ED) for DEM next to the normalized (ED-NORM2) ones, the results show parallel curves displaying similar slopes. In effect, both estimates detected similar increases in enzyme density. This reassures us that the same data lead to the same pattern of change when calculated from data with and without data units.

Comment: The hepatocytes recognized the phenobarbital problem, found a quantitative solution in the cell cytoplasm with enzyme densities and then scaled the solution to the required liver capacity.

6.2 General Point

Once the authors selected and tested the results for a significant difference, the experiment essentially ended. Either a change occurred (significantly different) or it did not (not significantly different). There was no data trail left to follow. By updating published studies, we restarted the experiments and gave the hepatocytes a chance to finish their stories. We now know that a cellular change involves far more than a simple binary event. To wit, did it change - yes or no? By pursuing a first principles approach we can learn enough of the basics to copy the cells to discover the details. Although a science can be driven by details or assumptions, the primer focuses largely on the details.

CHAPTER 7

MISCELLANEOUS PHENOTYPES

Summary

This chapter uses the case studies to focus on topics basic to the experimental process. Given the complexity of biology and the limitations of our experimental methods, we resorted to assumptions openly or tacitly. In the sciences, assumptions become the “loans” that eventually become due. For example, we borrowed statistics to solve the knotty problem of detecting changes in cells by assuming that a statistical difference meant a significant change. This assumption of a “significant change” evaporated when meta-analyses brought us to book: “Why most published research findings are false.” (Ioannidis, 2005). Although we can probably fix the correctness issue statistically, it still leaves the underlying assumption untouched. We still assume that a complex biological change has a simple statistical solution. Consequently, updating the literature became an attempt to repay the loan by checking the assumptions.

Although some investigators might still believe that a reductionist approach to detecting a biological change can work for us, it doesn’t work for biology. While we can see a biological change as the significant difference between two data points, cells cannot because they must change physically by literally growing into a new phenotype. Curiously, the most interesting part of a change may not be the before and after but what happens mathematically in between. This complex event becomes a basic understanding when we copy the way cells apply a first principles approach to change. The primary advantage of copying the hepatocyte’s approach to change is that it works most of the time because it’s based on a rule-based system designed by cells to be failsafe. When, for example, something doesn’t work experimentally, we can usually figure out why and then fix the problem by applying the rules built into the cells.

7.1.1 Case Study 1: Fasted Animals (Liver Parenchyma) – BBW77

Source: Update applied to original data from Blouin A., Bolender R.P., Weibel E.R. (1977) Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma. A stereological study. J Cell Biol 72(2):441–455.

Topic: Stereological analysis of the rat liver.

Update: Expand data, report results in adaptability and rules layers, normalize data, and analyze cell patterns.

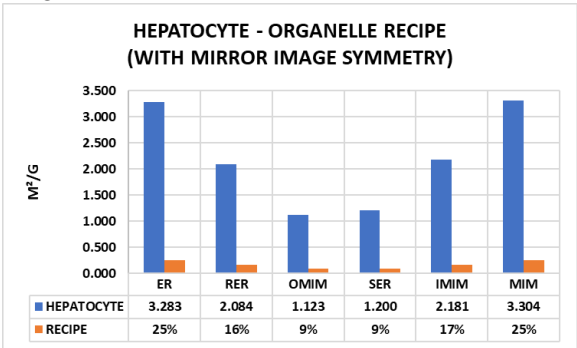
Dataset : Membrane surface areas for ER, mitochondrial, and other membrane compartments in each of the four parenchymal cell types: hepatocytes, endothelial cells, Kupffer cells, and fat-storing cells.

When combined, the control membranes of the parenchymal cells (hepatocytes, endothelial, Kupffer, and fat-storing) form distinct patterns that serve as the organelle recipes of the cell phenotypes (Blouin et

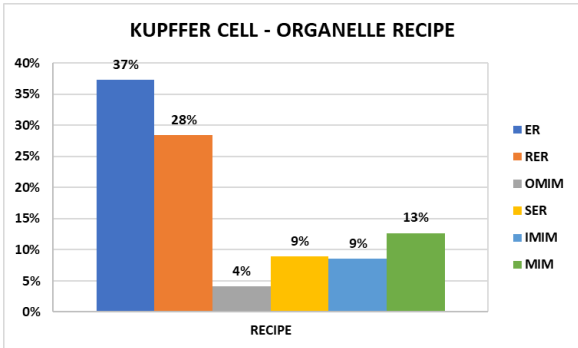
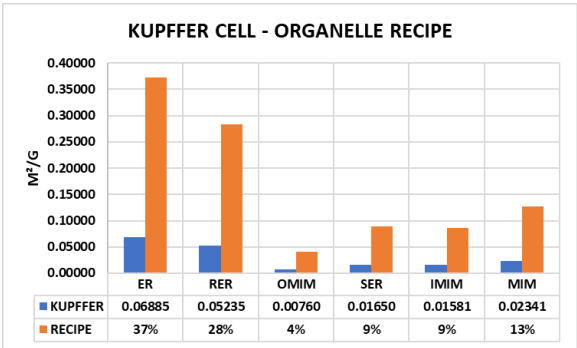
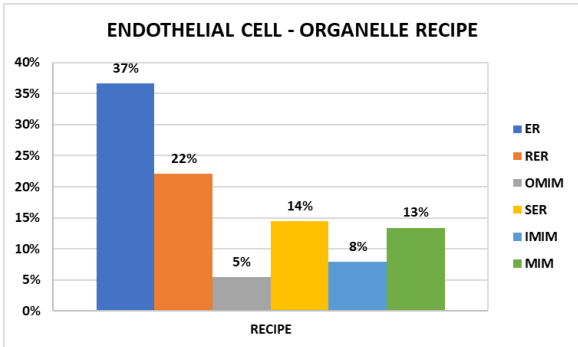
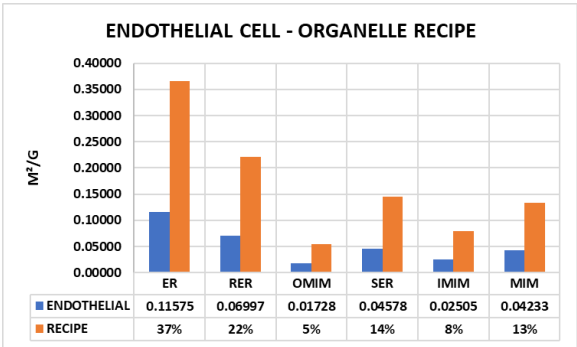
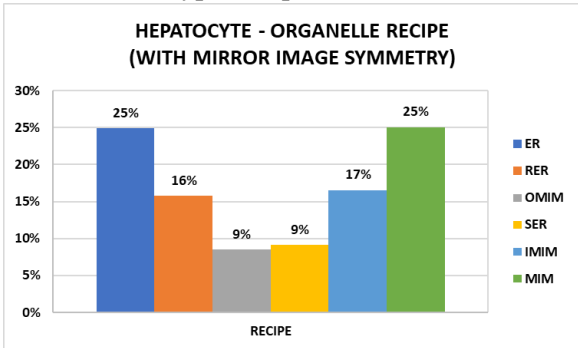
al.,1977; BBW77). In hepatocytes, for example, the surface area of the endoplasmic reticulum (ER) often matches the mitochondrial membranes (MIM), the rough-surfaced ER the inner mitochondrial membrane (IMIM), and the smooth-surfaced ER the outer mitochondrial membrane (OMIM). Furthermore, it appears that pairing membrane surface areas within the larger framework of symmetries identifies design principles hepatocytes apply when solving problems. Hepatocytes exhibit a remarkable plasticity that allows them to morph into special purpose phenotypes displaying new recipes with new properties. Similarly, endothelial, Kupffer, and fat-storing cells display recipes and changes consistent with the shifting functions of their phenotypes.

After looking at the properties of the individual cell types (Figure 7.1), we'll make cell to cell comparisons to identify their design principles.

Original Data



Control Phenotype Recipe



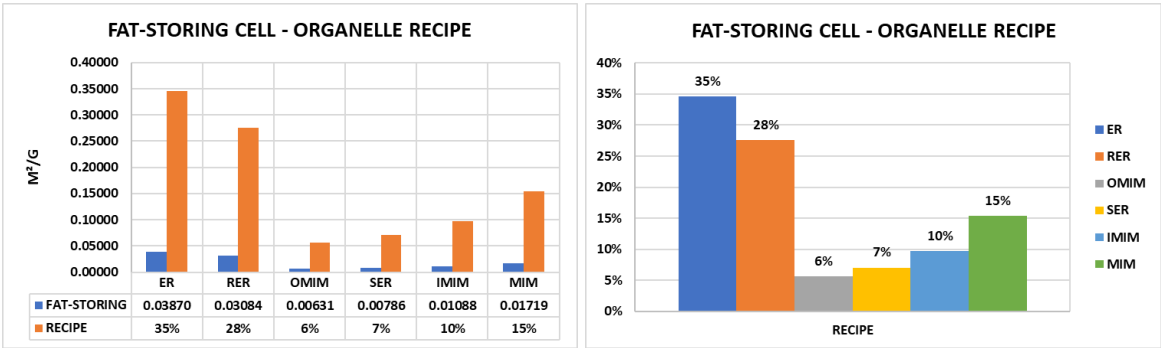
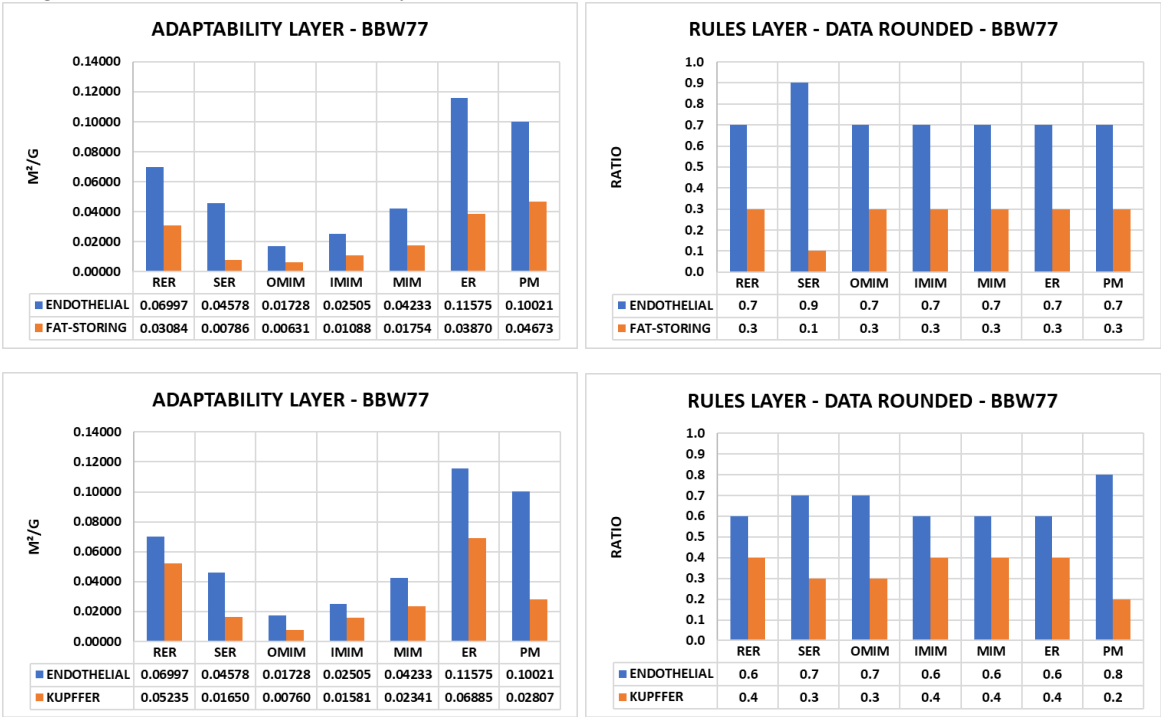


Figure 7.1 Organelle recipes identify their relative proportions. Changes in recipes appear in both adaptability and rules layers.

By translating the data of adaptability layers into ratios, we can identify the rules underlying the recipes (Figure 7.2). Since the rules define dimensionless ratios, they become prime candidates for checking the representativeness of the sampling methods and for demonstrating reproducibility. Reproducibility plays a key role throughout the experimental process by providing validity checks and locating methodological errors.

Both absolute and relative amounts of organelles influence the recipes and properties of cell types. This becomes evident by comparing organelle ratios calculated from pairs of cells. If we compare endothelial to fat-storing cells in the rules layer, for example, six of the seven organelle pairs displayed the same ratio (7:3). This means that one of the ways to design a cell type or to change its phenotype involves changing the rules related to the relative and absolute amounts of membrane organelles populating a cell. Notice that the endothelial vs Kupffer and Kupffer vs fat-storing data pairs share the same ratios for RER, SER, IMIM, MIM, and ER.

Original Data (Units) → Rules Layer (No Units)



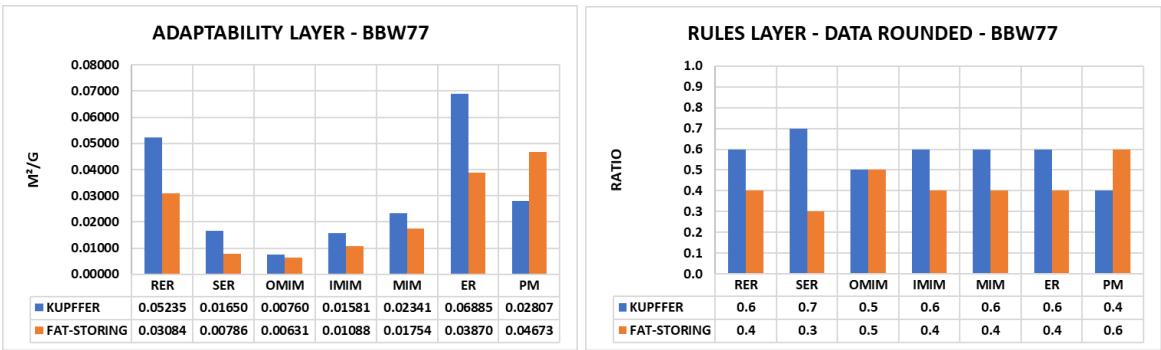


Figure 7.2 Relationships of organelle recipes occur within and between cell types. By forming ratios between the same organelle in different cells, cells become connected quantitatively and patterns appear.

Recall how the normalization process works (Figure 7.3). It assigns one to the largest value, zero to the smallest value, and then calculates values for all those in between. Normalizing a dataset has the advantage of comparing changes within the same range (0 to 1) and removing data units. Only the change remains, which we can capture as a polynomial equation. By summarizing a change with an equation, we obtain a complex piece of information that simplifies the task of finding rules and first principles.

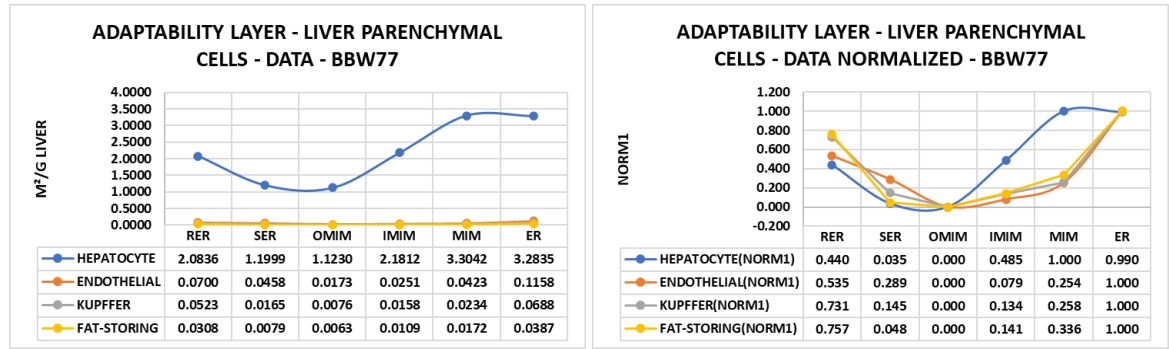


Figure 7.3 By normalizing results, organelles occurring in widely different amounts in different cell types can appear on the same plot.

In turn, normalized data become central to the task of assembling phenotypes from the heterogeneous publications that define the literature. A liver phenotype based on the membrane surface areas of parenchymal cell membranes, for example, conveys a complex pattern of interrelationships that change in response to internal and external inputs. In this case study, however, we encountered a technical problem. Using just six membrane compartments for the normalization led to the loss of information coming from the OMIM (0) and ER (1) data pair. Also dividing by zero created a loss of data. When unfolding the complexity of a change, for example, expanding the dataset by choosing a smaller time interval to increase the number of data points usually mitigates such problems. In practice, the minimum number of data points needed to produce a satisfactory result can sometimes be as few as ten.

By taking three cell types two at a time and forming ratios, we can see how the pairs of cell recipes compare (Figure 7.4). Notice that endothelial and fat-storing cells differed almost entirely by the absolute amounts of organelles, not their ratios. This identifies a fundamental design principle used to differentiate cells.

Rules Layer – Ratio Data



Figure 7.4 By relating the organelles of one cell type to that of another by calculating ratios, similarities and differences appear. For example, the organelle recipes of endothelial and fat-storing cells differ by only the relative amount of the SER. In effect, nearly identical organelle recipes produced completely different cells. However, we can imagine that the enzyme density recipes could be entirely different.

Summary: Biology appears to do everything by rule (Figure 7.5). By expressing the original data as ratios, we can see for example how biology uses a modular strategy to construct cells with unique properties by merely rearranging ratios of small whole numbers.

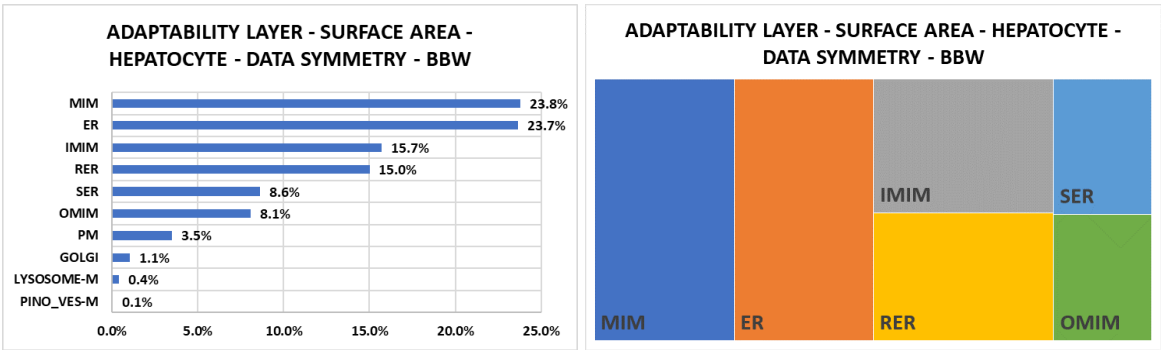


Figure 7.5 Cells display order observable as amounts and proportions. In turn, each membrane compartment defines a recipe of enzymes, both of which change when tuning or changing phenotypes. As we move through the cell's hierarchies of size, the rules layer moves with us.

7.1.2 Case Study 2: Removal of PB-induced Membranes from Hepatocytes) – BW73

Source: Update applied to original data from Bolender R. P., Weibel E. R. (1973) A morphometric study of the removal of phenobarbital-induced membranes from hepatocytes after cessation of treatment. J Cell Biol 56(3):746-761.

Topic: Removal of drug-induced ER membranes.

Update: Apply corrections, expand data, report results in adaptability and rules layers, and normalize data.

Dataset: Surface areas of the ER, RER, and SER membranes.

After the last of five daily injections of phenobarbital (100MG/KG/DAY/5 DAYS), the hepatocytes began to remove the drug-induced membranes and enzymes and replaced them with those of a control phenotype. ER membranes were related to a gram of liver and to the liver in the adaptability (Figure 7.6) and rules layers (Figure 7.7).

Original Data (M²/GRAM)

(M²/LIVER)

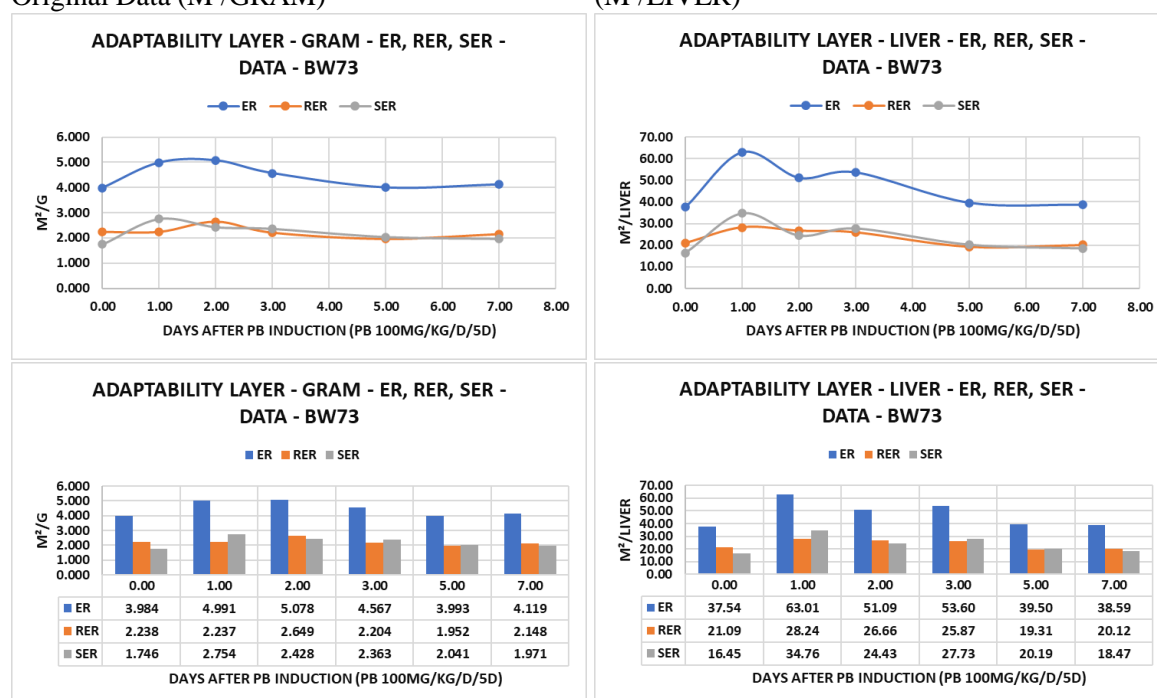


Figure 7.6 Changes in membrane surfaces related to a gram of liver and to the liver. Multiplying a gram data by the weight of the liver to get the liver values resulted in a slightly different set of curves.

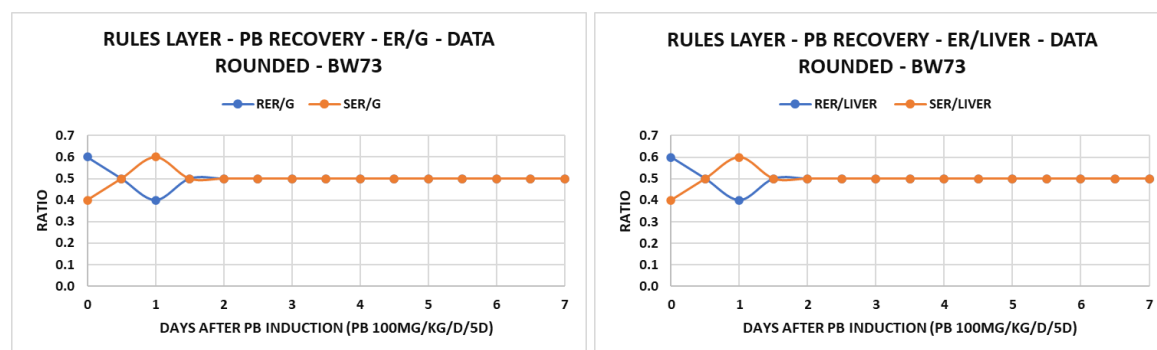
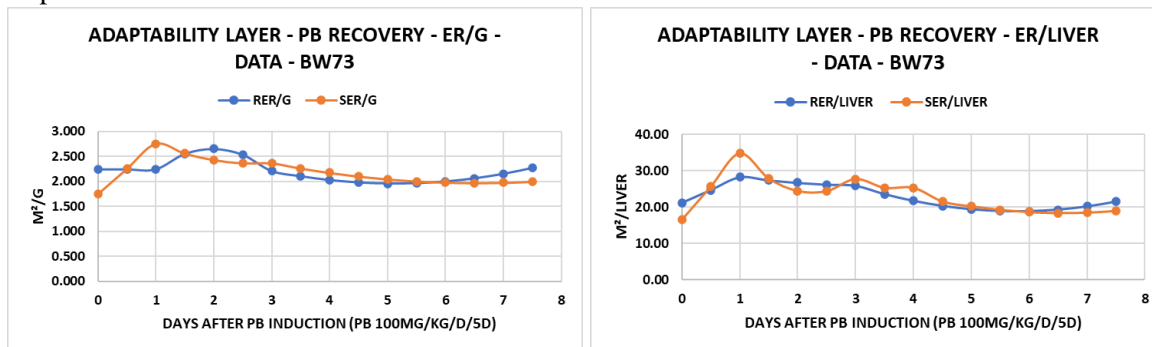


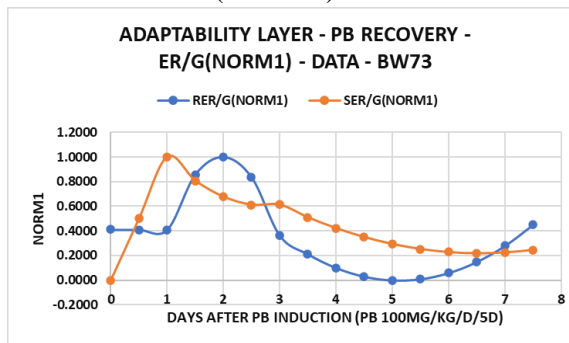
Figure 7.7 However, when calculating RER to SER ratios, both data references gave the same result. Multiplying a gram of liver by the liver weight changed the amounts but not the proportions of the parts.

When normalized and viewed in the rules layer, the RER:SER ratios are no longer the same when calculated from the gram and liver data (Figures 7.8, 7.9). Normalization doesn't change the changes, but it does change the relationship of one change to another. It restricts the changes to one data range (0 → 1), whereas the original data allow many ranges. Consequently, data related to a gram of liver and to the liver generate different ratios in the rules layer. For this reason, we routinely use the gram of liver to calculate enzyme densities.

Expanded Data



Normalized Data (NORM1) - GRAM



Normalized Data (NORM1) - LIVER

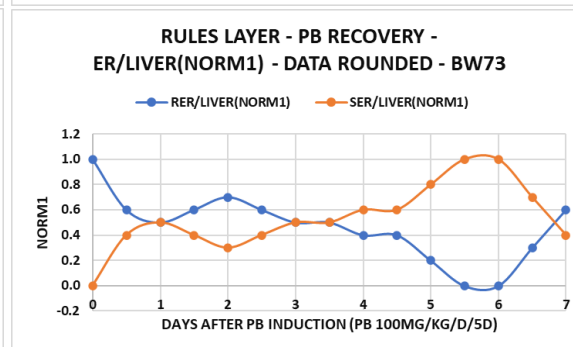
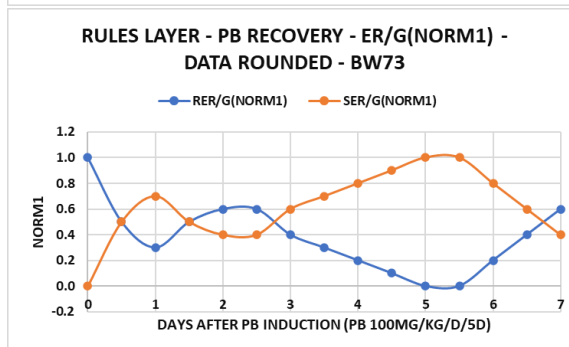
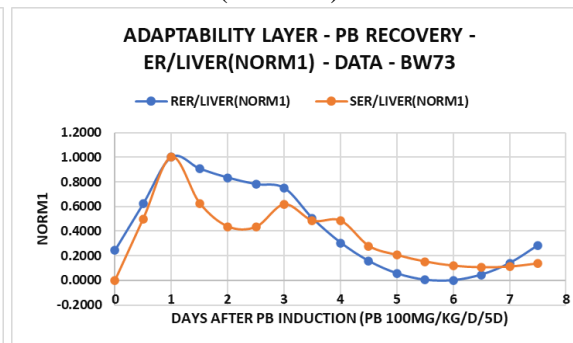


Figure 7.8 When using normalized data to generate ratios in the rules layer, the gram of liver and the liver produce different ratios and consequently different patterns. When calculating enzyme densities from normalized data, the original data was almost always related to a gram of liver.

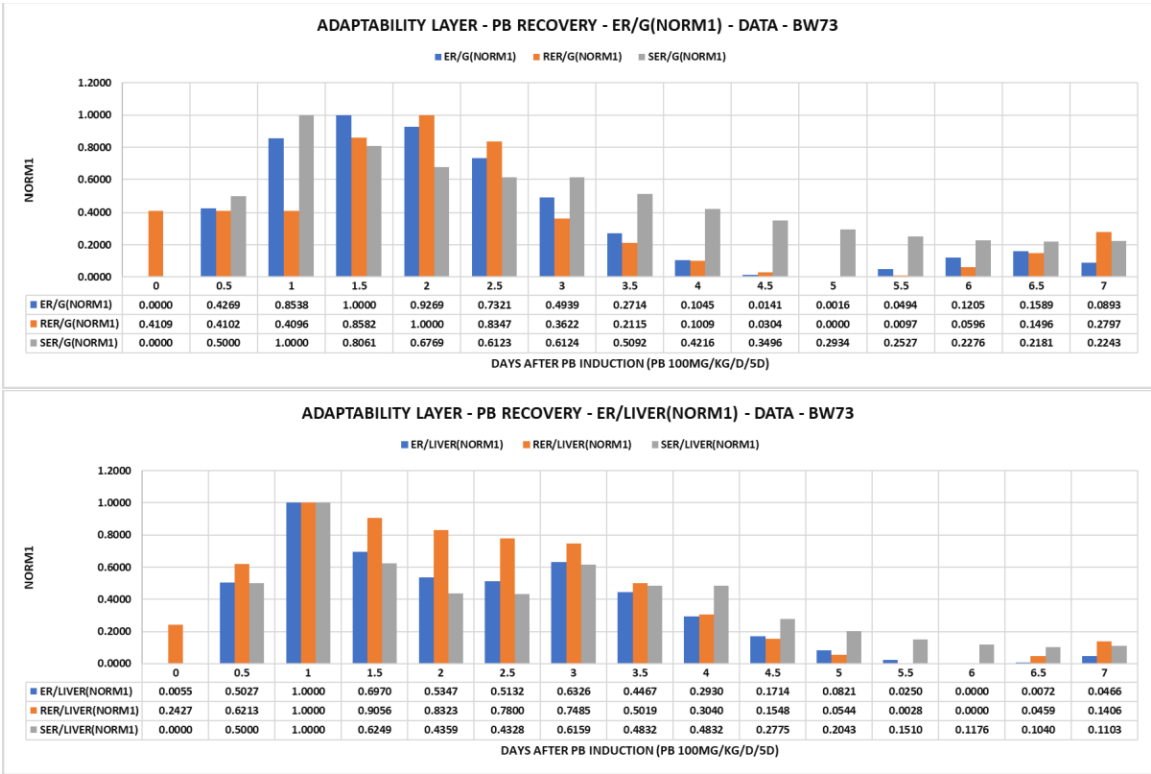


Figure 7.9 Patterns of normalized changes reflect the choice of data reference.

Summary: Using normalized data addressed two fundamental problems, aggregating data across publications and demonstrating reproducibility. Comparing absolute changes rarely works because both methods and local conditions routinely lead to different results. In contrast, comparing relative changes produced by normalization levels the playing field by allowing only one data range (0 → 1). When encountering two similar datasets, for example, we can test them for reproducibility by regressing one against the other to see how close the slope and R^2 of the resultant line are to one.

7.1.3 Case Study 3: Enzyme and Membrane Recoveries in Subcellular Fractions - BPLMW78

Source: Update applied to original data from Bolender R. P., Paumgartner D., Muellener D., Losa G., Weibel E. R. (1978) Integrated stereological and biochemical studies on hepatocytic membranes. I. Membrane recoveries in subcellular fractions. *J Cell Biol* 1978 May 1; 77(2): 565–583.

Topic: Morphological and biochemical analysis of the liver using analytical fractionation; test and confirm the postulates of biochemical homogeneity.

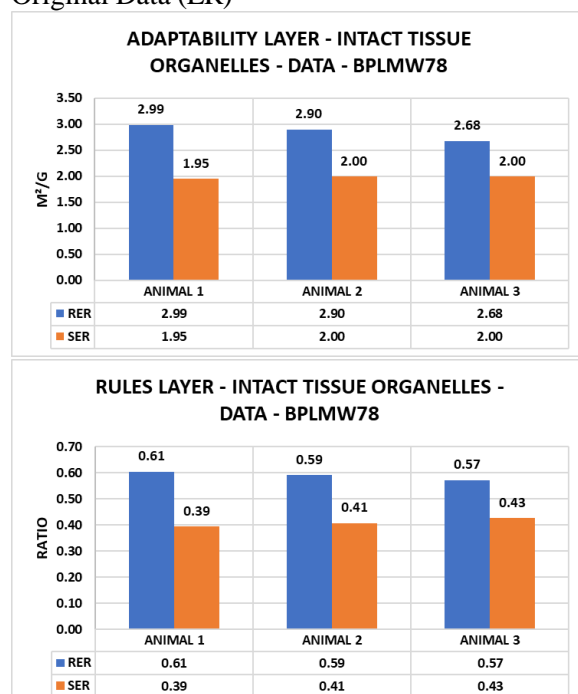
Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, calculate enzyme densities, and analyze patterns.

Dataset: Biochemistry: Glucose-6-phosphatase (G6PASE), cytochrome oxidase (CYOX), monoamine oxidase (MAO), 5'nucleotidase (5'NUC); Morphology: ER (RER, SER), mitochondria (MIM, OMIM, IMIM), GOLGI, and PM.

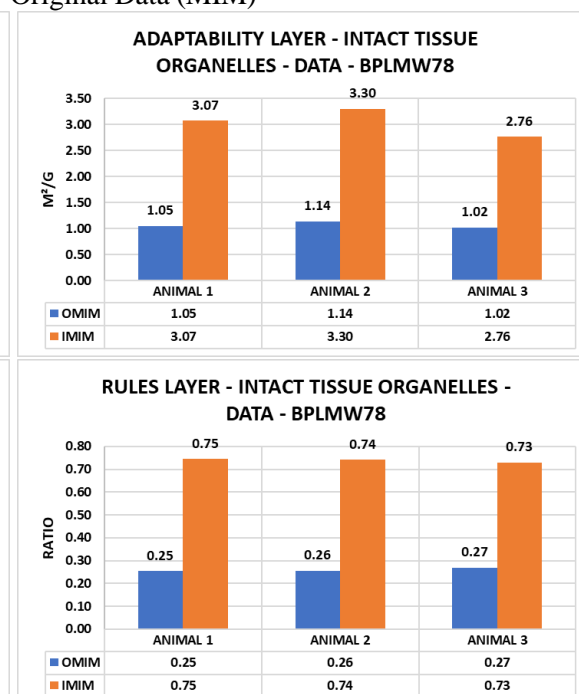
The original study showed that membrane surface areas estimated from intact liver tissue were reproducible in the tissue homogenate and fractions within the framework of analytical fractionation (de Duve, 1974). The membrane recoveries were comparable to those of the biochemical assays. Although the update started with the original data, it used a complexity model (copied from hepatocytes) to work up and analyze the results.

Original Data: To begin, we'll look at the ER and mitochondrial data of the individual animals (Figure 4.10). This will allow us to evaluate the reproducibility of the different experimental methods. Although the values differed slightly in the adaptability layer, the stereological estimates of the ER and mitochondrial membranes in the rules layer demonstrated homogeneous sampling. This created a level playing field for running tests of statistical significance should the need arise to demonstrate a difference between two data points. Currently, investigators don't distinguish between homogeneous and heterogeneous populations when running such tests, which might have contributed to our current statistical problems (Ioannidis, 2005). Populations originally homogeneous can become heterogeneous because of the methods.

Original Data (ER)



Original Data (MIM)



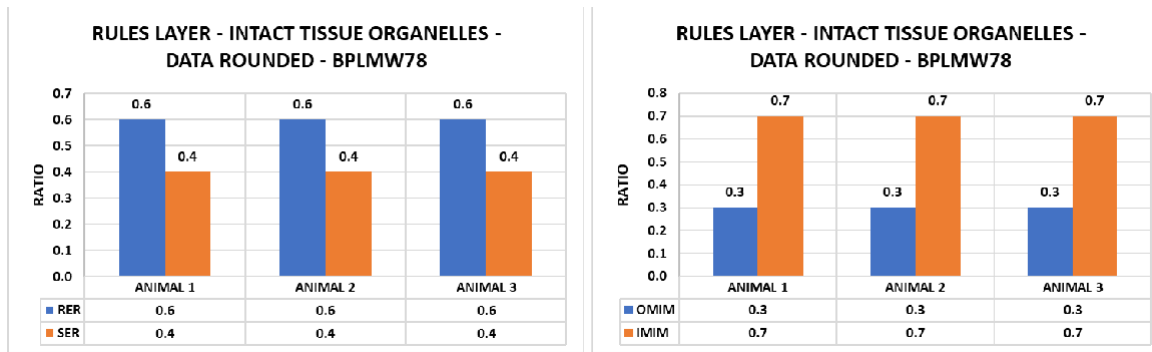


Figure 7.10 By expressing the data pairs from the adaptability data as ratios in the rules layer, we can evaluate the sampling. Notice the uniformity of results for all the membrane pairs (OMIM:IMIM, RER:SER) in the rules layer (data rounded). Such agreement suggests representative (homogeneous) sampling.

Using the membrane surface areas reported from individual animals, we can run local tests for duplicate patterns. Calculating ratios for the three animals taken two at a time produced three test results. The results were reproducible across the three animals (data rounded) except for the Golgi and plasma membranes (Figure 7.11). The miss with the Golgi and plasma membranes suggests under sampling.

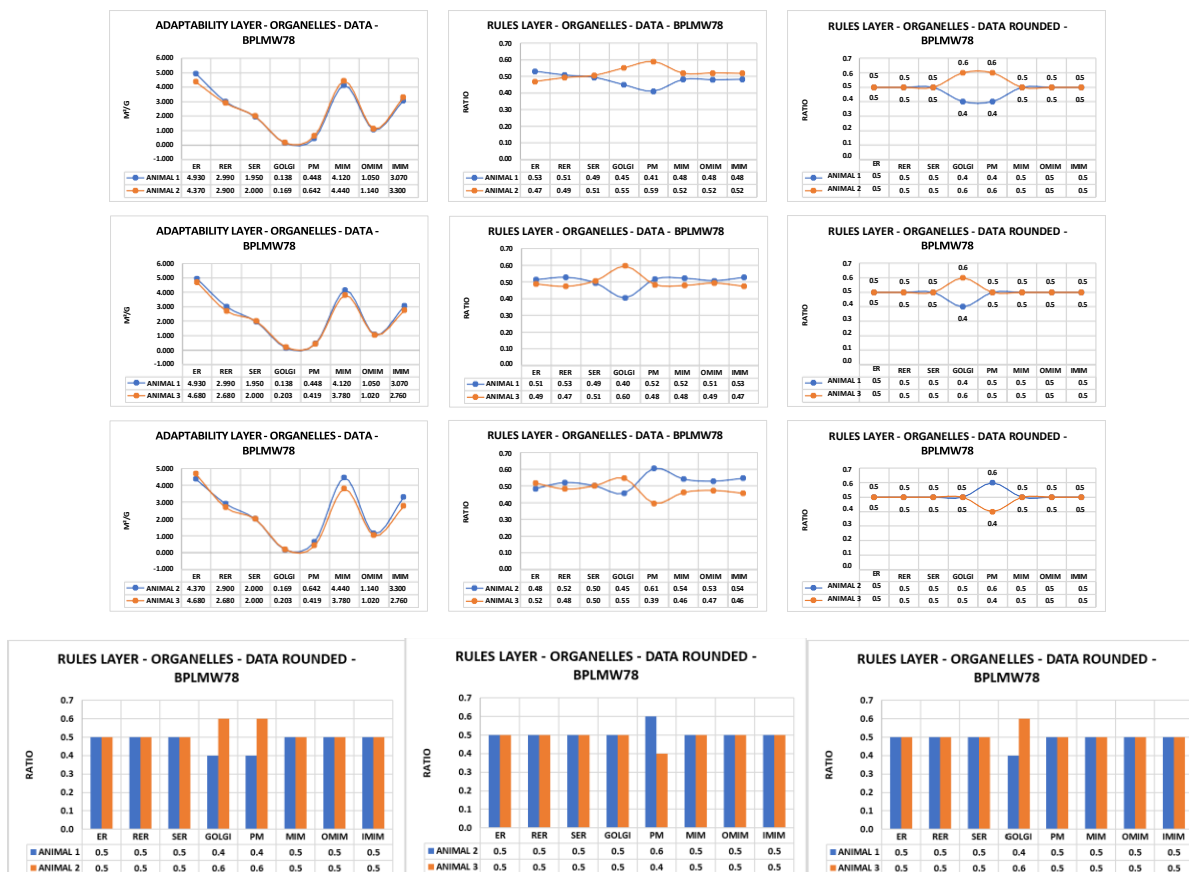


Figure 7.11 The histograms summarize the results indicating that the changes were largely the same (0.5:0.5). As expected, hepatocytes belonging to these three animals applied the same ratio rules to the ER and mitochondrial membranes, which means that applying the cell's approach to reproducibility worked. Since biology most likely follows the rules with the Golgi and plasma membranes as well, the miss suggests that these compartments (GO, PM) were either under sampled or not identified correctly. The point? Testing for reproducibility empirically at the level of individuals also seems to be testing for representative sampling (this caught the likely under sampling of the Golgi and plasma membranes).

If we remove the GOLGI and PM distractors and replot the results, the original data passed both the sampling and reproducibility tests (Figure 7.12). Note that a strategy based on testing raw data for sampling and reproducibility builds confidence in the experimental design and the results. It changes the reach of the study from simple to complex.

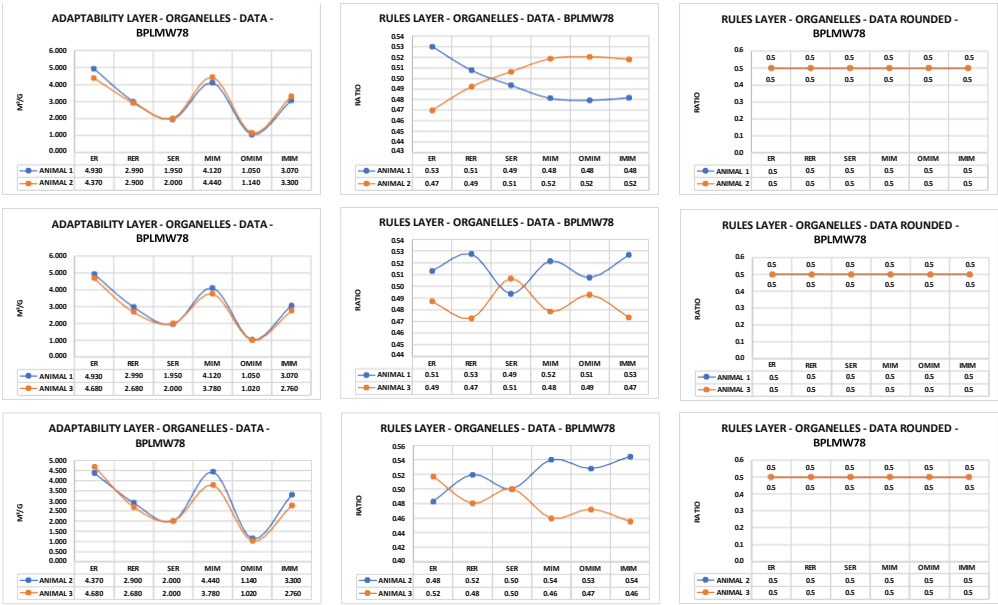
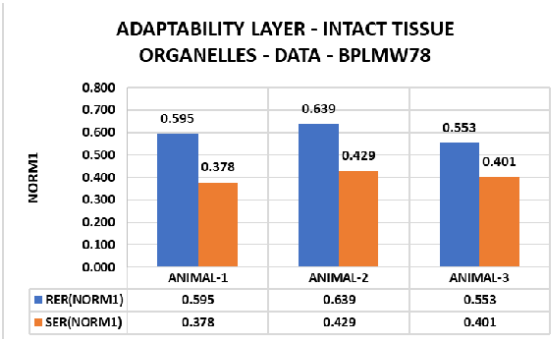


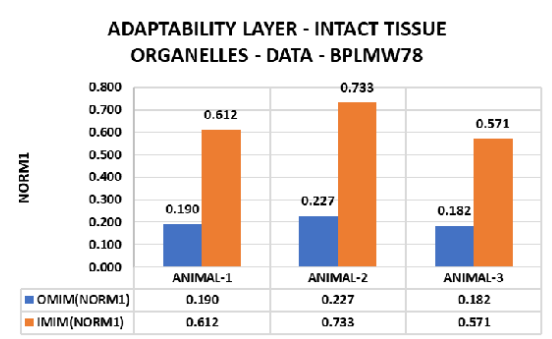
Figure 7.12 Forming and testing the data pair pairs of the three animals detected no differences (0.5:0.5). Notice the narrow range of the values on the y axis of the figures in the central column.

Normalized Data: With normalized data, however, the ratios can change because the numbers change (Figure 7.13). Observe that the RER:SER membranes stayed the same (0.6:0.4) but the mitochondrial membranes (OMIM:IMIM) shifted from 3:7 to 2:8.

Normalized Data (ER)



Normalized Data (MIM)



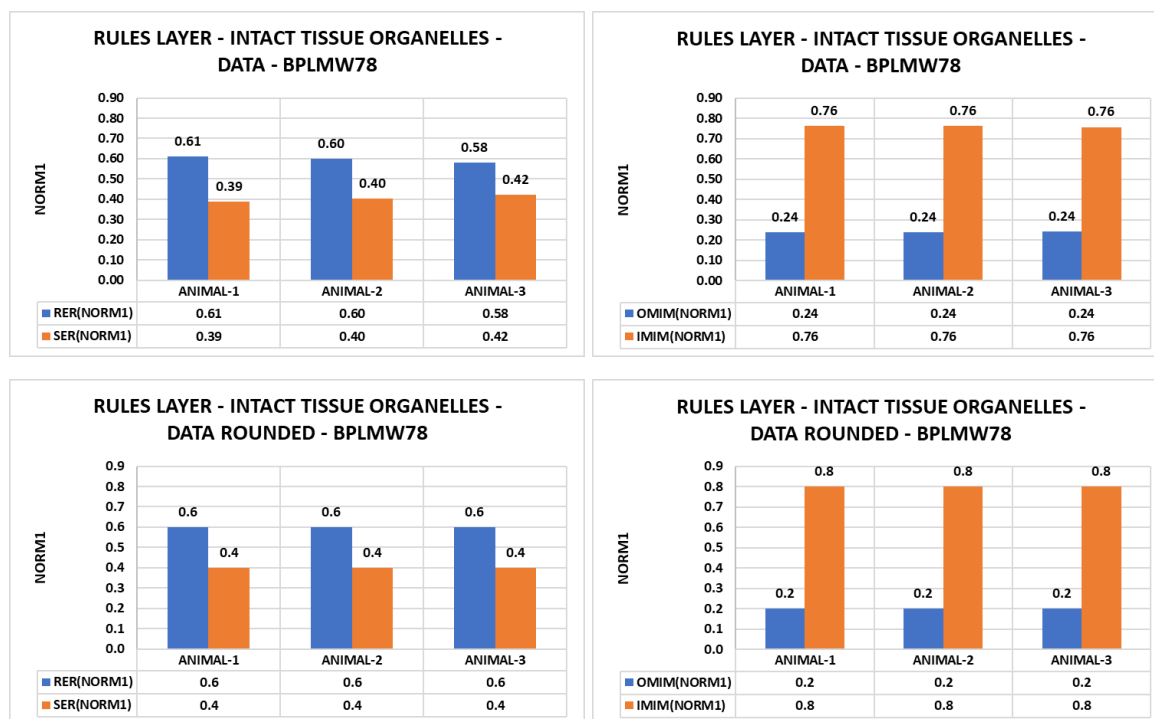


Figure 7.13 Normalized data can detect reproducibility and produce ratios different from those of the original data.

Enzyme Densities: Next, we can check the enzymes for patterns using the enzyme and membrane data expressed per gram of liver and as enzyme densities. Since an enzyme density (ED) supplies a constant reference (1 m^2), it serves as a universal reference for detecting and interpreting enzyme activities even when an enzyme has no connection to a membrane (e.g., a soluble enzyme). The four marker enzymes listed in Figure 7.14 specify their morphological locations. The enzymes densities, which include the four major membrane organelles, define the enzyme-membrane recipe of the hepatocyte. Such a dataset can often explain what happened, when, where, and how.

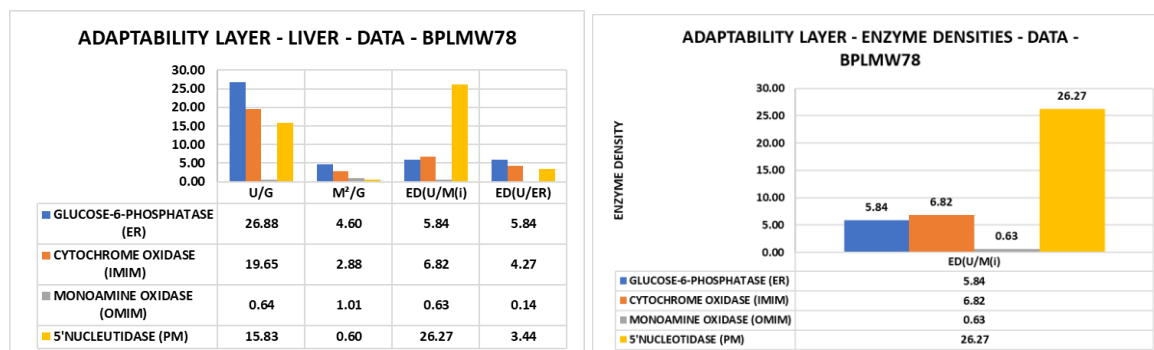


Figure 7.14 By combining the biochemical and morphological data, we can calculate the amount of enzyme activity associated with one square meter of a given membrane surface area ($ED = U/S$). In combination, the enzyme densities (ED) define an enzyme-membrane recipe involving four enzymes at four different locations. During a change, all four marker enzymes could have new values.

Enzyme-Enzyme Ratios: Figure 7.15 shows that the enzyme-enzyme ratios were the same (6:4) for three data pairs. In effect, hepatocytes made six units of G6PASE activity for every four units of CYOX and 5' NUC activity and six units of CYOX activity for every four units of 5' NUC activity. Notice how the hepatocytes linked the ER(G6PASE) to the IMIM(CYOX) and the IMIM(CYOX) to the PM (5' NUC). This ability to duplicate and superimpose ratios of enzymes and membranes appears repeatedly as a basic principle of design and pattern formation. The point? Duplicating results within and across animals becomes routine when a rule-based system drives the information management system of the cell.

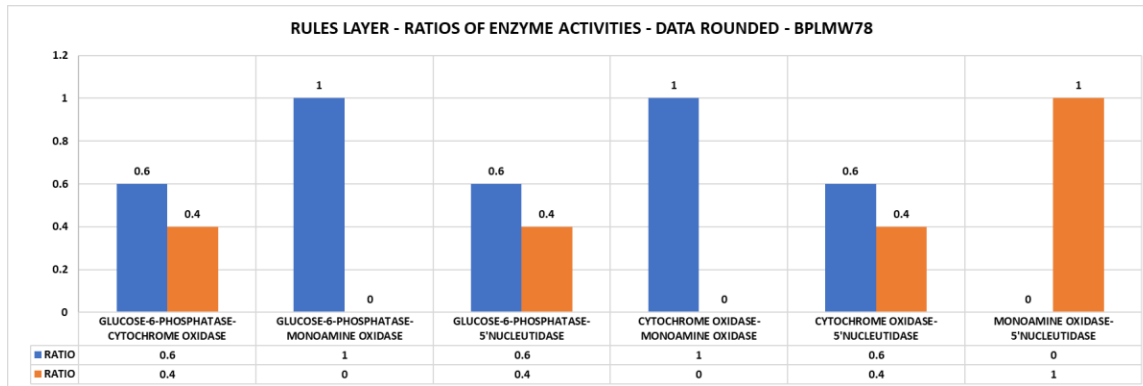


Figure 7.15 Relative changes in enzyme data pairs define relationships and connectivity at a primary level of complexity. While the 6:4 ratios reveal such patterns, the data pairs displaying a zero do not. The zeros appeared because one of the enzyme assays was too small to form a ratio equal to or greater than one. Normalizing the data usually removes this limitation.

Analytical Fractionation: Analytical fractionation represents a biochemical bookkeeping method that compares the amount of enzyme activity in the homogenate to the amount recovered in the fractions. It eliminates assumptions and unexpected surprises. For the update, we also want to know the membrane recoveries (intact tissue vs homogenate vs fractions). Since we know the enzyme densities of four marker enzymes (Figure 7.16), we can rearrange the enzyme density equation and solve for the surface area (m^2/g) of a given marker enzyme in each fraction ($S = U/ED$) and then check the results by calculating the morphological recoveries. If the balance sheet passes the morphology recovery test, then we have an empirical proof for the postulates of biochemical homogeneity. Additionally, we can determine the extent to which the P fraction (the microsomes) represent the total liver.

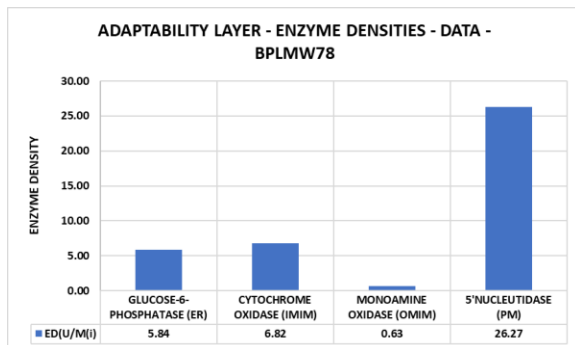


Figure 7.16 Enzyme densities (ED) relate units (U) of enzyme activity/gram of liver divided by a membrane surface area (S) also related to a gram of liver ($ED = U/S$). Recall: $ED = (U/G)/(S/G) = U/S$.

By measuring the enzyme activities of the four marker enzymes in each fraction (Figure 7.17), we can see where and to what extent the fractionation partitioned the enzyme-membrane complexes. Notice that the ER membranes (G6PASE-ER) concentrated in the microsomal fraction (P), mitochondrial membranes (MAO-OMIM, CYOX-IMIM) in the M fraction, and the plasma membranes (5'NUC- PM) appeared in the nuclear (N), heavy mitochondrial (M), light mitochondrial (L), and microsomal (P) fractions. The E and N fractions together constitute the homogenate (H) - the homogenized liver.

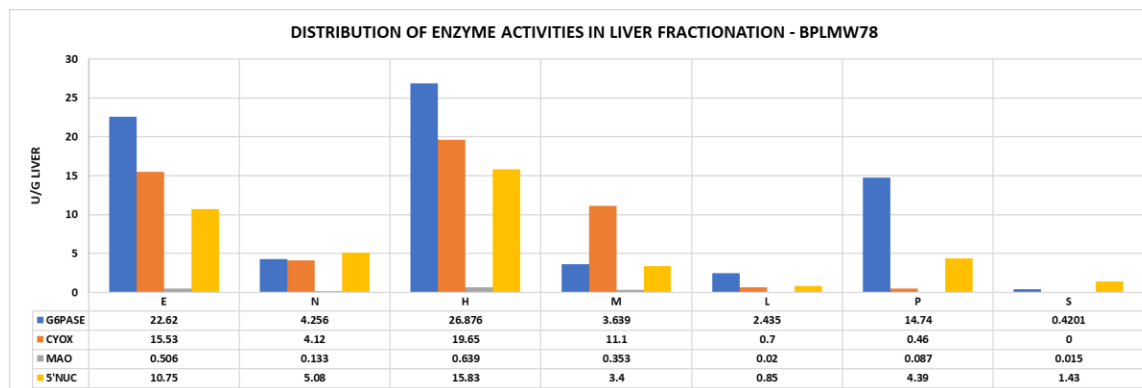


Figure 7.17 The E(extract) and the N(nuclear) fractions together represent the liver homogenate (H). In turn, the nuclear (N), mitochondrial (M) and light mitochondrial (L), microsomal (P), and supernatant (S).

Since we know the enzyme densities (Figure 7.16), and the enzyme activities (Figure 7.17), we can solve for the surface area of each membrane type in each fraction ($S = U/ED$) and in the E+N homogenate (Figure 7.18).

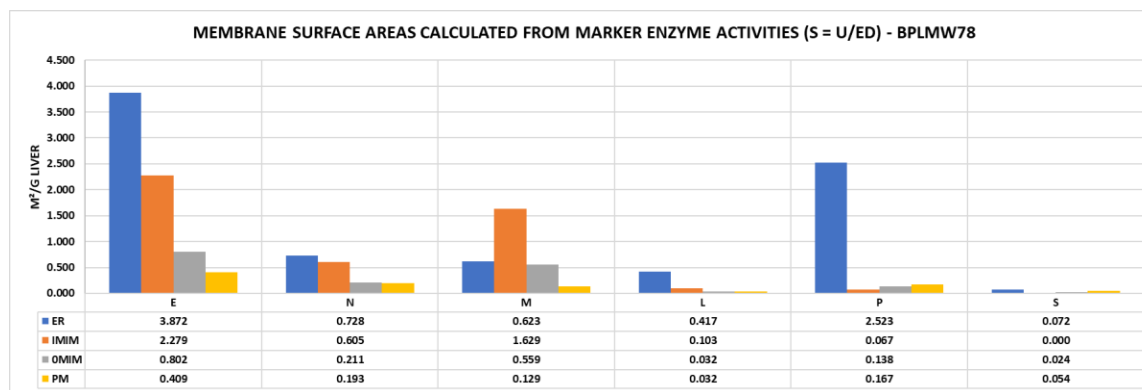


Figure 7.18 The histogram lists the distribution of four major membrane types that separated into named fractions using differential fractionation. While the microsomes represents the largest amount of the most ER, they account for only about 50% of the total. Soluble enzymes appear in the supernatant – without their membranes.

There was a problem. By including the soluble activity of 5' NUC of the supernatant (S) fraction in the calculations, the recovery of the plasma membrane (PM) was overestimated (115%) as shown in Figure 7.19. This was included to show that the data and recoveries were the same when calculated from either the homogenate or the fractions.

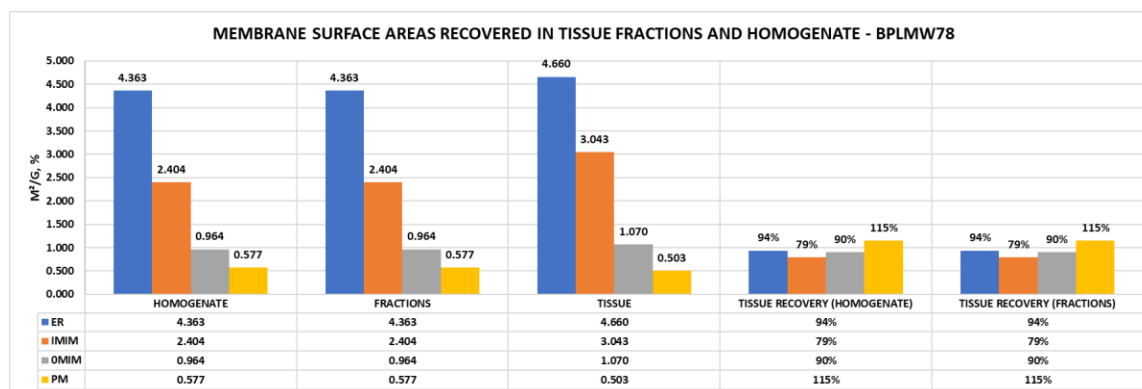


Figure 7.19 By applying the recovery method (analytical fractionation) of biochemistry to morphology, we can compare the recovery of specific membranes (ER, IMIM, OMIM, PM) in tissue fractions to the homogenate and to the intact tissue.

In short, what did the update of this paper tell us? When put to the test, the postulates of biochemical homogeneity (de Duve, 1971) successfully predicted a fundamental design principle of biology. The implication? By proposing that a relationship existed between cellular structure and function (enzymes mark a morphological location uniformly), the postulates of biochemical homogeneity opened the door to exploring biology quantitatively as a complexity. The enzyme density (ED = U/S) represents the postulate as a complex data type based on a constant data reference (one meter squared).

Comment: The postulates of biochemical homogeneity predicted a quantitative link between structure and function in hepatocytes (de Duve, 1964, 1974). The enzyme density tested the prediction empirically and found it to be correct. However, for the enzyme density to work, it required a solution to the long-standing stereological problem of section thickness as related to electron microscopy. Fortunately, that solution exists (Weibel and Paumgartner, 1978; Weibel, 1980). Since change in biology exists largely as complex relationships of structure to function, stereology in combination with biochemistry become principal players in establishing biology as a robust, quantitative science.

When both morphology and biochemistry are in play, the simpler and less expensive homogenate can usually get the job done. Collecting microsomes, which requires extra pipetting, centrifuging, decanting, calculating, waiting, and chances for making mistakes, can be avoided. Moreover, updating biochemical results with an interpretable reference (the liver) depends importantly on knowing how much of the original ER ends up in the microsomal fraction – for all the control and experimental data points. Publishing microsomal data – without recoveries – introduces an assumption that weakens the results. The reader never knows if the microsomal data represented the liver faithfully in the same way across multiple data points. In effect, it's much safer to treat fractionation data mathematically as a closed set. Complexity theory routinely encourages accountability by bundling reproducibility into experimental designs. Whenever possible, it avoids results with loose ends.

7.1.4 Case Study 4: Study ER Membranes vs Constituent Marker Enzymes – BPMLW80

Source: Update applied to original data from Bolender R. P., Paumgartner D., Muellener D., Losa G., Weibel E. R. (1980) Integrated stereological and biochemical studies on hepatocytic membranes. IV. Heterogeneous distribution of marker enzymes on endoplasmic reticulum membranes in fractions. J Cell Biol Jun;85(3):577-86.

Topic: Confirming the postulates of biochemical homogeneity by relating enzyme activities to membrane surface areas (calculating enzyme densities) within the framework of analytical fractionation.

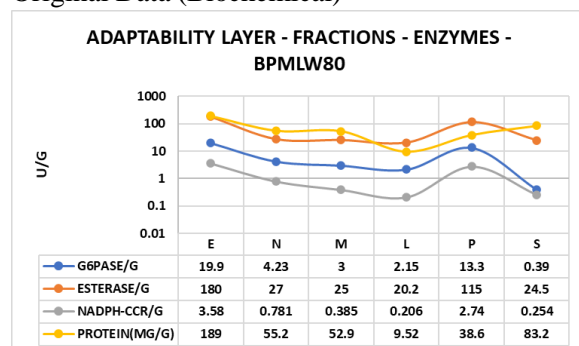
Update: Expand data, report results in adaptability and rules layers, include morphological and biochemical recoveries, normalize data, calculate enzyme densities [$ED = [(U/G)/(S/G)]$; $ED = ED(NORM2)$], analyze patterns, and report biological solutions (recipes).

Dataset: Biochemistry: Glucose-6-phosphatase (G6PASE), ESTERASE, NADPHCCR; Cytochemistry (glucose-6-phosphatase), Morphology: ER surface areas.

Since the earlier publication (Case Study 3: Bolender et al., 1978) showed promising membrane recoveries, the follow up study focused on identifying ER membranes cytochemically in the homogenate and fractions. The purpose of this study was to test the postulates of biochemical homogeneity (deDuve, 1964) by fractionating the liver, marking the ER membranes cytochemically (G6PASE), quantifying the ER membranes stereologically, and calculating tissue, homogenate, and fraction recoveries (Bolender et al., 1980; BPMLW80). The recoveries failed to supply the needed evidence because the cytochemical test missed too many ER membranes. Updating the study included rerunning the test for biochemical homogeneity with the same data but switching the experimental model from reductionism to complexity. Specifically, we want to know if different enzymes believed to be markers of the endoplasmic reticulum (ER) satisfy the postulates of biochemical homogeneity, which include unique locations and uniform distributions for each marker enzyme. Since the structural and functional properties of hepatocytes can vary across the liver lobule, the tests – for our purposes here - apply to averaged hepatocytes.

Reductionist Model: Figure 7.20 shows the enzyme activities assayed in the homogenate [E(extract)+N(nuclear)] and fractions: N(nuclear), M(heavy mitochondrial), L(light mitochondrial), P(microsomal), and S(supernatant). Notice that the microsomal fraction (P) accounted for slightly more than half of the total enzyme activity. Since marker enzyme activity existed in five fractions, studying the ER related changes in just one fraction – the microsomal – becomes subject to changes occurring in the remaining four fractions. Consequently, keeping track of all the changes by calculating recoveries becomes an important part of reporting microsomal results because it minimizes this uncertainty. Recall that the recoveries will compare the amount of the ER marker enzyme activity in the P fraction to the total liver homogenate (E+N) and to the sum of the fractions (N+M+L+P+S). We'll apply the same analysis to the ER membranes identified cytochemically and calculated from enzyme densities.

Original Data (Biochemical)



Recoveries (Biochemical)

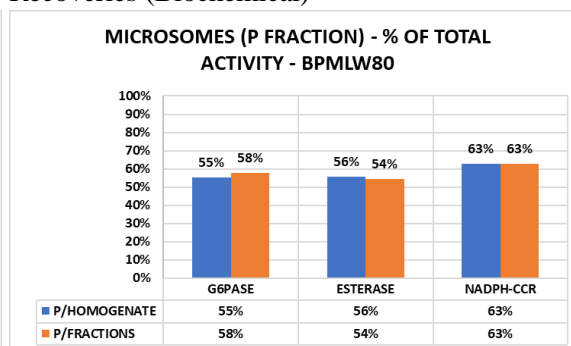


Figure 7.20 Using analytical fractional fractionation (de Duve, 1974), we can identify and compare the amount of the ER marker enzyme activity in the homogenate and fractions.

The original approach, which used G6PASE cytochemistry to identify the ER membranes, estimated the surface areas of the ER in the homogenate (E + N) and fractions (N + M + L + P), as shown in Figure 7.21.

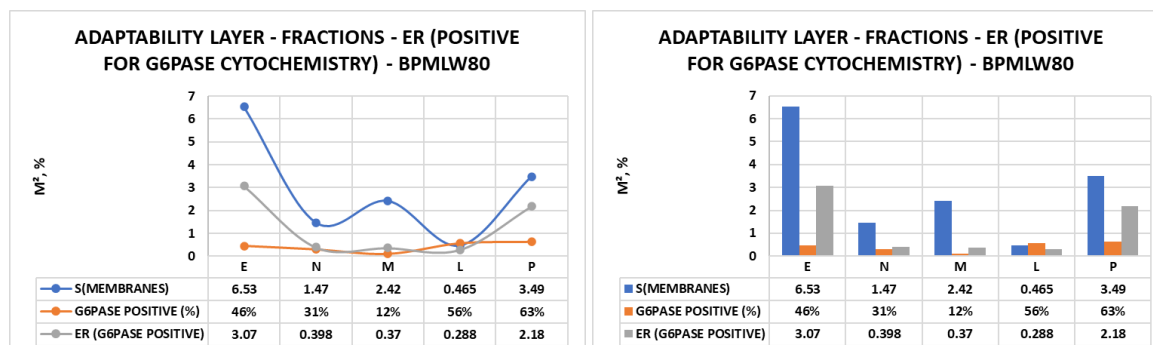


Figure 7.21 G6PASE cytochemistry identified ER membranes in the homogenate (E+N) and fractions (N, M, L, P).

Cytochemical identification of ER membranes in the homogenate and fractions gave 98.5% recoveries when relating fractions to the homogenate, but notably less when comparing both the homogenate and fractions to the intact tissue (T) estimates for the ER (Figure 7.22). Something seemed amiss.

Recoveries (Glucose-6-phosphatase Cytochemistry)

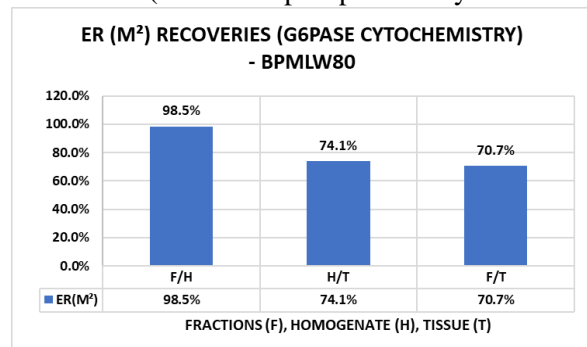


Figure 7.22 While fractions related to the homogenate gave the expected result ($\approx 100\%$), the recoveries for homogenate and fractions related to the intact tissue ($\approx 70\%$) were less than expected ($\approx 100\%$). With biochemical recoveries in the high 90s, it appeared that the cytochemical method used to identify the ER membranes in homogenate and fractions might have missed as much as 30% of the ER membranes.

Simplicity(Reductionism) vs Complexity (Copied from Biology): After dividing the enzyme activity of a fraction by the surface area of the ER (found by G6PASE cytochemistry), proof for the structure-function postulates of biochemical homogeneity did not appear. This result suggested that the cytochemical approach to detecting the ER in the homogenate and fractions either didn't work as imagined or that the postulates were incorrect. In short, applying a reductionist approach to a complex problem did not work as expected.

The update of the publication switched to complexity. It divided the biochemical activity of an ER marker enzyme by the surface area of ER estimated in the intact tissue to calculate the enzyme density directly from the original data [$ED = (U/G)/S/G$]. In turn, the enzyme density generated estimates for the

surface area of the ER in the homogenate and the fractions by dividing each enzyme activity by its ED: ($S = U/ED$). Now the recoveries fully supported the postulates of biochemical homogeneity (Figures 7.23, 7.24).

Original Data

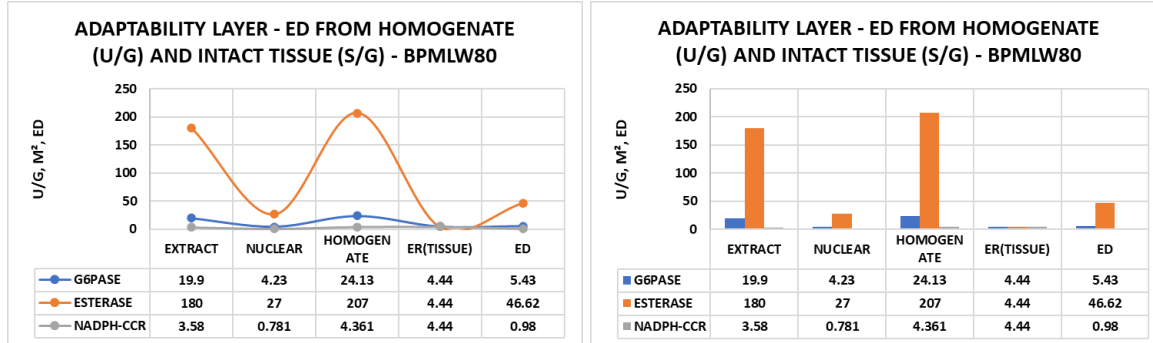


Figure 7.23 By dividing enzyme activities in the homogenate (E+N) by the surface area of the ER in intact tissue (both related to a gram of liver), we can obtain the enzyme densities (ED) for three ER marker enzymes (G6PASE, ESTERASE, AND NADPHCCR).

Biochemical Homogeneity Models: Cytochemical (Simplicity) vs Enzyme Density (Complexity)

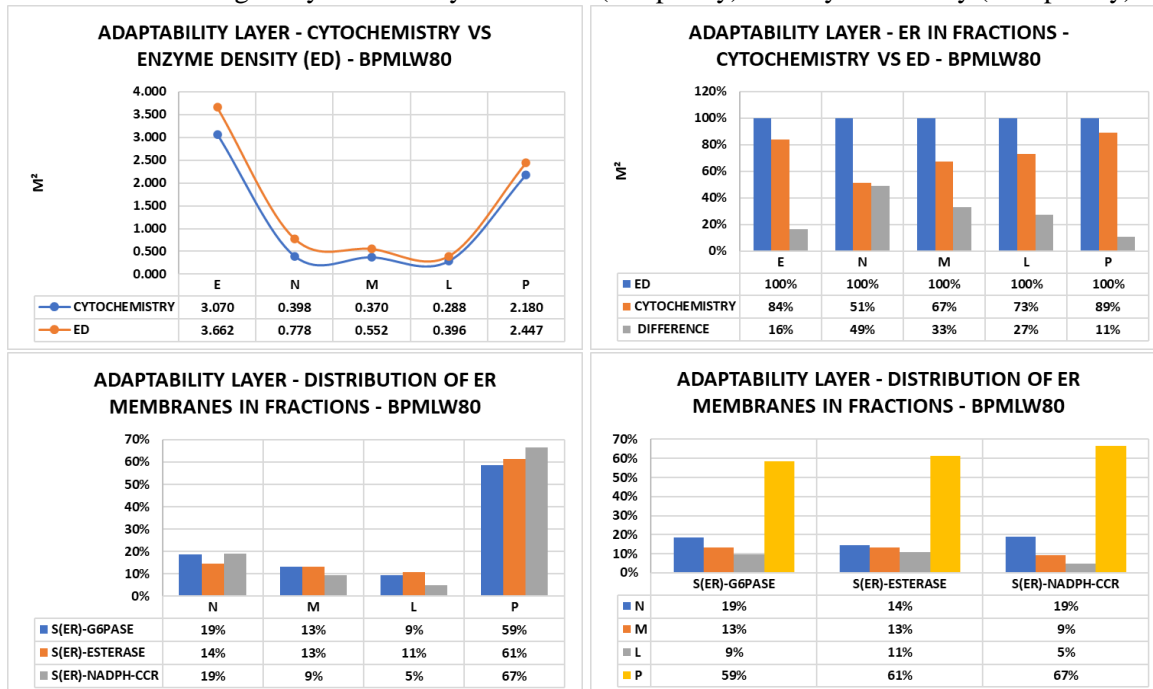


Figure 7.24 Comparing the results of models based on simplicity (assumption) and complexity (prediction). When we assumed that cytochemistry could identify all the ER membranes in the homogenate and fractions, we had no way to check the results. The update tried a second time to confirm the postulates of biochemical homogeneity (de Duve, 1964, 1974) by expressing the relationships of structure to function as enzyme densities ($ED = U/S$; $S = U/ED$). This time it worked, and the recoveries confirmed the postulates empirically.

Now let's look more closely at the details of the complexity model. Notice in Figure 7.25 that the surface areas calculated from units of enzyme activity and the enzyme density ($S = U/ED$) gave similar but not identical results. Moreover, since the microsomal fraction (P) captured roughly 60% of the control ER membranes, how do we know if it captures the same proportion of the ER membranes when collecting

experimental data points? We don't. Unless we calculate the recoveries for each experimental data point, 60% becomes the default assumption. If the assumption is incorrect, then the results carry unknown methodological errors. Regrettably, the biological literature abounds with such unknowns largely because of its reductionist roots. Why? Because we're asking our theory structure to do something it's not designed to do. For biological studies, reductionism can identify a simple difference between two parts statistically, but it cannot detect a complex change occurring between the same two parts unless the investigator previously demonstrated the change biologically. The point? Using a reductionist model, demonstrating a cellular change statistically produces a catch 22.

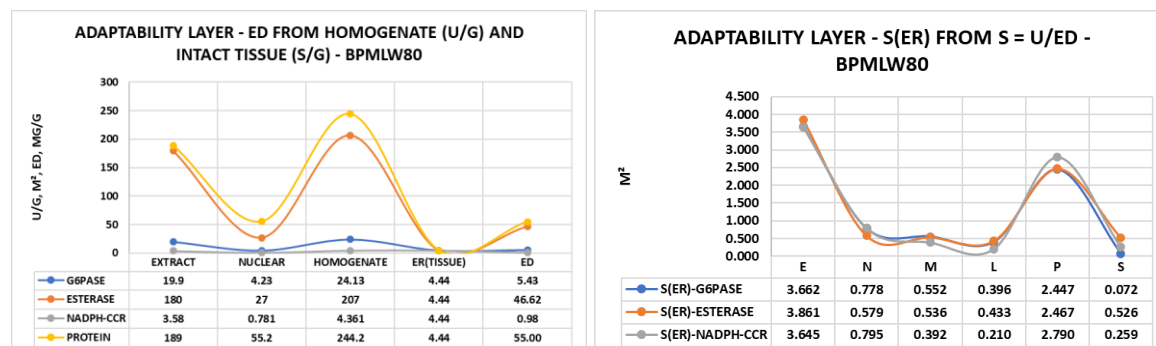


Figure 7.25 Enzyme densities calculated from (1) enzymes assayed in the homogenate and from (2) membrane surface areas estimated in the intact tissue can turn enzyme activities assayed in homogenates and fractions into membrane surface areas.

The Recoveries: The recoveries, shown in Figure 7.26 tell the story. The biochemical recoveries, which divided the activities of the fractions by that of the homogenate, came reasonably close to the expected result of 100%: 96%, 102%, and 100%.

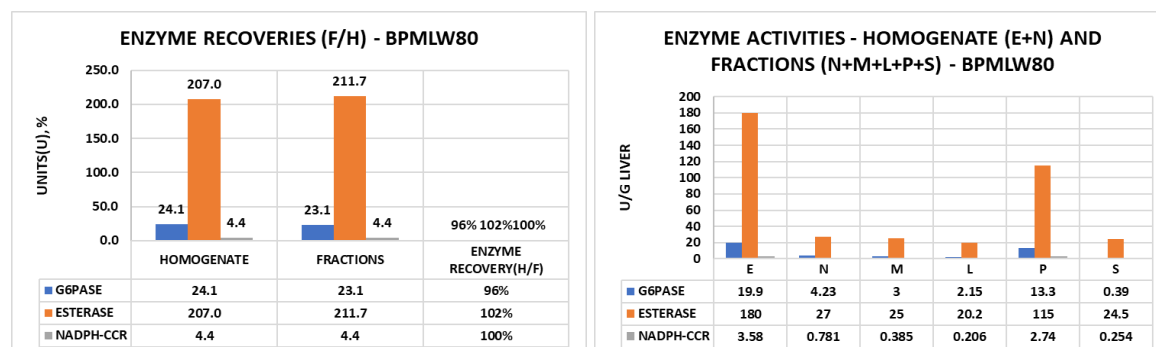


Figure 7.26 Enzyme recoveries and activities based on analytical fractionation.

At first, whoever, the membrane recoveries didn't perform as well as shown in Figures 7.27 and 7.28. Why? The soluble activities of the enzymes in supernatant (S) fraction were included in the homogenate, but not in the fractions because there were no membranes in the S fraction. By removing the incorrect contributions of the supernatant to the homogenate, the membrane recoveries now resemble those of the enzymes: 96%, 103%, and 100%

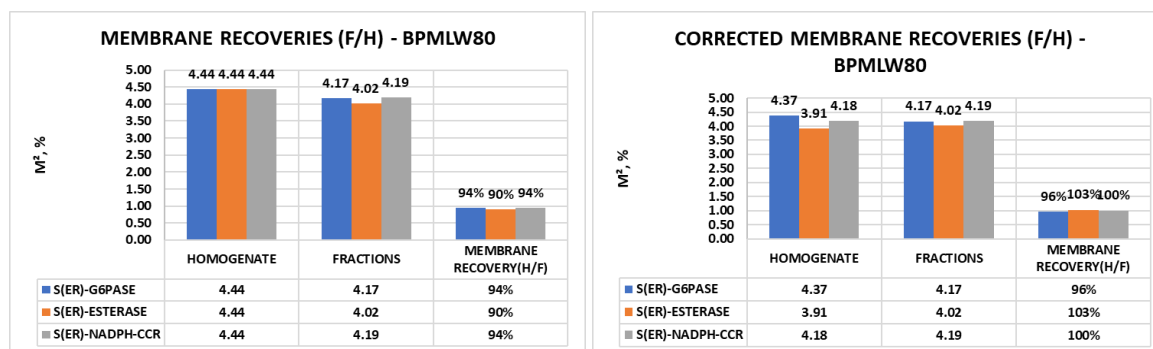


Figure 7.27 Membrane surface area (ER) recoveries before and after correcting for the enzyme activities in the supernatant.

Point? By updating the theory structure from simplicity to complexity, demonstrating biochemical homogeneity becomes a routine task. The strategy of copying the way biology relates biochemical function to membrane surface area provides a mathematical link (the enzyme density) between structure and function. Reproducibility came from recoveries (H/T) derived from predictions ($S = U/ED$).

As biology becomes a data driven science based on rules and first principles, understanding how to detect and interpret biological changes becomes a first order of business. When biology changes, it changes relationships of structures to functions that appear to us as multilayered complexities (phenotypes).

Membrane Recoveries

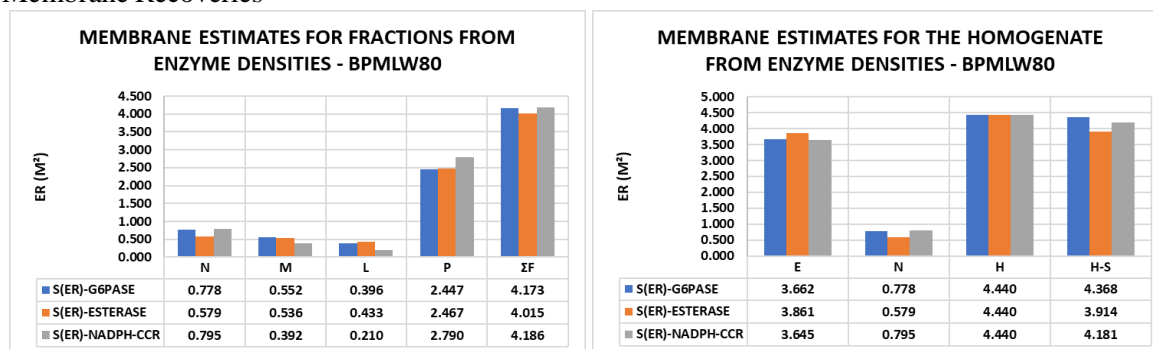


Figure 7.28 Data used to make corrections for the soluble enzyme activities in the supernatant.

Basic Principle: Biochemical Homogeneity

The postulates of biochemical homogeneity (deDuve, 1974), which provided the theoretical cornerstone for cell and molecular biology, allow us to detect and interpret the many and complex interactions of molecules, organelles, cells, and genes within a coherent mathematical setting. Cells change by altering the rules and recipes of biochemical homogeneity by creating new relationships of structure to function on their way to solving specific problems. Biology is in the business of knowing what's happening, when, where, and how across its vast hierarchies of size. Copying the way biology changes, for example, puts us on the path to defining biology from its first principles, a process that begins by taking biology apart (reductionism), putting it back together (forward engineering), and taking it apart a second time (reverse engineering) according to rules defined by cells. By understanding how cells solve problems by becoming the solution, we can update our experimental methods and literature with the confidence and encouragement that comes directly from the source.

Comment: A biological change, which gradually alters the relationships of many interacting variables, applies rules based on biology's first principles. For example, the cellular changes we're trying to detect and interpret derive from at least four primary changes including membrane surface areas, enzyme activities, enzyme densities, and enzyme-membrane recipes. When a cell changes, it remodels its phenotype by changing these variables to create the new structure-function recipes needed to solve the existing problem. Copying the cell's approach to problem solving will eventually lead to the algorithms driving the optimizing routines of complex biological changes. Since amoeba and slime molds have already shown such an optimizing prowess (Zhu et al., 2018, Farhad et al., 2023), hepatocytes should be just as capable or even more so. The point? Cells change by rules and complex algorithms based on first principles. Moreover, cells do not appear to have structured their approach to change around a requirement based on demonstrating a significant difference between two isolated data points. Instead, proof of a biological change comes from rules, algorithms, and reproducibility. Everything distills down to a basic truth. Cells change by becoming the solution.

Pathology Studies (Bile Duct Ligation, Etcetera)

7.1.5 Case Study 5: Secondary Biliary Cirrhosis (Bile Duct Ligation) – KKSGR92

Source: Update applied to original data from Krahenbuhl S., Krahenbuhl-Glauser S., Stucki J., Gehr P., Reichen J. (1992) Stereological and functional analysis of liver: Mitochondria from rats with secondary biliary cirrhosis: Impaired mitochondrial metabolism and increased mitochondrial content per hepatocyte. Hepatology 15(6):1167-1172.]

Topic: Bile duct ligation as a model for secondary biliary cirrhosis.

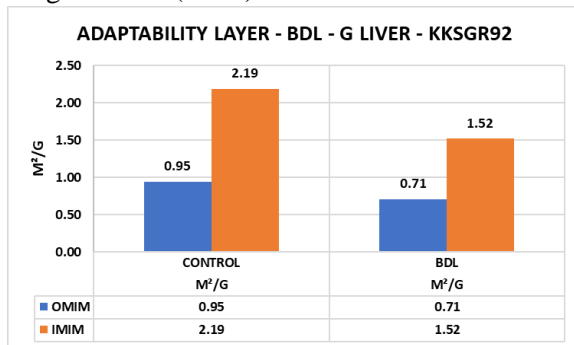
Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, calculate enzyme densities [$ED = [(U/G)/(S/G)]$], analyze patterns, and report biological solutions (as recipes).

Dataset: Biochemistry: ATPASE, cytochrome oxidase (CYOX), monoamine oxidase (MAO); Morphology: mitochondria (MIM, OMIM, IMIM).

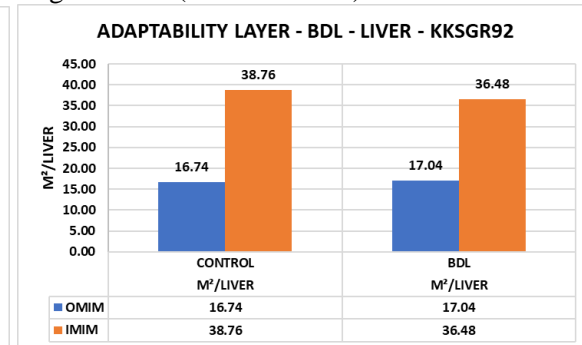
The results of this study led to the conclusion that mitochondrial function in rats with secondary biliary cirrhosis became impaired (Krahenbuhl et al., 1992; KKSGR92). The update added three new pieces of information: (1) the amount of change detected depended on the data reference, (2) in the rules layer, BDL (bile duct ligation) did not change the data pair ratios of the membranes (\leftrightarrow) but changed the ratios of CYOX (\uparrow) and MAO (\downarrow), and (3) BDL changed the enzyme densities of CYOX (\uparrow) and MAO (\downarrow). Given the time allotted to the experiment, the hepatocytes recovered or exceeded the capabilities lost to the BDL except for monoamine oxidase (MAO).

Data Related to a Gram of Liver: Experimental results routinely become a function of the data reference. Mitochondrial membranes related to a gram of liver and to the liver routinely detect different changes. As expected, Figure 7.29 shows that both references (per gram and per liver) displayed the same ratios in the rules layer (OMIM:IMIM - 0.3:0.7).

Original Data (Liver)



Original Data (Gram of Liver)



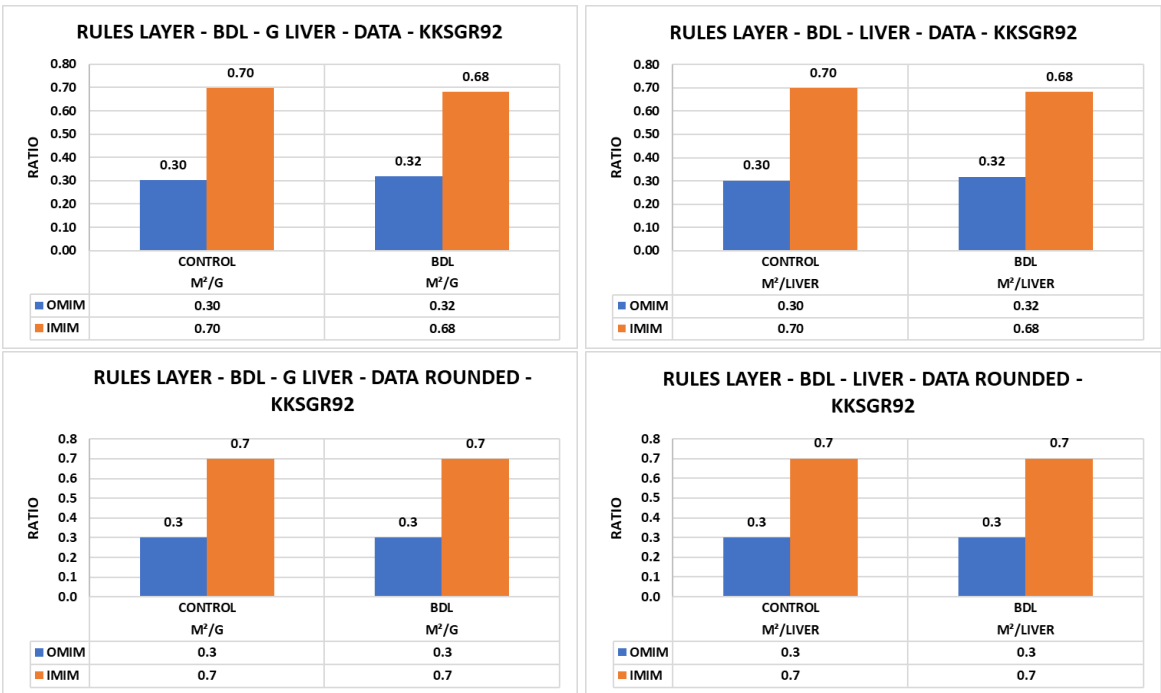
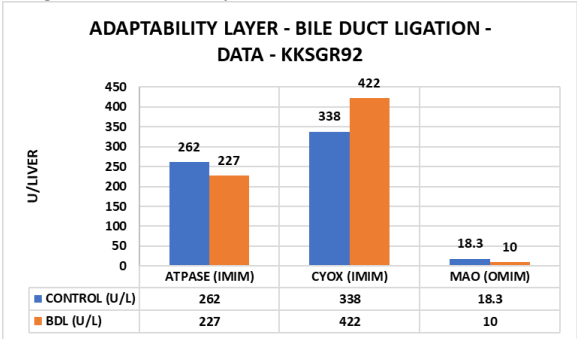


Figure 7.29 When detecting changes, they can be absolute (adaptability layer) and relative (rules layer). Although the surface areas of both membrane compartments increased, the membrane ratios (OMIM:IMIM) remained constant.

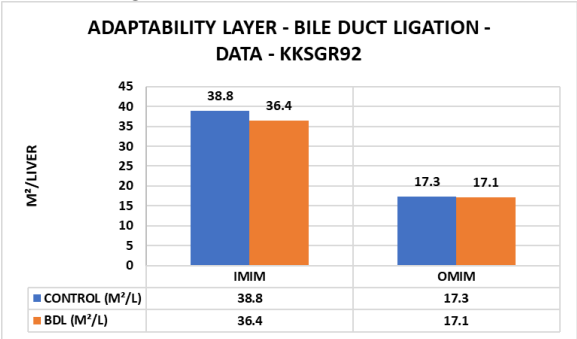
Data related to the Liver: When attempting to detect changes in the liver, several variables come into play including enzyme activities, membrane surface areas, enzyme densities, and the number of hepatocytes in a gram of liver. The authors, who applied corrections for section thickness and compression, also avoided the data reference error (the cell packing problem) by relating results to the liver.

Here, the update considers three changes related to the liver: enzyme activities, membrane surface areas, and enzyme densities. The ratios in the rules layer indicated changes in enzyme activity for cytochrome oxidase (\uparrow), and monoamine oxidase (\downarrow), but not for ATPase (\leftrightarrow). Notice in Figure 7.30 that the surface areas and ratios of the mitochondrial membranes carrying these enzymes remained unchanged.

Original Data (Enzymes)



Original Data (Membranes)



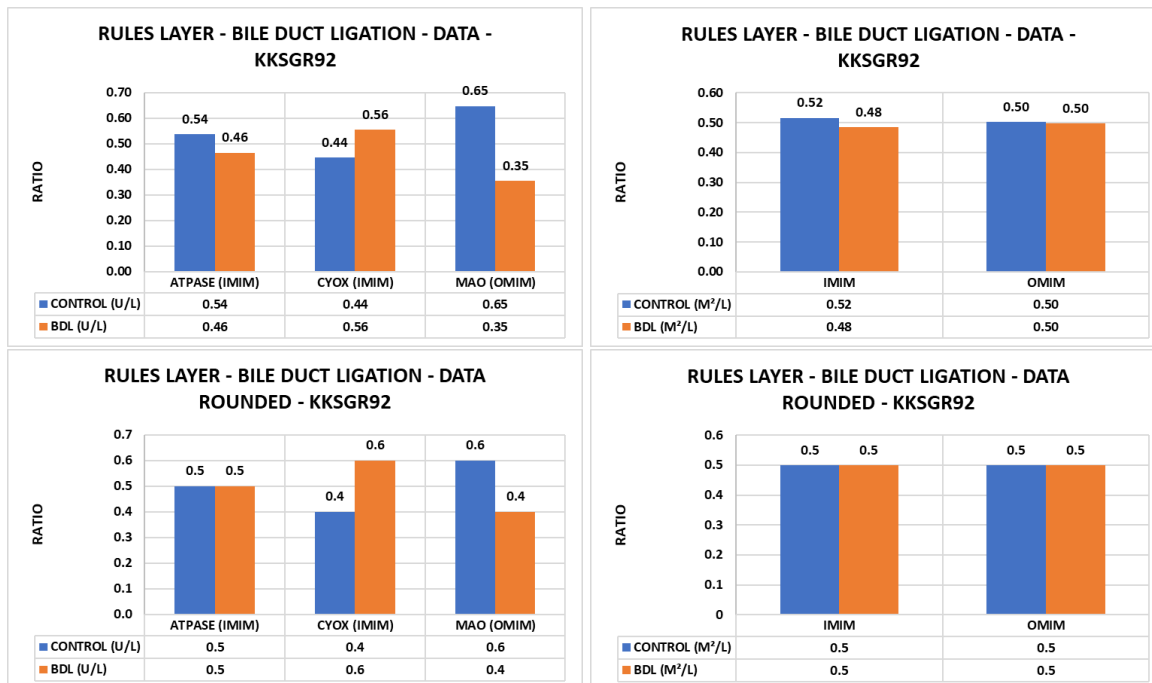
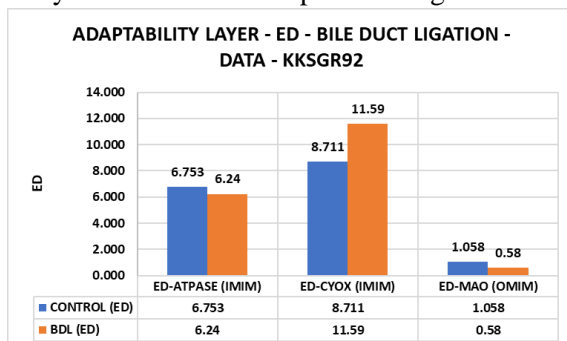


Figure 7.30 Detecting and interpreting a biological change requires information from both the enzymes and the membranes supporting the enzymes. The figure shows changes in the proportions of enzyme activities but not similar changes in their supporting membranes.

The study wanted to know how the hepatocytes dealt with the deficit of energy problem caused by bile duct ligation (cirrhosis). The hepatocytes had three options. They could increase one or more of the following: (1) the surface areas of the mitochondrial membranes, (2) the mitochondrial enzyme activities, and (3) the enzyme densities. What did they do?

In response to the bile duct ligation (BDL), the hepatocytes redefined the enzymatic recipes of their outer and inner mitochondrial membranes. Figure 7.31 summarizes the results. Cytochrome oxidase became more concentrated on the IMIM, while monoamine oxidase became less concentrated on the OMIM. The ATPase associated with the inner mitochondrial membrane remained neutral.

Enzyme-Membrane Recipe: ED-Original Data = $[(U/G)/S/G] = U/S$



Note the difference between the EDs (left) and their ratios (below).



Figure 7.31 By calculating the enzyme densities, we can see that the hepatocytes responded to the bile duct ligation by increasing the activity (concentration) of the cytochrome oxidase enzymes on the IMIM and decreasing the activity of monoamine oxidase on the OMIM. The new enzyme-membrane recipe defined the cells response and explained the results of the experiment.

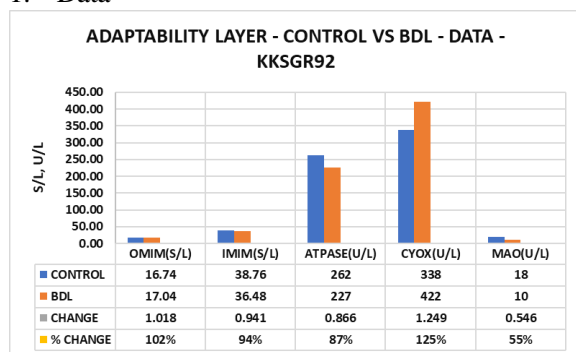
The outcome of this study becomes satisfying because it explained to us how the hepatocytes used its mitochondria to manage the bile duct ligation problem.

Summary

Results of a two data point study (Control vs Experimental): In practice, analyzing the results of an experiment consists of working our way down a checklist to see how the hepatocytes solved a problem. After five weeks of bile duct ligation, the hepatocytes figured out how to rework their mitochondrial membranes and marker enzymes to generate a new phenotype (bile duct ligation) capable of recovering near control-like membrane and enzyme recipes. The hepatocytes apparently fixed everything except for the monoamine oxidase activity, which remained reduced at 55% of the control value. The piece of data missing from the study was the ER, which presumably supplied most of the mitochondrial parts.

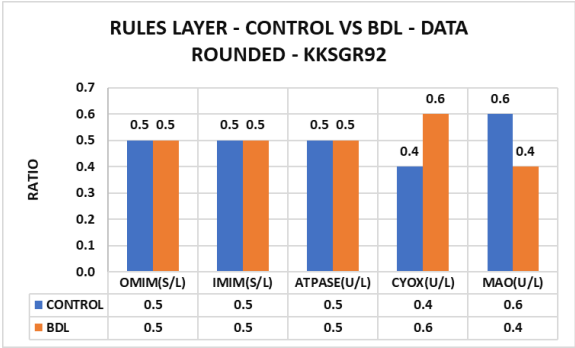
Review Exercise

1. Data



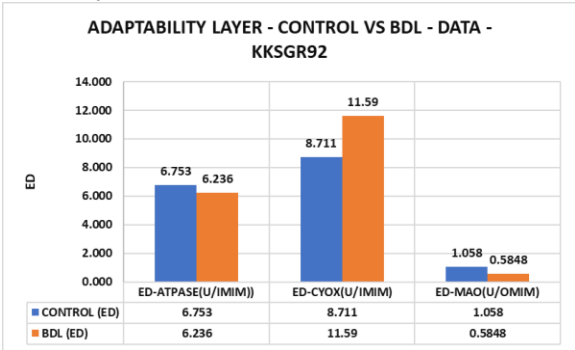
Data were correctly related to the liver to avoid the cell packing problem.

2. Data Rules (Ratios)



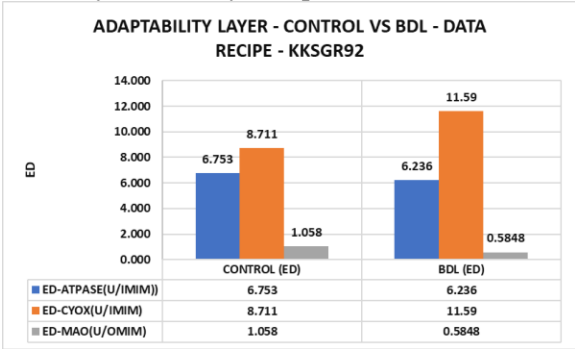
Cytochrome oxidase increased but monoamine oxidase decreased. Notice the switched symmetry of the enzyme changes.

3. Enzyme Densities



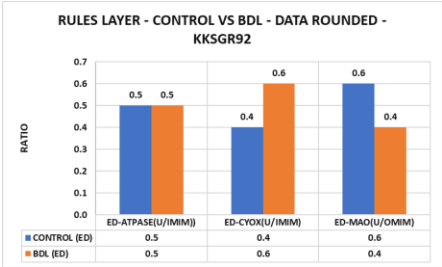
Cytochrome oxidase was the only enzyme that increased its activity per one square meter of membrane surface area (IMIM).

4. Enzyme Density Recipes



The enzyme density of CYOX increased, but not the surface area of the IMIM. This might suggest that new mitochondria replaced the originals.

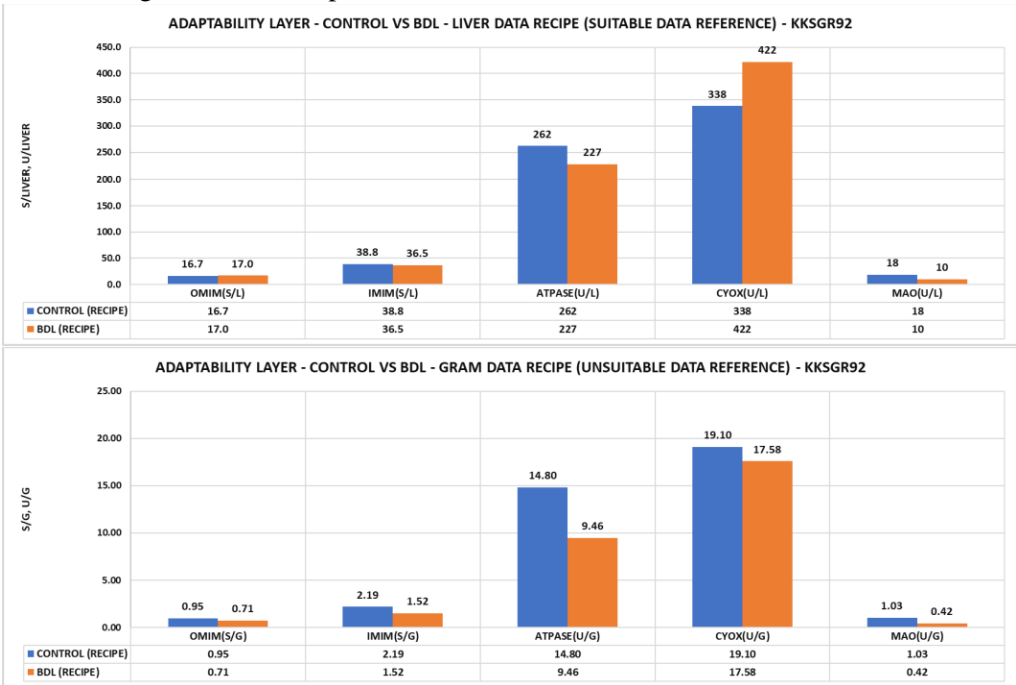
5. Enzyme Density Rules



To get the 6:4 ratio, the monoamine oxidase activity decreased in the BDL group. Recall that the OMIM has direct connections to the SER.

Notice below (6.) that using the gram reference produced substantially different results because the ligation data most likely came from a different number of cells.

6. Working Solution (Recipes)



Comments: The transition from dealing with one variable at a time to many variables changing simultaneously becomes a challenge for the beginner. In contrast, cells orchestrate changes involving thousands or more parts routinely because they know the rules, need access to many variables to solve problems, can call or assemble critical algorithms, maintain vast information networks, and excel at doing clever things by bridging classical and quantum mechanics. Since the case studies tell the same change story repeatedly, the reader will soon discover that knowing and applying the basics of a biological change is preferable to suffering the bewilderment of not knowing what to do after demonstrating a significant difference between two data points.

7.1.6 Case Study 6: Mitochondrial Response to CCl₄-Induced Cirrhosis.) – KRZGS90

Source: Update applied to original data from Krahenbuhl S., Reichen J., Zimmermann A., Gehr P., Stucki J. (1990) Mitochondrial structure and function in CCl₄-induced cirrhosis in the rat. *Hepatology* 12:526-532.

Topic: Morphological and biochemical responses of hepatocytes to CCl₄-induced cirrhosis.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, calculate enzyme densities [(ED = [(U/G)/(S/G)]); ED = [(U/G)/(S/G)](NORM1); ED(NORM2)], analyze patterns, and report biological solutions (recipes).

Dataset: Biochemistry: ATPASE, cytochrome oxidase (CYOX), monoamine oxidase (MAO); Morphology: MIM (OMIM, IMIM).

Animals exposed to a combination of phenobarbital and carbon tetrachloride experienced liver damage, which the study identified as cirrhosis (Krahenbuhl et al., 1990; KRZGS90). In short, the hepatocytes faced the problem of compensating for a loss in liver function. Did they succeed in reestablishing the previously normal metabolic capability of the liver? Yes and no. The total capacity of three enzymes increased, but the surface areas of the mitochondrial membranes decreased. To compensate for the loss of mitochondrial membranes, the hepatocytes increased the mitochondrial enzyme densities (enzyme activity/m² of membrane). But how exactly did the hepatocytes attempt to solve the liver problem quantitatively?

Figures 7.32 to 7.35 show the cirrhotic liver with diminished mitochondrial membrane surface areas, increased amounts of enzyme activity, and substantially increased enzyme densities.

Original Data

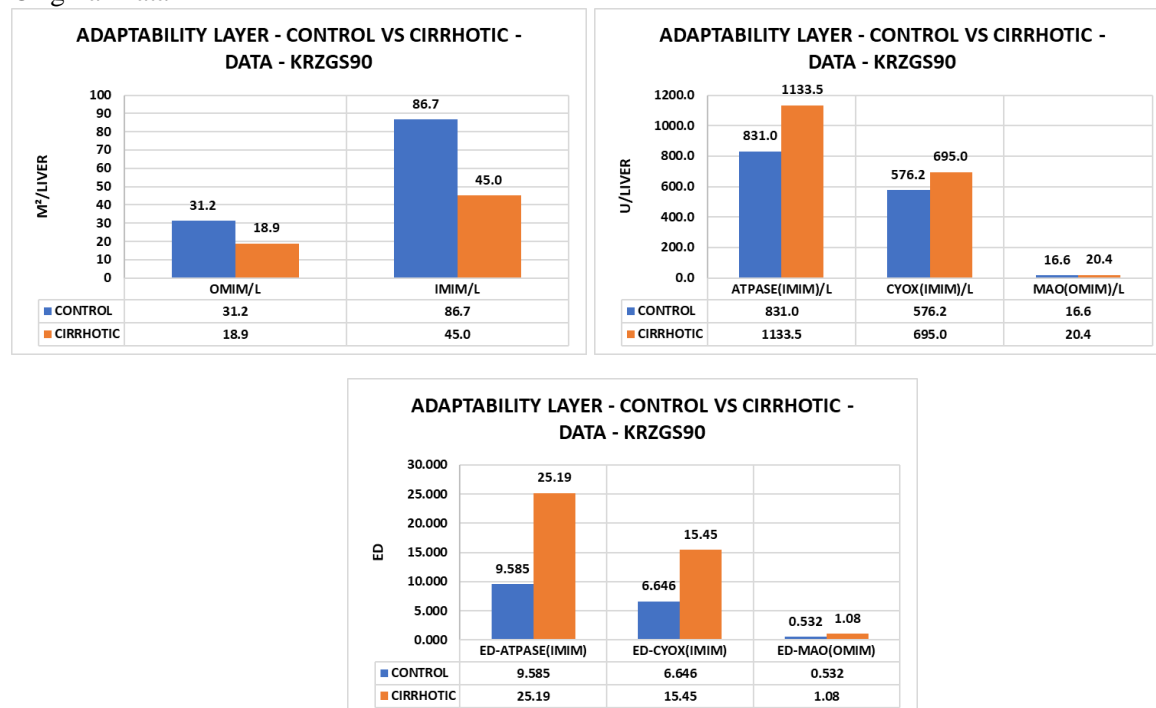


Figure 7.32 In response to the liver damage, the enzyme activities increased, the membrane surface areas decreased, and the enzyme densities of the mitochondrial membranes increased.

Rules Layer – Ratio Data

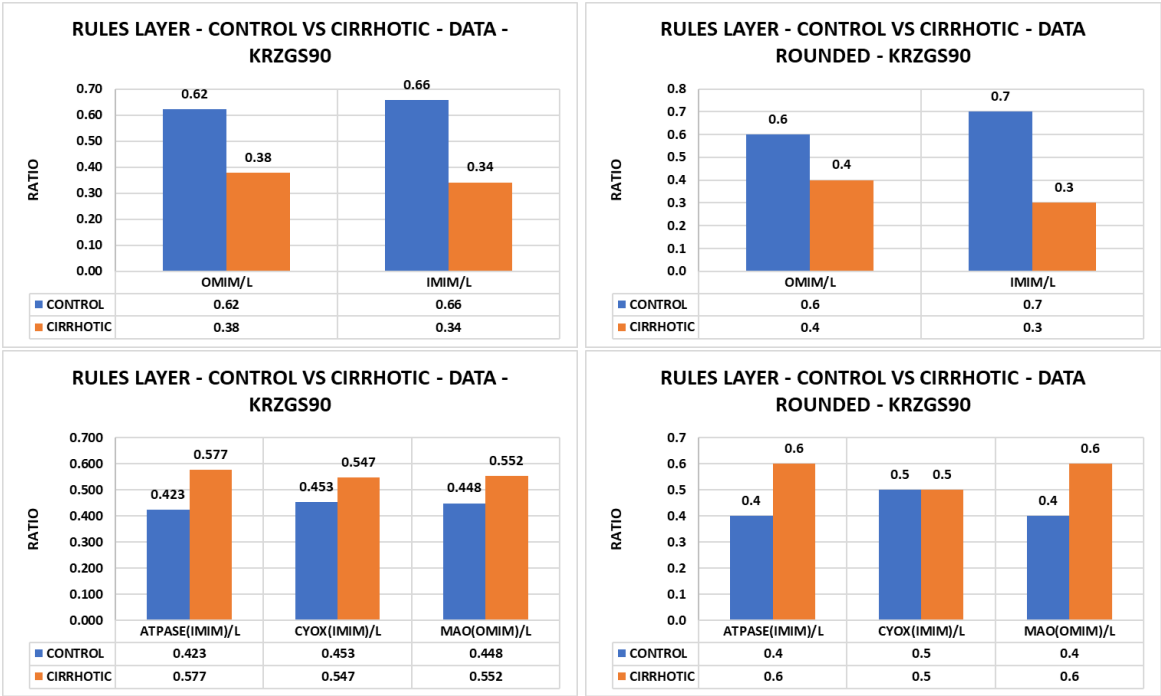


Figure 7.33 The membrane ratios reflect the membrane losses (control vs cirrhotic – 6:4 for the OMIM and 7:3 for the IMIM). ATPase and MAO followed the same rule (4:6 – an increase) but CYOX stayed the same (5:5 – no change). Notice that by relating data to the liver, we can form data pair ratios using data from the control and experimental animals because the liver contains all the cells.

Since biology changes by altering relationships of structure to function, the enzyme densities tell the story of what happened. To compensate for the losses of liver function and membrane surface area, the hepatocytes attempted to solve the problem by more than doubling the enzyme densities of the mitochondrial membranes. Moreover, the liver capacity increased beyond that of the control for all three enzymes. Since the experimental model (CCl₄-induced cirrhosis) limited the ability of the hepatocytes to produce additional mitochondrial membranes, it solved the problem by increasing the enzyme activities.

The point? There exists a preferred state – the one represented by the control phenotype – initially lost to cirrhosis but then regained by changing the membrane recipes. A common problem-solving strategy used by hepatocytes when they suffer a loss in capacity is to change the structure-function mix to reestablish the capacity of the normal state. This often involves applying different solutions to the same problem by using different amounts, combinations, and relationships of morphology to biochemistry. Given the quantity of parts and connections in play, the number of possible combinations becomes large.

Figure 7.34 summarizes the increases in enzyme densities expressed as percentages and ratios (Figure 7.35).

Experimental Changes (%)

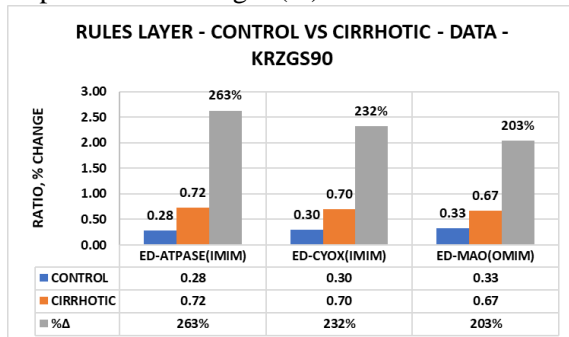


Figure 7.34 The changes (in percentage) indicate that the enzyme densities more than doubled for the cirrhosis model.

Enzyme-Membrane Recipe: $ED\text{-Original Data} = [(U/G)/S/G] = U/S$

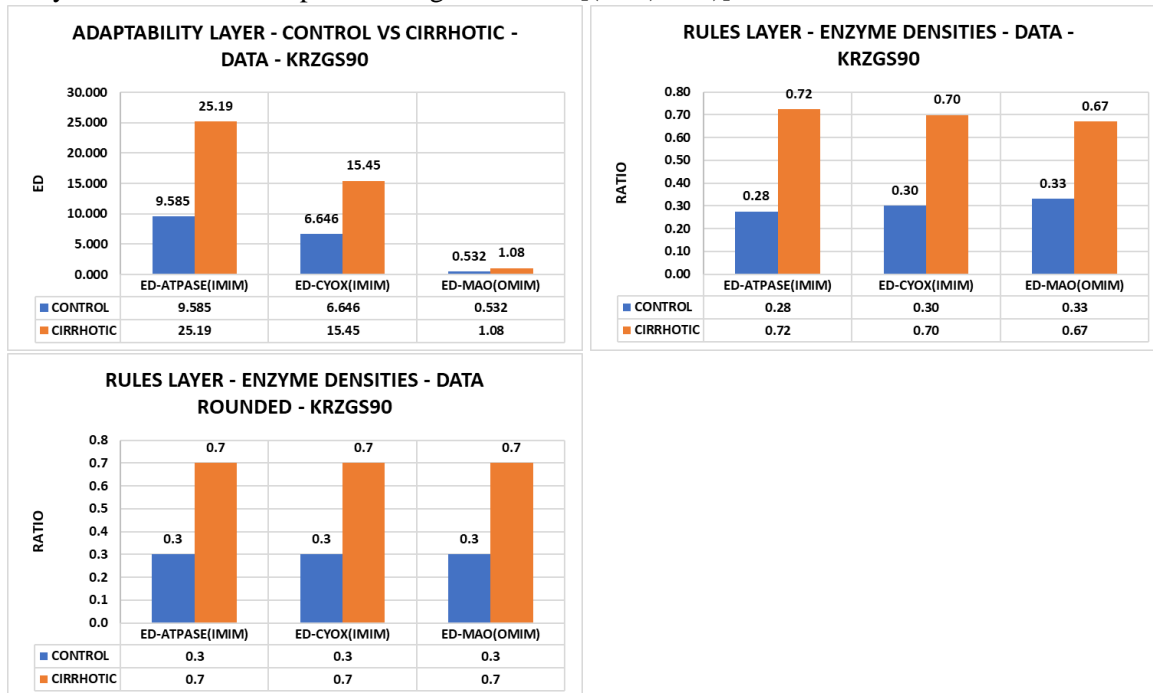


Figure 7.35 All three enzyme densities shared the same control to experimental ratios. Recall that generating ratios from control and experimental data carries the assumption that the number of cells filling a gram of liver did not change. By calculating the enzyme densities using data related to the liver, one can avoid the cell packing problem of the gram of liver reference.

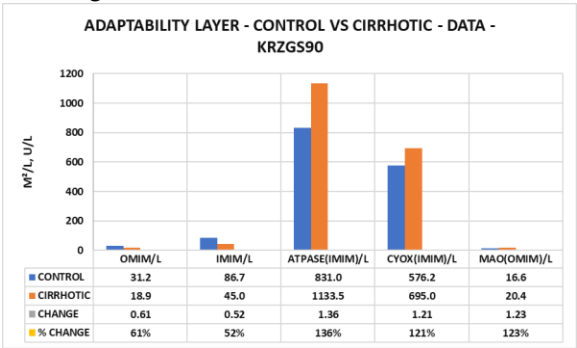
When applying complexity theory to experimental biology, attention to detail becomes essential. Understandably, this becomes a problem for beginners who can quickly become overwhelmed when asked to keep track of so many things changing in so many directions at the same time.

One solution involves working through a list of items in a checklist exercise, which involves little more than moving from one item to the next. The following exercise uses the included data to figure out how

the hepatocytes managed the problem created by the CCl₄-induced cirrhosis. What questions remain unanswered? What data would you need to answer them?

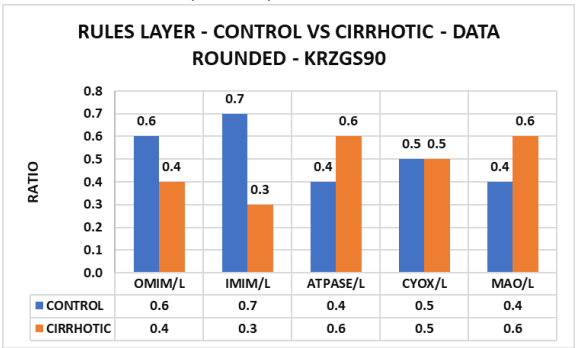
Review Exercise

1. Original Data



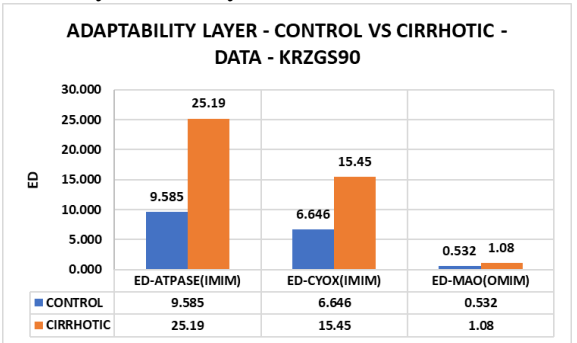
Why do some values increase while others decrease?

2. Data Rules (Ratios)



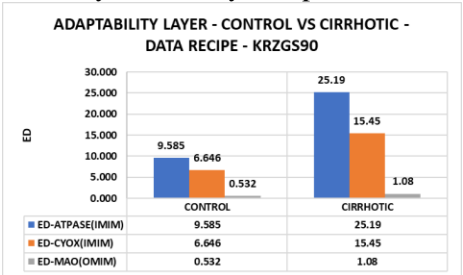
Only ATPASE and MAO have the same ratios. Why?

3. Enzyme Density



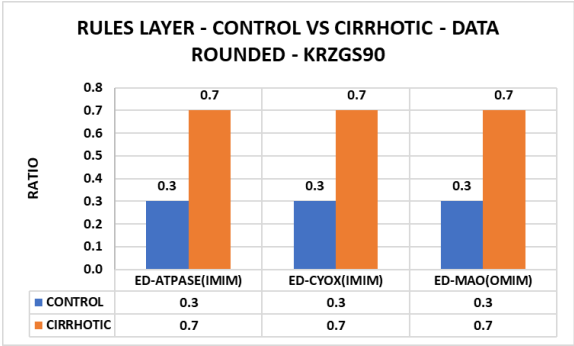
Enzyme densities (U/M²) roughly doubled. Why?

4. Enzyme Density Recipe



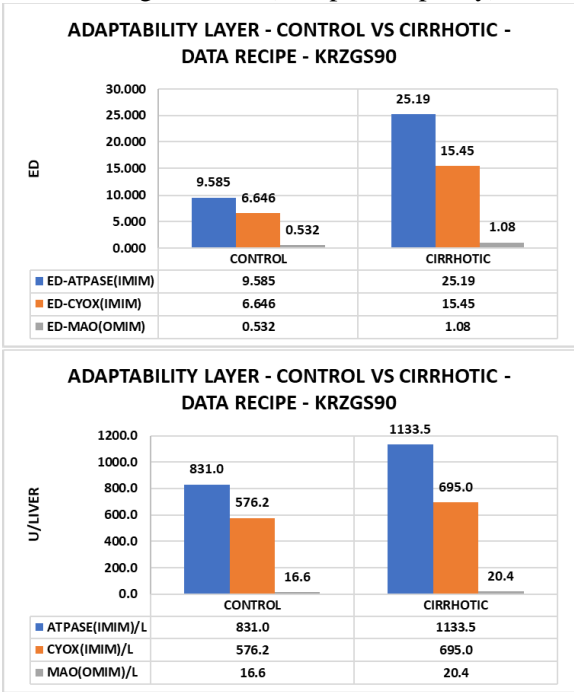
What do the enzyme density recipes tell us about the solution?

5. Enzyme Density Rules



Notice that the ED ratios were the same for the three enzymes.

6. Working Solution (Recipe + Capacity)



Did the hepatocytes succeed in reestablishing the control state given the parts in play?
Yes, by exceeding the capacity of the control.

Comment: Note that a change in marker enzyme activity can result from two different events: (1) a change in a membrane surface area and (2) a change in the amount of activity associated with a unit of membrane surface area.

7.1.7 Case Study 7: Influence of Mechanical Cholestasis on Hepatocytes – DER77

Source: Update applied to original data from Denk, H., Eckerstorfer R., Rohr H. P. (1977) *The endoplasmic reticulum in the rat liver cell in experimental mechanical cholestasis. Correlated biochemical and ultrastructural-morphometric studies on structure and enzyme composition. Exptl and Molecular Pathology* 26:193-203.

Topic: Morphological and biochemical analysis of experimental cholestasis.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, calculate enzyme densities [(ED = [(U/G)/(S/G)]); ED = [(U/G)/(S/G)](NORM1); ED(NORM2)], analyze patterns, and report biological solutions (recipes).

Dataset: Biochemistry: G6PASE, UDP-BILIRUBIN GLUCURONYL TRANSFERASE, CYTOCHROME P450, CYTOCHROME B5, AMINOPYRINE-N-DEMETHYLASE, BENZOPYRENE HYDROXYLASE, CYTOCHROME P450, FERRICYANIDE REDUCTASE (NADPH), CYTOCHROME C REDUCTASE (NADH), LIPID PEROXIDATION (NADPH), STEARYL CO A DESATURASE (NADPH). Morphology: ER (RER, SER).

Denk et al., 1977; DER77) used mechanical cholestasis (ligated the bile duct) to trigger a host of structural and functional changes in hepatocytes. Uncorrected, cholestasis typically leads to cirrhosis, and loss of liver function. Since the liver will try to manage the cholestasis problem by recovering the lost function, the primary question for the update focuses on the liver capacity – before and after bile duct ligation. This will tell us if and how the hepatocytes solved the ligation problem.

Figure 7.36 identifies decreases in enzyme activities for the ligated animals accompanied by a compensatory increase in the ER surface area. By looking at the original (Figures 7.36) and updated data (Figure 7.37), can we explain how the hepatocytes solved the problem? No, because the liver weights were missing.

Original Data

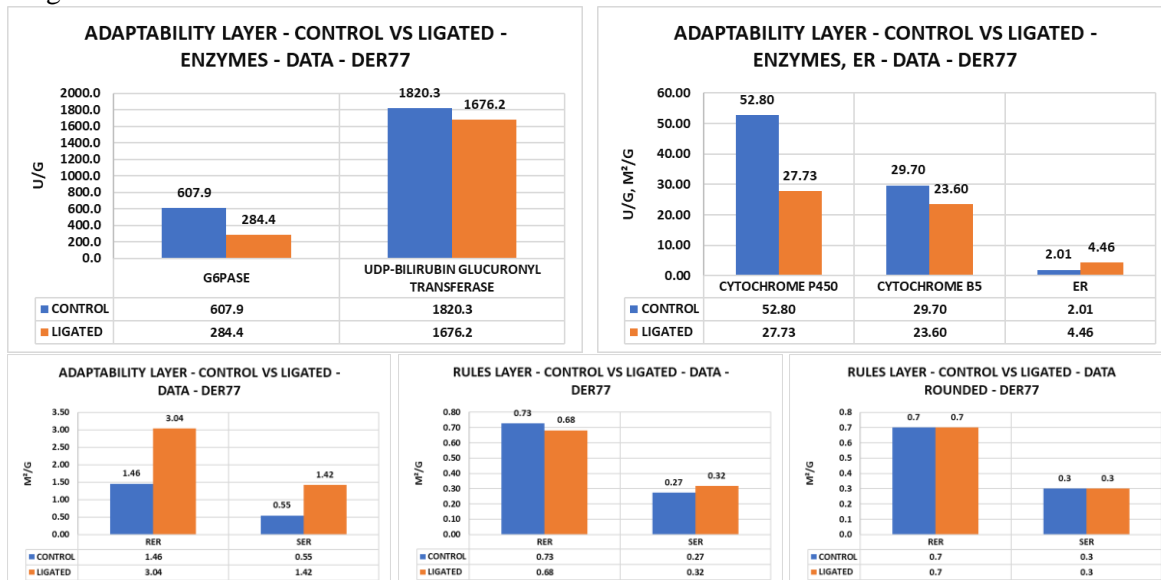


Figure 7.36 In response to the ligation, the surface areas of RER and SER membranes increased by different amounts in the adaptability layer but continued to share the same ratio (7:3) in the rules layer. Since the biochemical ratios come from the control and ligated animals, we have created a problem of interpretation. Without data related to the liver, we must assume that the number of hepatocytes per gram of liver before and after ligation remained the same. Unfortunately, unchecked assumptions can produce wobbly data and results.

ED-Original Data = [(U/G)/S/G)] = U/S

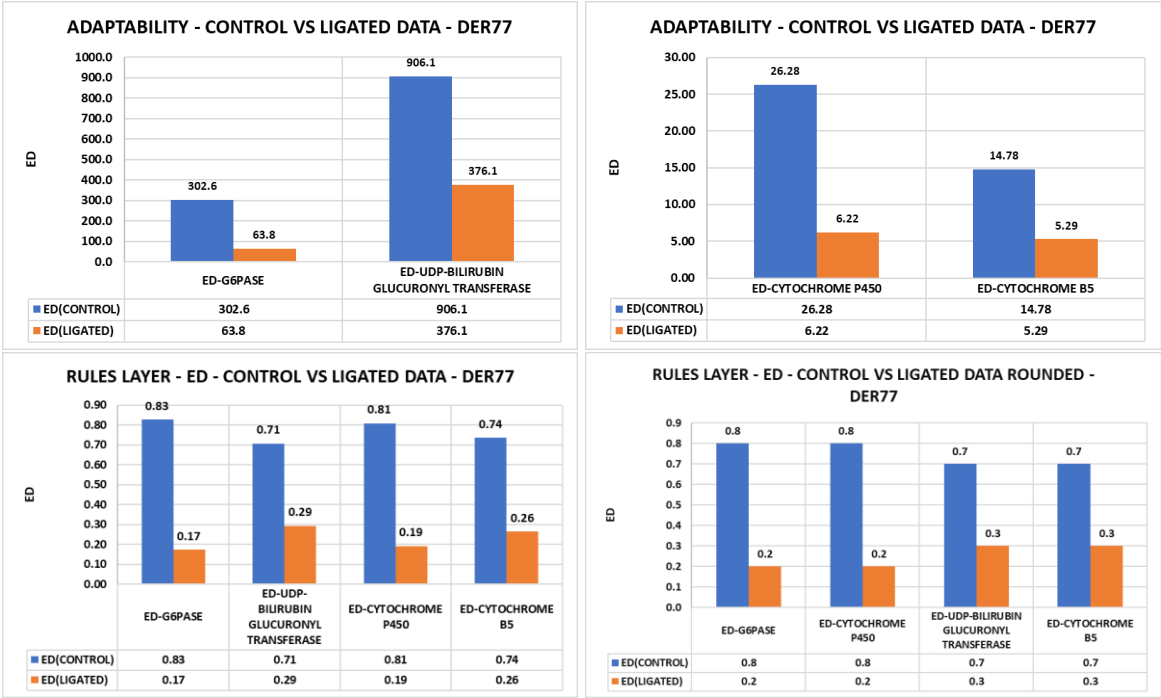
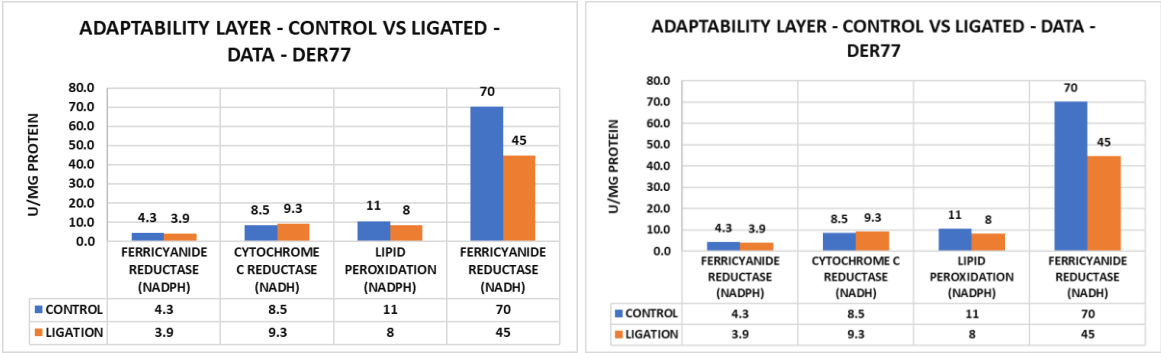


Figure 7.37 Bile duct ligation (cholestasis) decreased the ratios of the enzyme densities quite substantially. Moreover, the four enzyme densities formed two subgroups with similar ratios (e.g., 7:3, 8:2), which suggests linked data pairs. The enzyme densities (ED) begin to explain what happened. While hepatocytes increased the ER surface area, they were unable to do the same for the associated enzymes. Since one calculates enzyme densities independently for the control and experimental data points, they don't incur the risk of ambiguity produced by the cell packing problem.

A second set of results, which included microsomal enzymes associated with the electron-transport pathways, showed variable patterns of change when related to a mg of microsomal protein (Figure 7.38). However, these ratios are subject to the cell packing problem. This explains why the updates routinely used data pair ratios calculated from the same animals and data references.



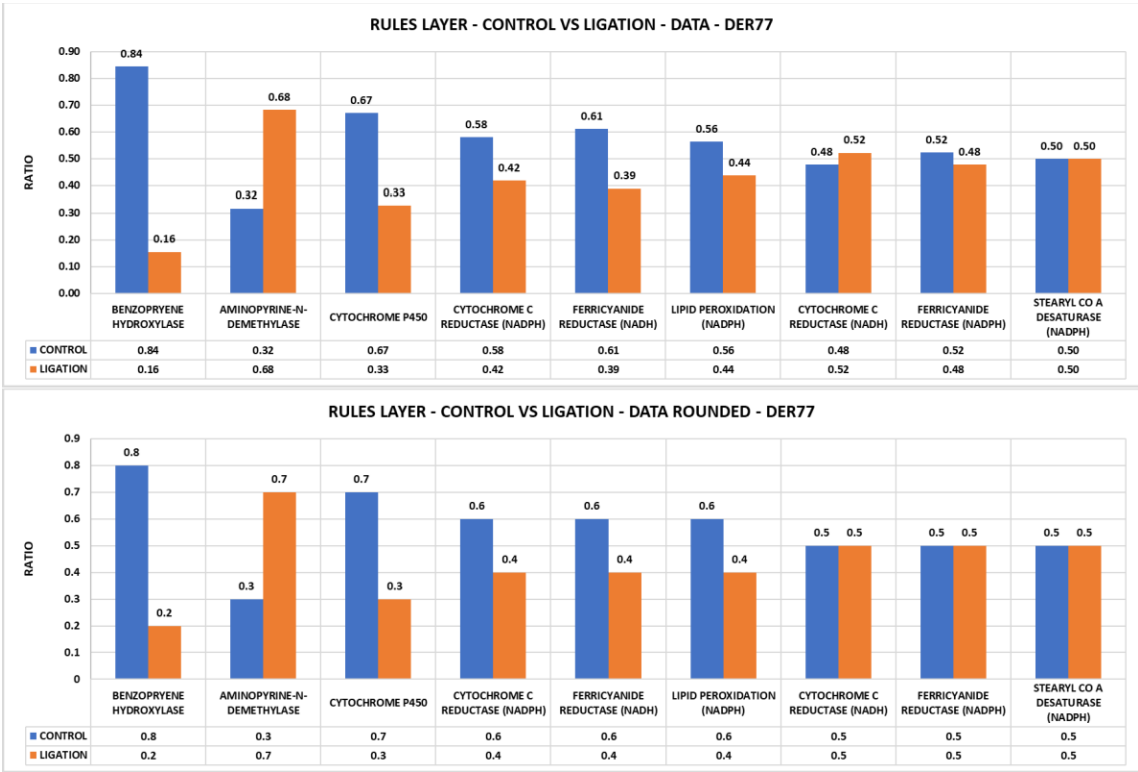


Figure 7.38 When viewed in the rules layer, the enzymes formed distinct subgroups identified by the shared ratios (0.6:0.4 and 0.5:0.5). Moreover, the ratios resemble the patterns encountered when reverse engineering phenotypes. The 0.6:0.4 and 0.5:0.5 enzymes appear to have reached a solution whereas the remaining three enzymes were still a work in progress.

Comment: Understanding the basics of a biological change protects investigators from making risky assumptions unintentionally. Copy the priorities of biology. The organism depends on the liver, the liver on the hepatocytes, the hepatocytes on the organelles, the organelles on the molecules, and everything on the rules. Interpreting a change in hepatocytes, for example, cannot establish the effect of the change without the total liver capacity.

7.1.8 Case Study 8: Human Liver in Cholelithiasis (Needle Biopsies) – KFLGJO78

Source: Update applied to original data from Koch M. M., Freddara U., Lorenzini I., Giampieri M. P., Jezequel A. M., Orlandi F. (1978) A stereological and biochemical study of the human liver in uncomplicated cholelithiasis. Digestion 18:162-77.

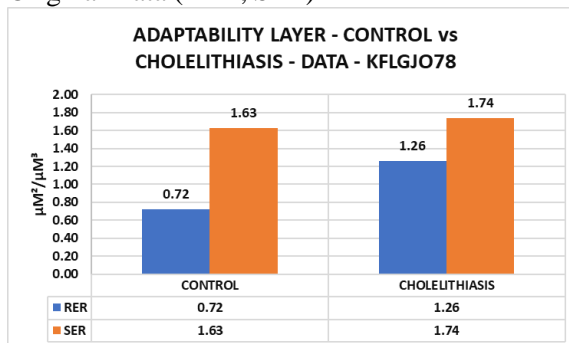
Topic: Morphological changes in cytoplasmic membranes estimated from biopsies.

Update: Apply corrections, report results in adaptability and rules layers, calculate EDs, and analyze results.

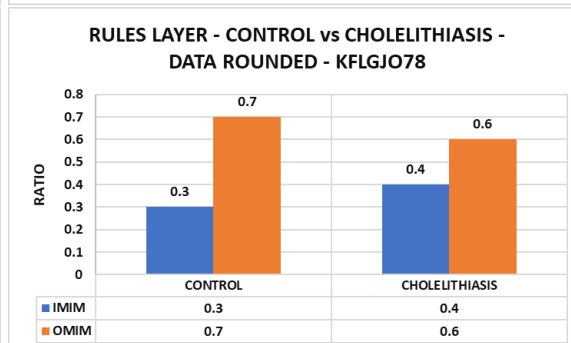
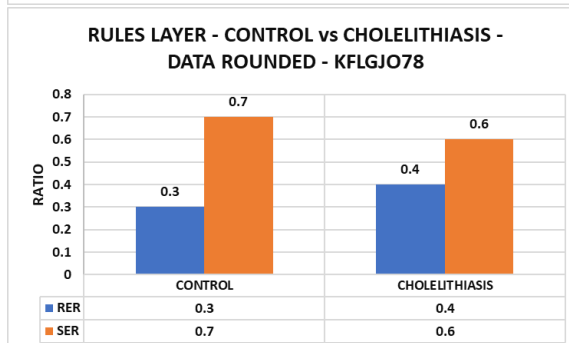
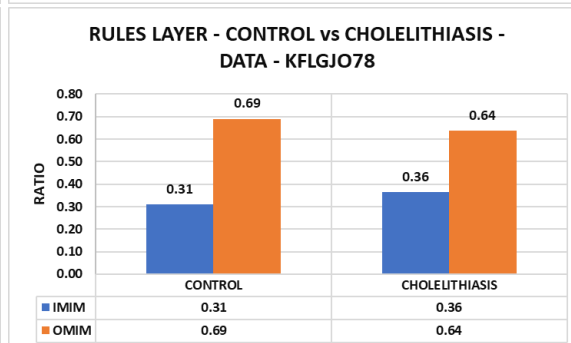
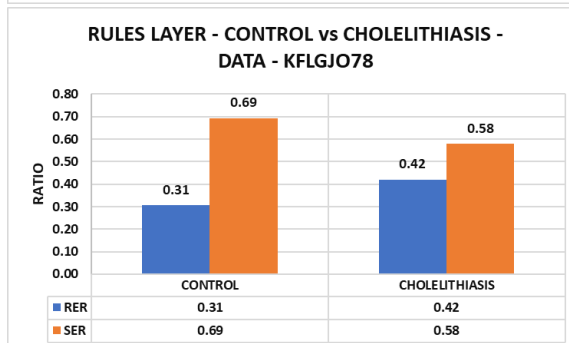
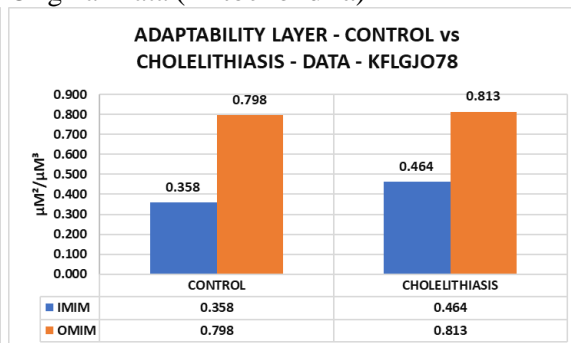
Dataset: Biochemistry: NADPHCCR; Morphology: Surface area estimates for ER, RER, SER, MIN, OMIM, IMIM and EDs calculated for the ER.

Using needle biopsies, Koch et al., (1978; KFLGJO78) studied patients with uncomplicated cholelithiasis (gallstones) using stereology and biochemistry. The patients included 8 normal subjects and 21 with radiolucent gallstones (11♂, 12♀). After applying the section correction, we can recalculate the results and see how the patterns of the hepatocytes changed in response to cholelithiasis (Figure 7.39).

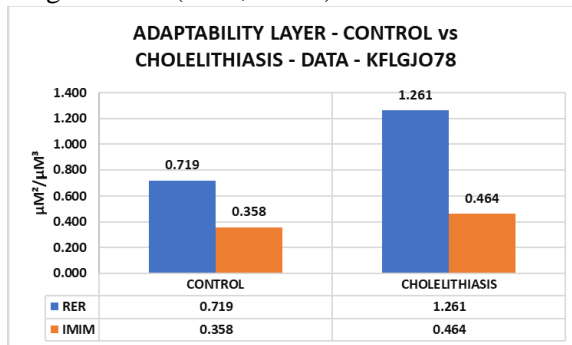
Original Data (RER, SER)



Original Data (Mitochondria)



Original Data (RER, IMIM)



Original Data (SER, OMIM)

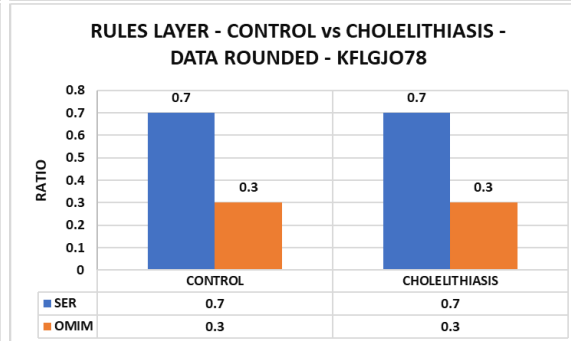
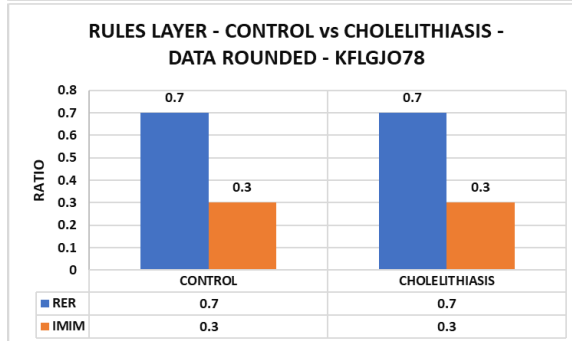
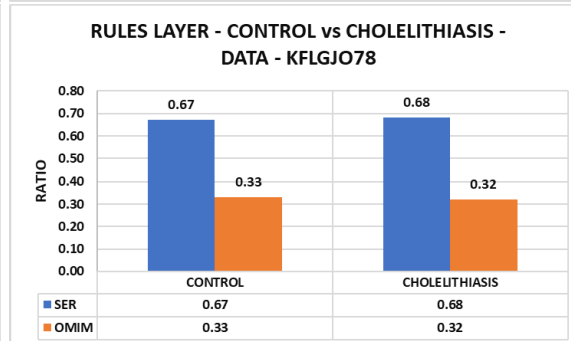
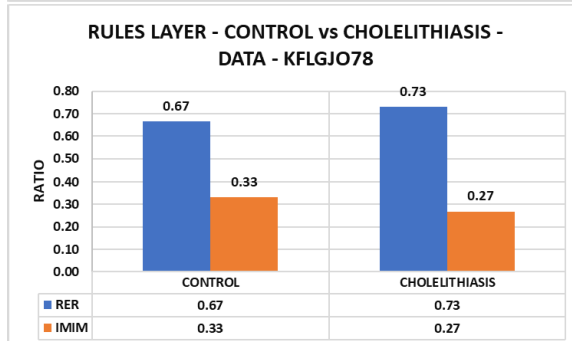
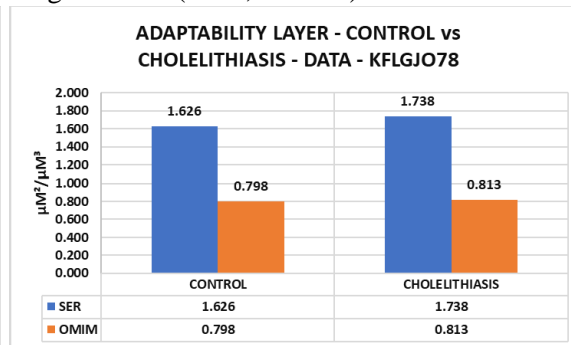


Figure 7.39 By forming data pairs, we can calculate the data pair ratios. The data pair ratios explain how cholelithiasis changed the rules.

Figure 7.40 summarizes the updated results. The first thing to notice is that the control hepatocytes used mirror image symmetry and data pair ratios to organize their organelles. The hepatocytes in the patients with cholelithiasis, however, changed the rules by switching to the 0.4:0.6 ratio for the RER:SER and IMIM:OMIM ratios. Now comes the hard question. How were the hepatocytes able to change the adaptability values in the patients with cholelithiasis to get the new ratio (4:6) without disrupting the 0.7:0.3 ratios of the RER:IMIM and SER:OMIM?

This means that if a hepatocyte entangled two parts, by knowing the value of one part (and the ratio rule), it would immediately know the value of the other part.

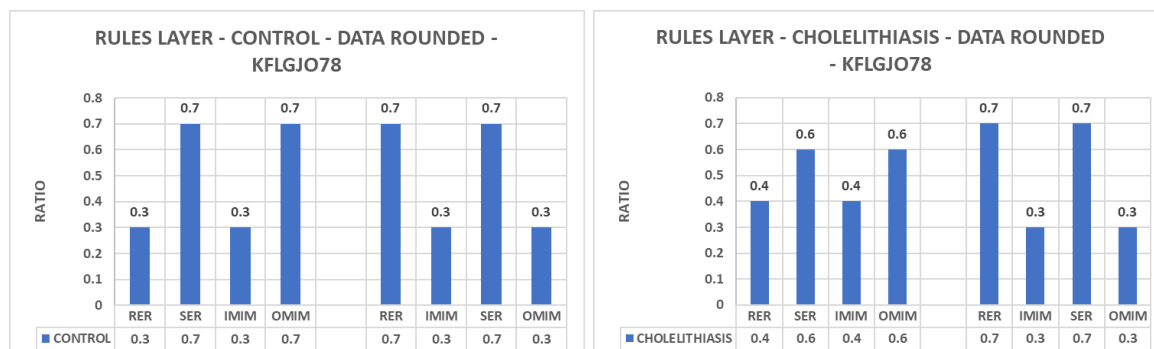


Figure 7.40 In patients with cholelithiasis, hepatocytes can change the values of their organelles to form new ratios without disrupting the same organelles forming other ratios (paradox?). One explanation might involve classical entanglement. If real, this observation might be part of the algorithm cells use to optimize outcomes – or something else.

If we know the rule and have a value for one of the two parts, do we automatically know the missing value? If we set up and solve an equation in Excel, did it work? No, the answer was off by 14%.

$$X = (0.719 \cdot 0.3) / 0.7 = 0.308143$$

	VALUE	RATIO		IMIM	EXPECTED	MISSED BY
RER	0.719	0.7		0.308143	0.357883	14%
IMIM	X	0.3				

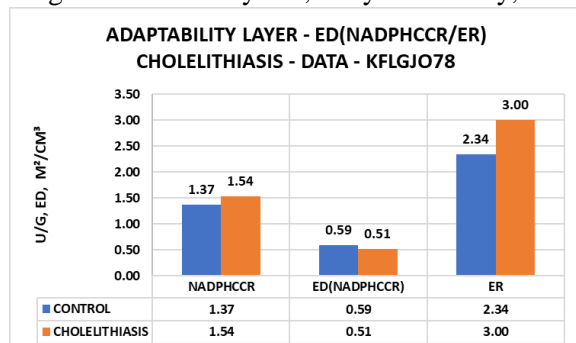
If we add back a few digits and try a second time, it worked. The point? It's better to use non-rounded data when predicting missing data.

$$X = (0.719 \cdot 0.332) / 0.667 = 0.357883$$

	VALUE	RATIO		IMIM	EXPECTED	MISSED BY
RER	0.719	0.667		0.357883	0.357883	0%
IMIM	0.358	0.332				

Can the enzyme densities begin to explain why the hepatocytes changed their RER:SER and IMIM:OMIM ratios? Figure 7.41 suggests that cholelithiasis had little effect on the enzyme density but seems to have increased the ER surface area.

Original Data – Enzymes, Enzyme Density, ER



Rules Layer

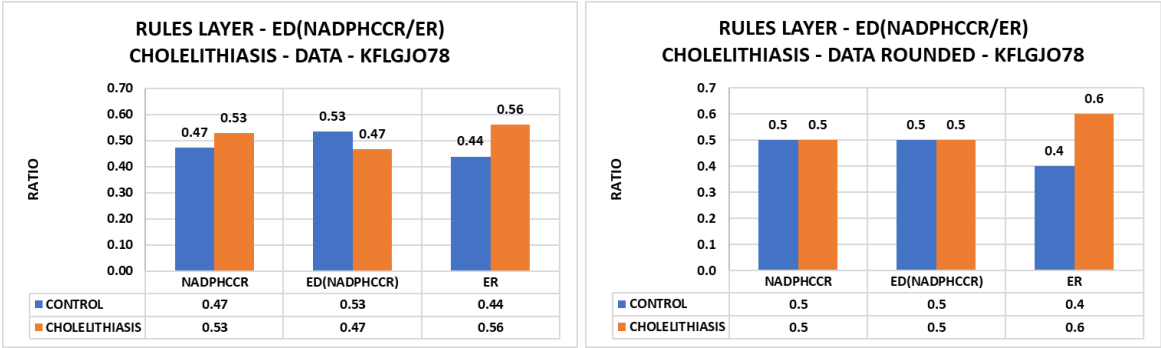


Figure 7.41 Ratios in the rules layer (data rounded) identify a rule change only in the ER. Comparing the increase in the ER surface area (28%) to the slight decrease in the enzyme density (15%), suggests that the ER provided the largest response to the cholelithiasis.

Comments: How did the hepatocytes use their enzymes, membranes, and enzyme densities to manage the problem created by the gallstones? They increased their RER surface area (synthesizing capacity) and to a lesser degree the amount of IMIM (energy supply). Since the enzyme activity and enzyme density of NADPHCCR remained largely unchanged, other unstudied enzymes may also have benefited from the increases in membrane surface areas. **Caution:** The enzyme density calculations were based on mixed data references (U/g vs S/cm³) and they were not normalized. However, the results provided a rough estimate.

7.1.9 Case Study 9: Hepatocytes After Total Biliary Obstruction (TBO) – JSMAO76

Source: Update applied to original data from Jones A. L., Schmucker D. L., Mooney J. S., Adler R. D., Ockner R. K. (1976) Morphometric analysis of rat hepatocytes after total biliary obstruction. *Gastroenterology* 71:1050-1060.

Topic: Bile duct ligation as an experimental model for cholestasis in central and portal hepatocytes.

Update: Apply corrections, report results in adaptability and rules layers, and analyze results.

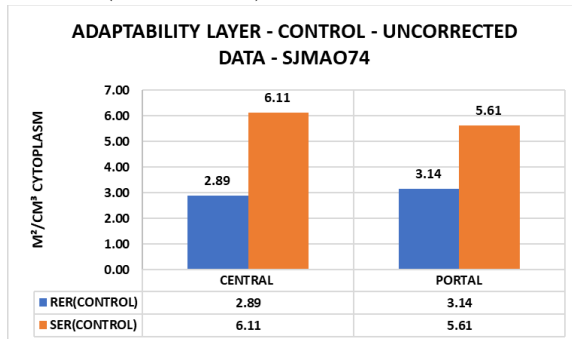
Dataset: Surface area estimates for ER, RER, SER, MIN, OMIM, and IMIM.

The functional unit of the liver defines a lobule consisting of hepatocytes wherein blood enters at the periphery (portal) and exits at the center (central). According to their age and position within the liver lobule, hepatocytes can display different structural and functional properties. The update of the study (Jones et al., 1976; JSMAO76) considers estimates for the RER and SER before and after total biliary obstruction (TBO) – uncorrected and corrected for section thickness and compression. First the uncorrected data and then the corrected.

Uncorrected Results: The uncorrected data showed a preponderance of SER at both locations (central and portal) and the biliary obstruction produced a loss of both RER and SER membranes (Figure 7.42). The ratios of SER to RER (0.5:0.5) in both control and TBO animals at both locations (portal and central) were the same (Figure 7.43).

Original Data

Control (Uncorrected)



Total Biliary Obstruction (Uncorrected)

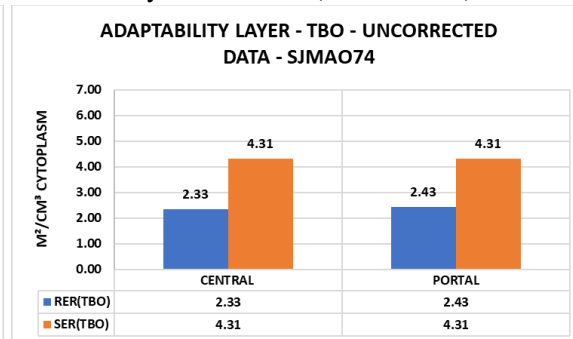
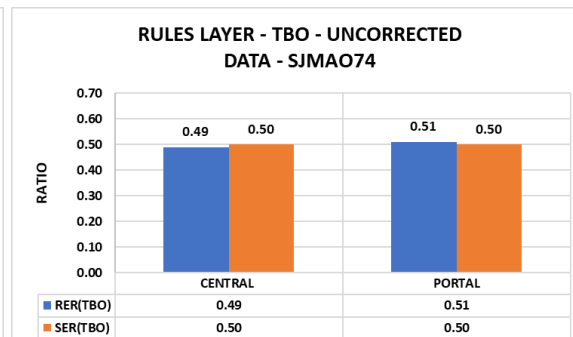
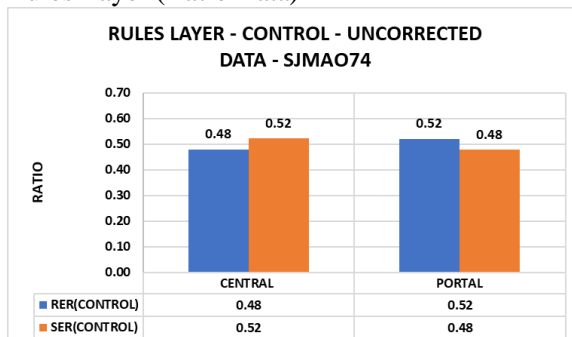


Figure 7.42 Uncorrected estimates for membrane surface areas suggested changes to the surface areas of RER and SER membranes in both central and portal hepatocytes following total biliary obstruction.

Rules Layer (Ratio Data)



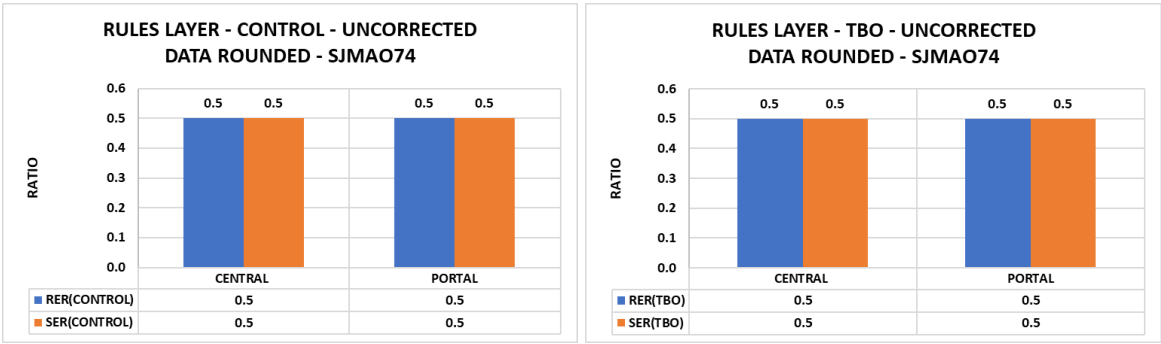
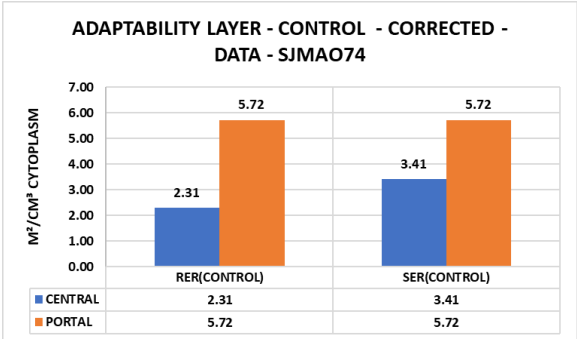


Figure 7.43 When comparing groups (control vs TBO) - at both central and portal locations - the uncorrected estimates for RER and SER found no changes to the proportion of these two membrane compartments.

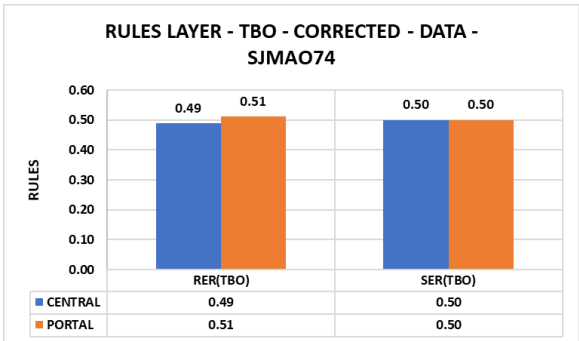
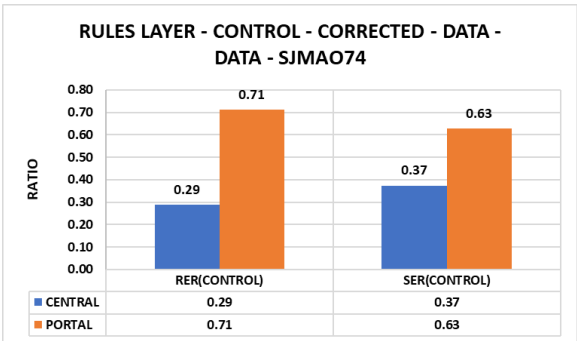
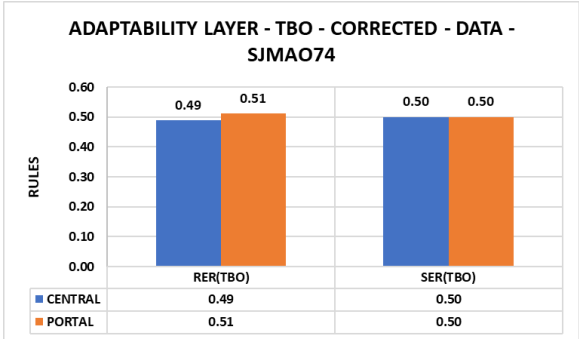
The same dataset with section corrections applied produced different results (Figure 7.44). In the adaptability layer, the portal hepatocytes had more RER membranes than those of the central ones. However, TBO resulted in equal amounts of membranes at each location but with diminished surface areas. Across the liver lobule (central to portal), the ratios of the rules layer went from 0.3:0.7 for the RER and 0.4:0.6 for the SER in the controls to 0.5:0.5 for both the RER and SER in the TBO group. In effect, TBO diminished both the amounts and differences between the central and portal hepatocytes. Without the enzyme densities and total liver values, this is as far as the story goes. Regrettably, we don't know how or if the hepatocytes solved the TBO problem. On the other hand, we now know what parts were missing experimentally.

Original Data

Control (Corrected)



Total Biliary Obstruction (Corrected)



Rules Layer (Ratio Data)



Figure 7.44 When comparing the data rounded groups (control vs TBO), estimates for the RER and SER - at both central and portal locations - found changes for the control RER and SER but not for the experimental (TBO) RER and SER.

Comment: Updating the literature by correcting estimates for membrane surface areas often leads to new results and interpretations.

Biopsy Studies

To be unbiased, stereological estimates based on liver biopsies assume that the sampling protocol meets the requirement of representative sampling (i.e., all parts of the liver have an equal chance of becoming sampled). Since the biopsies came from the livers of anesthetized animals *in situ*, the sampling requirement was by necessity waived. Fortunately, the major source of heterogeneity in the liver parenchyma appears to come from differences in portal and central hepatocytes, which can appear in the same biopsy.

7.1.10 Case Study 10: Liver Biopsies: Wedge Vs Needle) – HGWP73

Source: Update applied to original data from Hess F. A., Gnagi H. R., Weibel E. R., Preisig R. (1973) Morphometry of dog liver: Comparison of wedge and needle biopsies. Europ J Invest 3: 451-458.

Topic: Morphological changes in RER, and SER estimated from biopsies.

Update: Apply corrections, expand data, report results in adaptability and rules layers, and analyze patterns.

Dataset: Morphology: RER, SER.

The authors (Hess et.al., 1973) applied morphometry (i.e., stereology) to tissue collected with needle and wedge biopsies. Since both types of biopsies came from the same animals, one would expect similar estimates for the RER and SER surface areas. Unfortunately, such agreement did not appear. Instead, the update suggested that the estimates might have been under sampled (unrepresentative) and/or each biopsy method carried a different set of methodological errors. Nonetheless, we can use the new data from the rules layer to look for answers.

Since ratios define the relationship of one part to another, they define the rules of individuals, which in turn define the rules of the population. However, a designated population may contain outliers that could turn a homogeneous population of individuals into a heterogeneous one. Can you see where this is going? If we use reproducibility at the level of individuals as a test for homogeneity, then a finding of homogeneity could indicate – provisionally – that the sampling was representative.

Figure 7.45 shows that data from the adaptability layer suggest that both the needle and wedge biopsies came from heterogeneous populations.

Original Data

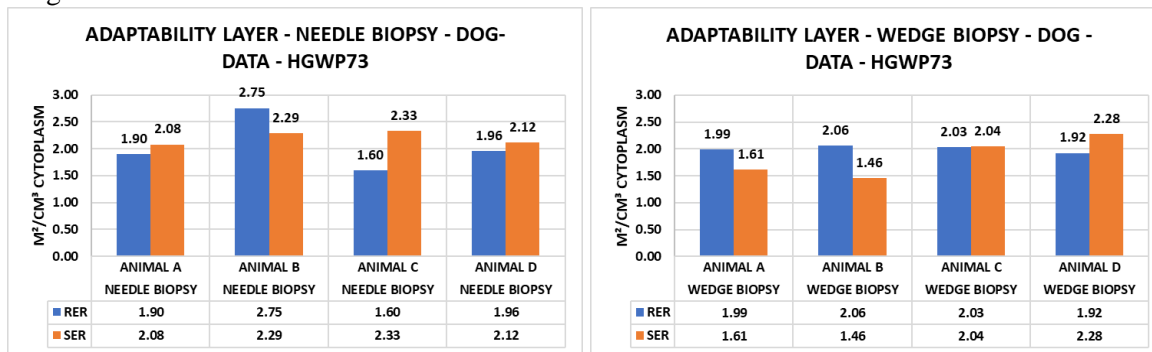


Figure 7.45 The results in the adaptability layer suggested that both the needle and wedge biopsies came from a heterogeneous population of animals. The RER was either larger or smaller than the SER.

If representative sampling existed for needle and wedge biopsies (Figure 7.46), one might expect to find identical ratios in the rules layer. Since this did not appear (only animal D shared the same result for both methods), an assumption of representative sampling would seem hasty. Either the animals, samples, or both might not meet the sampling requirements.

Rules Layer (Ratio Data)

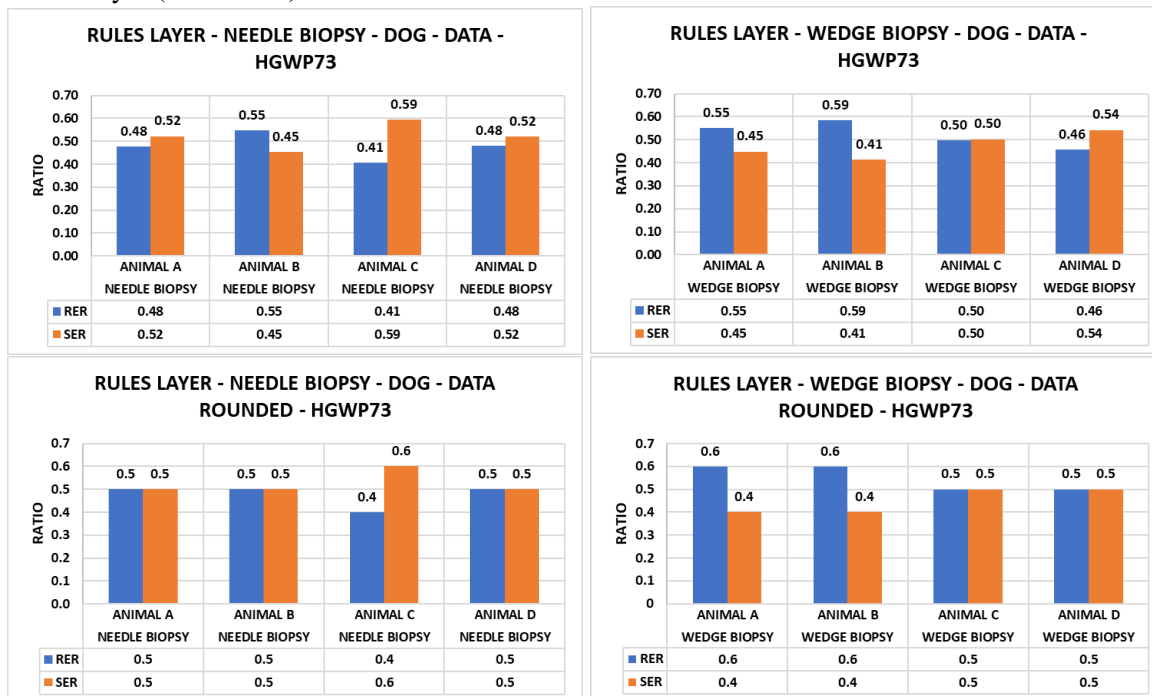


Figure 7.46 Moreover, when viewed in the rules, notable differences appeared between the needle and wedge biopsies. Since both estimates came from the same animals, both ratios (needle and wedge) should have been the same. They were not except for animal D (0.5:0.5).

For a population to be representative, shouldn't it be 100% homogeneous with respect to the rules? Would a finding less than 100% homogeneity in the rules layer point to a sampling problem? Since biopsies fail to meet the basic requirement of representative sampling (all parts sampled with equal probability), the variability of the results points to nonrepresentative sampling.

A careful reading of the experimental methods revealed the most likely source of the inconsistency problem (Figure 7.47). The estimates for animal A came from two biopsies, animals B and C from one biopsy, and animal D from 5 biopsies. In effect, animal D appears to have delivered the most reasonably sampled data point. None the less, trying to explain the results of this paper led to a strategy based on applying reproducibility as a check for homogeneous sampling. Since it's a quick and easy test to apply, it offers authors a way to reinforce biopsy results that cannot meet the requirements of representative sampling.

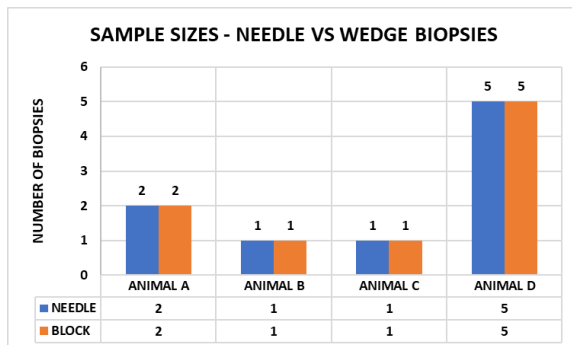


Figure 7.47 Inadequate sampling can change the ratios in the rules layer.

Comment: When published data represent estimates, representative sampling becomes essential because it's a basic assumption of the experimental design. Stereology includes sampling methods designed specifically to collect representative samples (for sample calculations see Bolender et al., 1993).

7.1.11 Case Study 11: Human Liver: Needle Biopsies – RLGGB76

Source: Update applied to original data from Rohr H. P., Luthy J., Gudat F., Oberholzer M., Gysin C., Bianchi L. (1976) Stereology of liver biopsies from healthy volunteers. Virchows-Arch A Patholol Anat His. 371(3): 251-263.

Topic: Morphological changes in cytoplasmic membranes estimated from biopsies.

Update: Apply corrections and report results in the adaptability and rules layers.

Dataset: Morphology: Surface area estimates for ER, RER, SER, MIN, OMIM, and IMIM.

For this study of biopsy data (Rohr et al. (1976; RLGGB76), the update compared results before and after applying corrections for section thickness and compression. The study included four human subjects - each providing a single biopsy.

Figure 7.48 shows the results before and after correcting the original data for section thickness and compression. Curiously, the ER and mitochondria displayed an unexpected pattern, wherein the RER mirrored the surface area of OMIM and the SER the IMIM. Usually, but not always, the RER tracks with the IMIM and the SER with the OMIM. Note that the rules derived from the data pair ratios showed one result for the uncorrected (7:3) data but a different one for the corrected data (4:6).

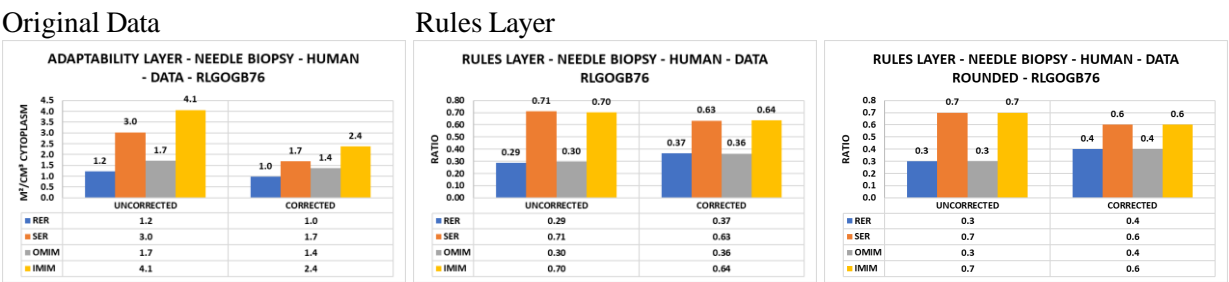


Figure 7.48 Correcting stereological estimates changed the results absolutely and relatively.

Comment: Once again, these results underscore the importance of applying section thickness and compression corrections to stereological estimates of membrane surface areas.

7.1.12 Case Study 12: Human Liver (Needle Biopsies) – W-27 – RKSBBT78

Source: Update applied to original data from Roessner A., Kolde G., Stahl K., Blanke G., van Husen N., Themann H. (1978) Ultrastructural morphometric investigations on normal human liver biopsies. Acta Hepato-gastroenterol 25:119-123.

Topic: Morphological changes in cytoplasmic membranes estimated from biopsies.

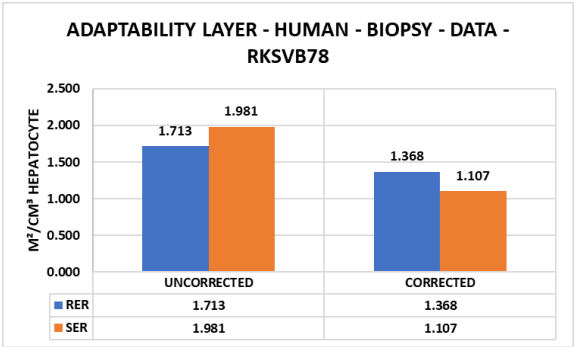
Update: Apply corrections, report results in adaptability and rules layers, and analyze the results.

Dataset: Morphology: Surface area estimates for ER, RER, SER, MIN, OMIM, and IMIM.

Roessner et al., (1978) estimated the surface areas of the ER (RER, SER) and mitochondrial (OMIM, IMIM) membranes with liver biopsies collected from 14 normal patients. The update compares the results before and after applying corrections for section thickness and compression.

Figure 7.49 shows that estimates for ER and mitochondrial membranes led to notably different results when uncorrected for section thickness and compression. When corrected, no difference (0.5:0.5) became a difference (0.6:0.4) for the RER:SER ratio and one difference (0.3:0.7) became another (0.4:0.6) for the OMIM:IMIM ratio.

Original Data
ER Membranes



Original Data
Mitochondrial Membranes

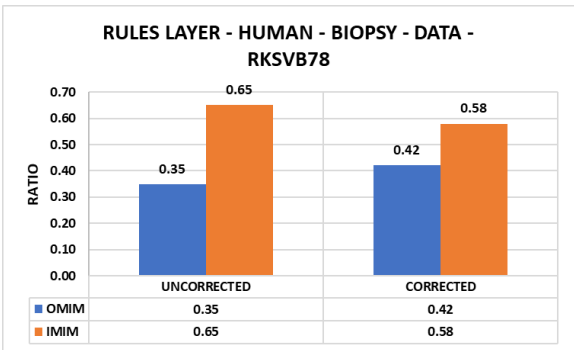
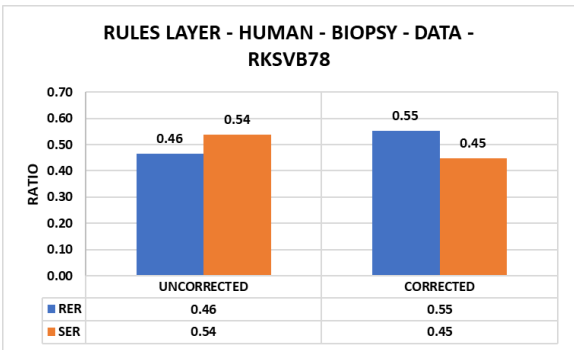
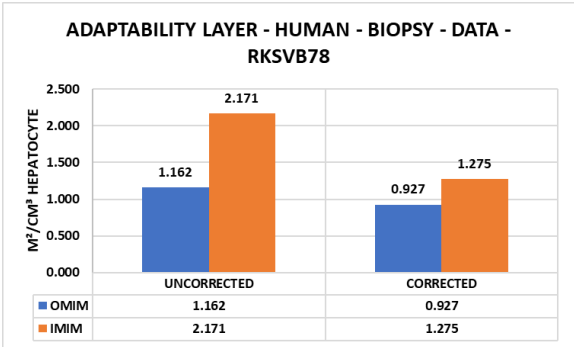




Figure 7.49 Stereological estimates based on uncorrected and corrected data continue to produce different results in both the adaptability and rules layers.

When reporting data from human liver biopsies studies tend to collect one biopsy from several individuals and then average the results. Figure 7.50 compares the results from two similar studies. The authors of the current RKSVB78 study included results from fourteen subjects whereas the one from Rohr et al., (1976 – RLGGB76) used four. The two studies gave mixed results. The figure demonstrated reproducibility for the mitochondrial membranes, but not for those for the RER and SER. Two issues may exist: (1) the protocols used to identify membranes in electron micrographs may vary and (2) data may come from either homogeneous or heterogeneous populations. The approach used in Case Study 10 (Hess et al., 1973) suggested that biopsy results benefit from working up results for every individual (to evaluate the underlying population) before calculating averages. Since data collected from biopsies cannot meet the requirements of unbiased sampling, ratios can at least offer reproducibility as a provisional measure of correctness. In any case, publishing raw data would seem to become an important consideration when working with biopsies.

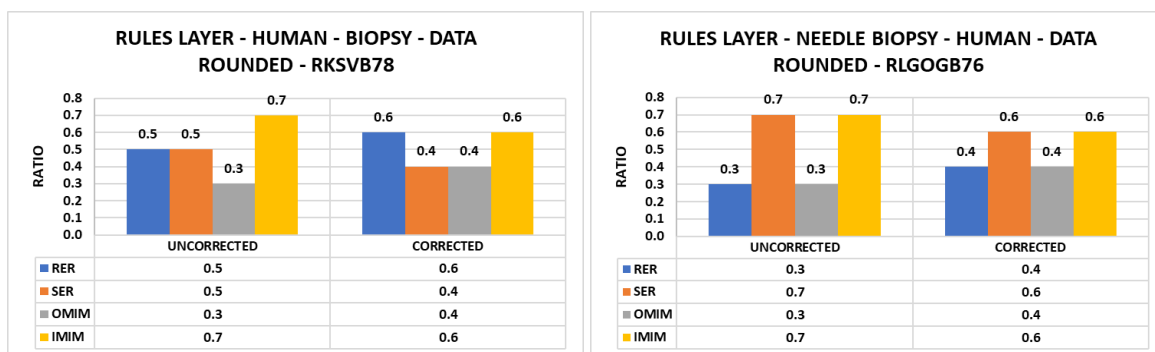


Figure 7.50 Estimates uncorrected for section thickness and compression overestimate membrane surface areas depending on the sizes and shapes of the parts in relation to the section thickness (Weibel and Paumgartner, 1978). The RKSVB78 plot averaged biopsy data from 4 patients and RLGGB76 from 14 volunteers. Without the raw data, we can do little to reconcile the differences between the results for the RER and SER.

Comment: The task of a primer is to steer the beginner through the mine fields of errors created by our research methods. By learning to detect changes by copying the way biology changes, for example, reproducibility automatically becomes a fundamental part of our experimental models. Reproducibility checks sampling identifies persistent patterns, links similar studies, and can confirm biological changes.

Case 15: Developmental changes in the ER of hepatocytes (Needle Biopsy) – DSMF76 (CW33)

Source: Update applied to original data from De la Iglesia F. A., Sturgess J. M., McGuire E. J., Feuer G. (1976) Quantitative microscopic evaluation of the endoplasmic reticulum in developing human liver. *An J Path* 82(01): 61-70; For liver weights Coppoletta and Wolbach, 1933.

Topic: Morphological changes in ER, RER, and SER during development.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, and analyze patterns.

Data Set: Morphology: ER (RER, SER).

De la Iglesia et al., (1976) followed the early development of hepatocytes in normal patients ranging in age from two months to eighteen years. Sixteen liver biopsies provided the data. After applying corrections for section thickness and compression, the intermediate changes seen below in the adaptability layer returned to the initial values fifteen years later. However, when viewed in the rules layer the same data showed distinct changes in the ratios of RER to SER. (Figures 7.51, 7.52).

Original Data

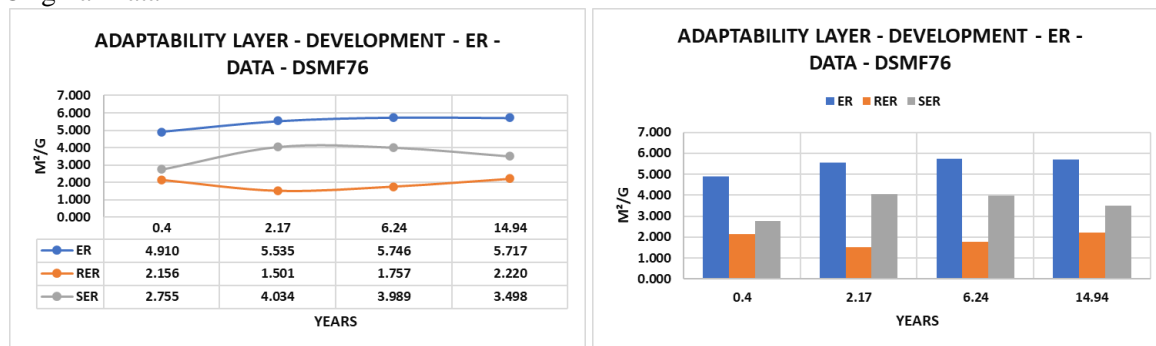


Figure 7.51 During development, changes in organelles can signal changes in rules.

Rules Layer (Ratio Data)



Figure 7.52 In the rules layer, the RER and SER appear to be oscillating between two ratios: 0.4:0.6 and 0.3:0.7. Such fluctuations often occur during growth and may represent a general pattern (Bolender, 2016).

Unfortunately, using a gram of liver reference to detect changes carries the risk of comparing data coming from different number of cells filling the gram (or cm³). Although shifting reference from a gram of liver to the entire liver solves this problem, the paper didn't include liver weights. Since we can substitute liver

weights published in the literature (Coppoletta and Wolbach, 1933), a mashup provided a workaround (Figure 7.53).

By combining data coming from the two publications, we now have a structural framework for reinterpreting biochemical data coming from the liver during the first 12 years of human development.

Original Data

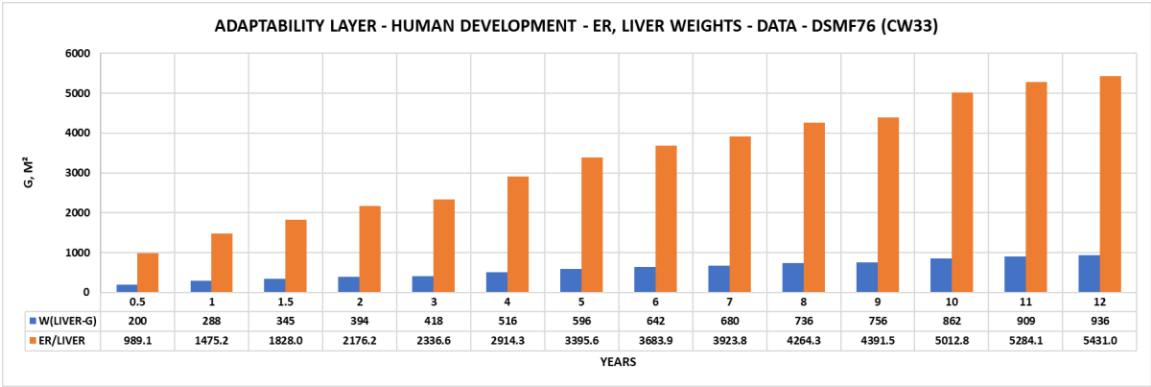
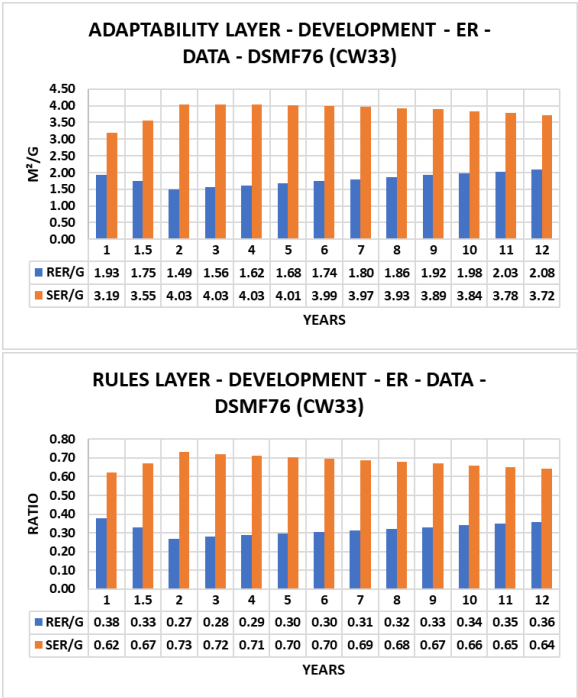


Figure 7.53 Liver weights were adapted from Coppoletta and Wolbach (1933).

By relating the same data to a gram liver and to the liver, we can see how changes in the number of hepatocytes filling a gram of liver influences the results (Figure 7.54). The per liver data show persistent increases in RER and SER membrane surface areas whereas the per gram of liver do not. As expected, both references gave the same ratios in the rules layer. In effect, the update allowed us to mitigate the effects of two major methodological biases (section thickness/compression and cell packing/gram).

Per Gram



Per Liver



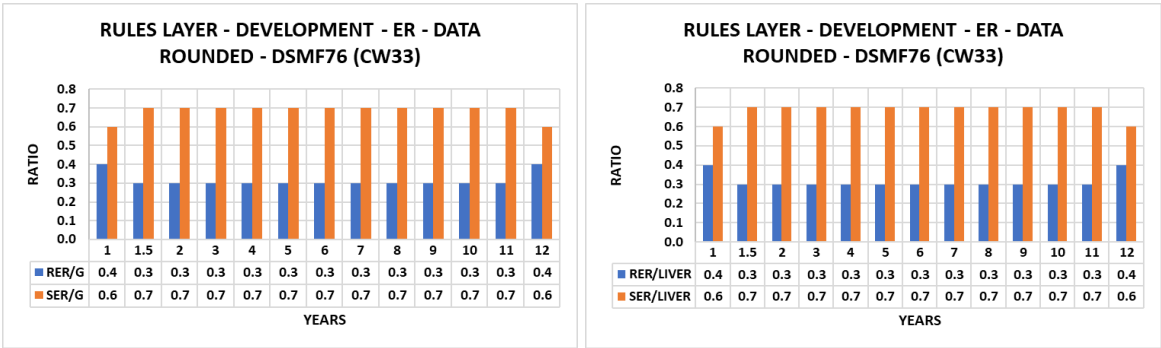
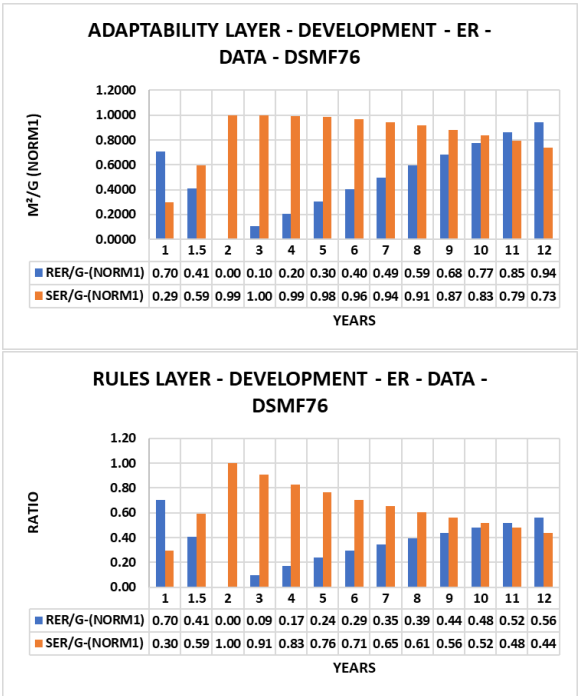


Figure 7.54 Changes in hepatocytic RER and SER during early human development display different slopes (adaptability layer) but identical ratios (rules layer) when related to a gram of liver and to the liver.

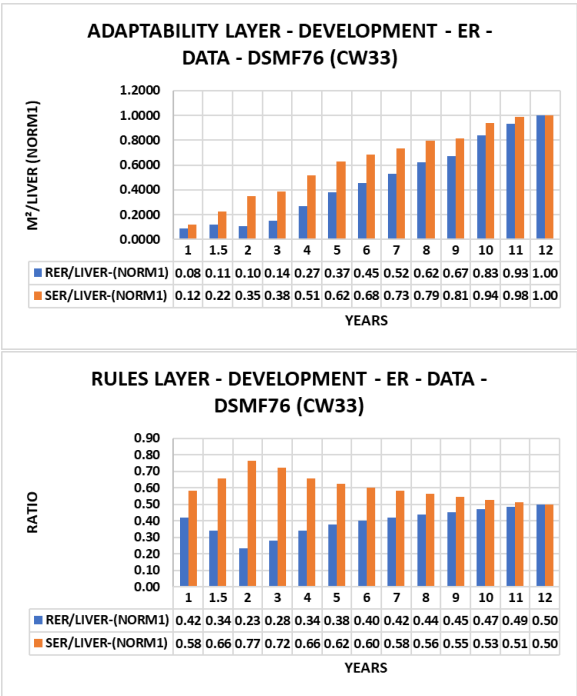
By normalizing the updated data (Figure 7.55), we can see how the RER and SER change proportionately. Notice that the two references (gram and liver) deliver different results in both the adaptability and rules layer. Why is this important to know? Since forward and reverse engineering biology includes reconnecting phenotypic and genetic changes, working out the relationships of cells to organelles to enzymes to RNAs to genes becomes an exercise in reconnecting two sets of different changes collected with different methods.

Normalizing the data offers a standardized interpretation of structural changes related to a gram of liver and to the liver (Figure 7.55).

Normalized Data (NORM1)
Per Gram



Per Liver



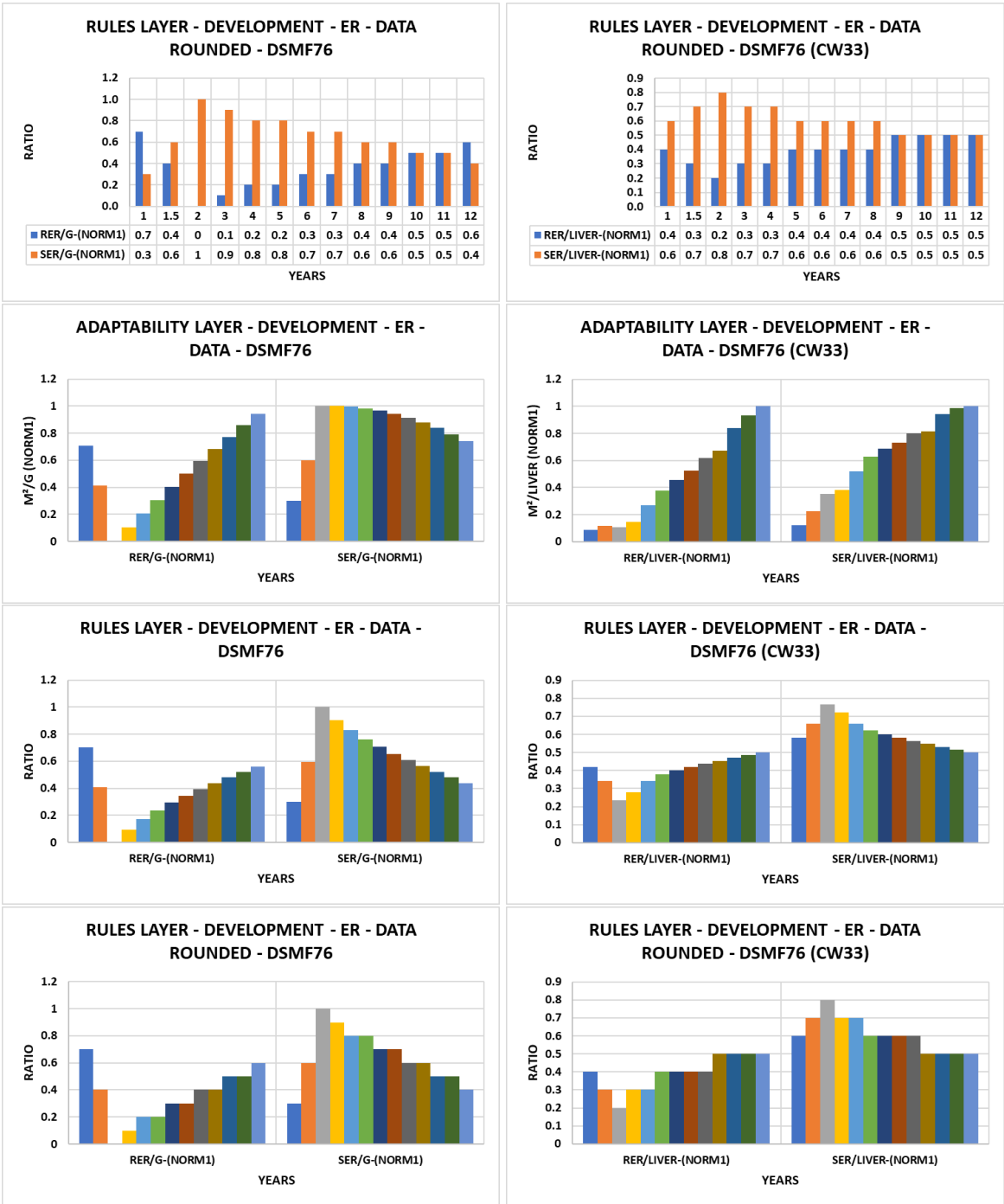


Figure 7.55 When normalized, data related to the gram and to the liver produce different results. In effect, a given dataset can produce different results with multiple interpretations.

A normalized version of the starting original data completes the dataset (Figure 7.56).



Figure 7.56 The normalized version of the original data shows changes in the ER surface area related to the gram and to the liver. Notice that the same data can produce both similar and different results. It all depends on how many hepatocytes can fit into a gram of liver. When hepatocytes change, they can change their average volume (a mere shape change will do it), which changes their cell count in the gram, but typically not the liver (assuming cell numbers remain essentially constant).

Comments: Why connect phenotypes to genotypes? Because we want to know which one is responsible for what at a time when complex problems are being analyzed and solved. Cells, which derive their capabilities from universal principles, no doubt run on mathematical algorithms that define vast systems of interconnected information. Is everything hard coded in modules that can be assembled into solutions or do cells compute their way to optimal outcomes by solving problems computationally step-by-step? Perhaps the noncoding portions of the DNA (e.g., ncRNAs) provide the solutions. At this point of our understanding, we at least know that cells have bridged the gap between classical and quantum mechanics (e.g., photosynthesis and vision). Moreover, it would not be surprising to discover that cells run their information systems in house with quantum devices using a yet to be defined type of cell intelligence (CI). Knowing that cells are smart is one thing, knowing where the smarts come from is something else.

Miscellaneous Studies

7.1.13 Case Study 13: Effects of Food Additives on Hepatocytes – DMF75

Source: Update applied to original data from De la Iglesia F. A., McGuire E. J., Feuer G. (1975) Coumarin and 4-methylcoumarin induced changes in the hepatocytic endoplasmic reticulum studied by quantitative stereology. *Toxicology* 4:305-314.

Topic: Morphological effects of various food additives on the surface areas of RER and SER membranes.

Update: Apply corrections, expand data, report results in adaptability and rules layers, and analyze patterns.

Dataset: Morphology: ER (RER, SER).

De la Iglesia et al., (1975 – DMF75) studied the effects of food additives on the surface areas of the SER and RER. The update looks at the results before and after applying the section related corrections. Figure 7.57 adds the ratio data to support the conclusion that the food additives had no effect on the RER and SER membranes of hepatocytes.

Original Data (Uncorrected)



Original Data (Corrected)

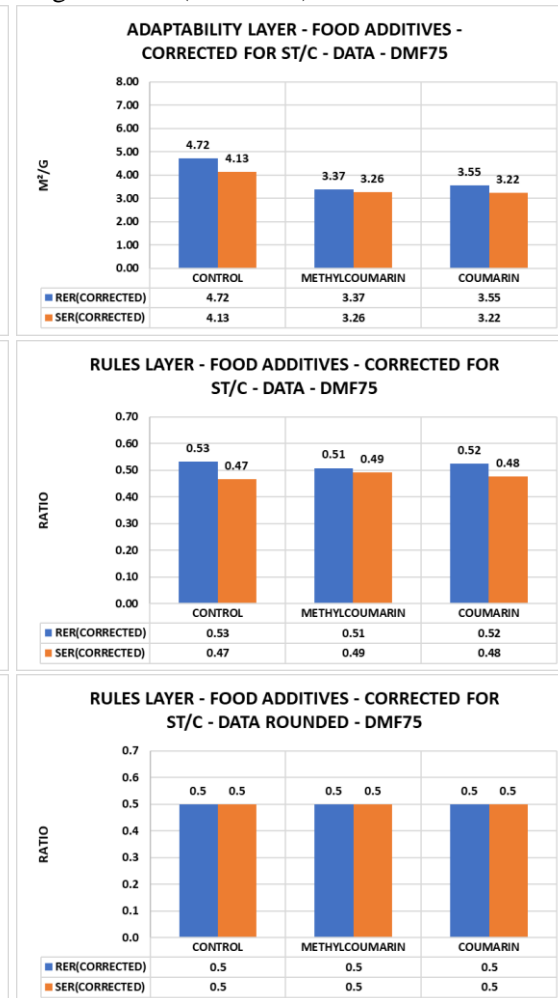


Figure 7.57 Without the corrections for section thickness and compression, the original results produced notably different data and results.

7.1.14 Case Study 14: Aging of Centrolobular Hepatocytes – SJM74

Source: Update applied to original data from Schmucker D. L., Jones A. L., Mills, E. S. (1974) *Morphometric analysis of the ultrastructural changes in the liver of aging rats. J Gerontology* 29:506-513.

Topic: Aging in the central cells of the liver lobule.

Update: Apply corrections, report results in adaptability and rules layers, and analyze results.

Dataset: Surface area estimates for the RER and SER in centrolobular hepatocytes.

Updating the study of Schmucker et al. (1974) included making corrections (section thickness and compression) and adding new data for the rules layer (Figures 7.58, 7.59). Results from centrolobular hepatocytes in the rules layer showed that the ratio of RER to SER in young adults (0.4:0.6) changed to (0.5:0.5) in the retired breeders. Such a shift appears to be a characteristic of aging.

Original Data

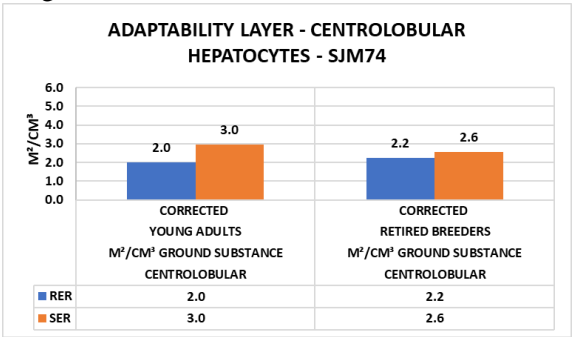


Figure 7.58 In the adaptability layer, aging affects the relative amounts of RER and SER in centrolobular hepatocytes.

Rules Layer (Ratio Data)

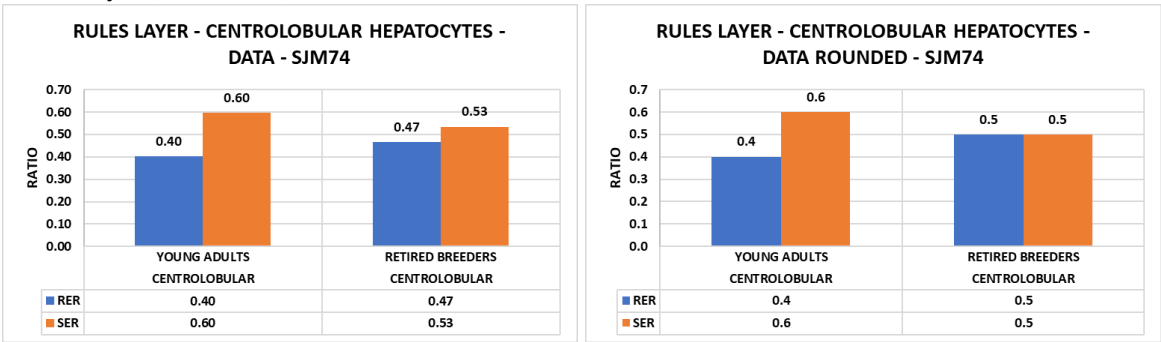


Figure 7.59 Results from centrolobular hepatocytes in the rules layer showed that the ratio of RER to SER in young adults (0.4:0.6) changed to (0.5:0.5) in the retired breeders. Such a shift appears to be a quantitative marker of aging.

A later publication (Schmucker et al. 1978 – SMJ78) reproduced the results of the current study using data from the rules layer (Figure 7.60).

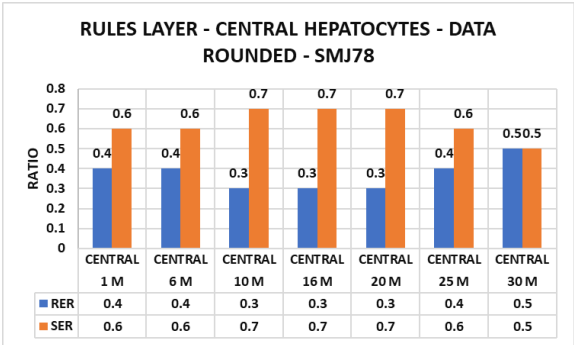


Figure 7.60 Thirty-month-old adults duplicated the results shown in Figure 7.59.

Comment: We can use the ratios of the rules layer to demonstrate reproducibility across publications.

CHAPTER 8

PHENOTYPE – LIVER LOBULE

Summary

The arrangement of hepatocytes in liver lobules exposes them to different gradients of oxygen and nutrition, which can affect relationships of cell structure to function in health and disease. From an experimental perspective, the setting is complex because hepatocytes can respond differently according to (1) exposure, (2) timing, and (3) position within the liver lobule. After updating the published data to understand the problems and imagine solutions, a model-based approach to separating cells mathematically seemed a logical next step.

8.1 Lobular Hepatocytes

The liver includes several lobes each consisting of hexagonal lobules. Blood enters a lobule at its periphery (portal) through branches of the hepatic artery and portal vein and exits through a central vein at the center of each lobule. Hepatocytes arranged in parallel with the vascular elements exchange constituents with the blood. As the blood courses through the lobule, hepatocytes alter the composition of the blood and can display different structural and functional properties across the liver lobule. Since the properties of the hepatocytes within the lobule can change in response to local events and aging, detecting and reproducing results remains a challenging task when working with incomplete datasets.

Given the positional relationship of structure to function within the liver lobule, the challenge of the experimental model becomes one of associating the total liver activity of an enzyme with two or more subpopulations of hepatocytes. Using a first principles approach, one can approach such a problem with a model based on writing and solving pairs of linear equations simultaneously. Knowing when, where, and under what conditions to look for lobular changes becomes a key part of the solution.

Since stereological methods can provide detailed structural data consistent with a first principles approach, we can use morphology as a mathematical platform for detecting and interpreting complex biological changes. Recall that stereological data come from electron micrographs of intact tissue whereas biochemical data derive from tissue homogenates and tissue fractions such as microsomes. First, we'll look at the lobular heterogeneity of hepatocytes and then suggest an experimental model designed to allocate enzyme activities to structural and functional subpopulations of hepatocytes defined by their organelles.

8.1.1 Case Study 1: Lobular Hepatocytes – L68

Source: Loud A. V. (1968) A quantitative stereological description of the ultrastructure of normal rat liver parenchymal cells. *J Cell Biol* 37: 27-46.

Topic: Morphological comparison of membranes in the periportal, midzonal, and central hepatocytes of the liver lobule.

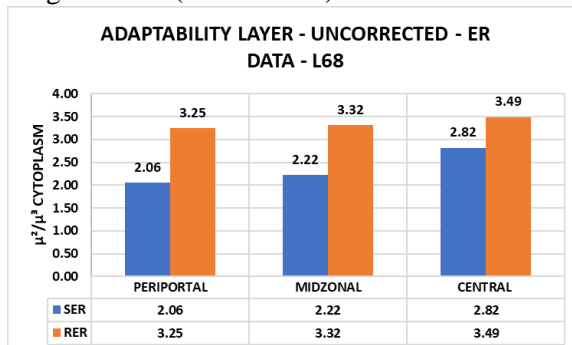
Update: Apply corrections, report results in adaptability and rules layers and analyze the results.

Dataset: Morphology: ER (RER, SER), mitochondria (MIM, OMIM, IMIM).

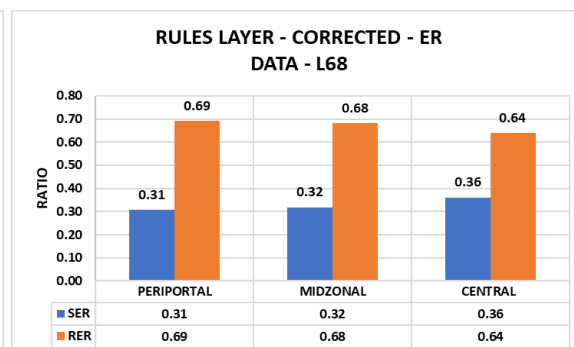
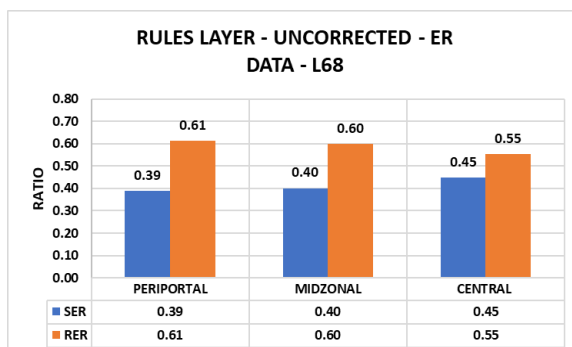
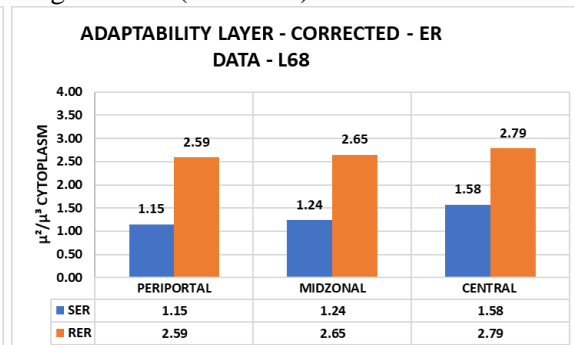
Hepatocytes can display heterogeneous properties according to their position within the liver lobule. In this study, Loud (1968; L68) described three zones including peripheral (portal), midzonal, and central - arranged functionally in order of the blood flow. After applying corrections in the adaptability layer for section thickness and compression (Weibel and Paumgartner, 1978), the update calculated ratios for the membrane organelles in the rules layer. Note that the rats were five months old.

The updated data in Figures 8.1, 8.2, and 8.3 illustrate the effects of correcting stereological estimates for biases (section thickness and compression) in adaptability and rules layers. Figure 8.1, for example, detected a likely change in the rules layer (corrected) that was otherwise undetected (uncorrected). Moreover, Figure 8.4 offers rule-based evidence that at least two populations of hepatocytes exist in five-month-old rats. Of course, the missing biochemical data might have added a third population.

Original Data (Uncorrected)



Original Data (Corrected)



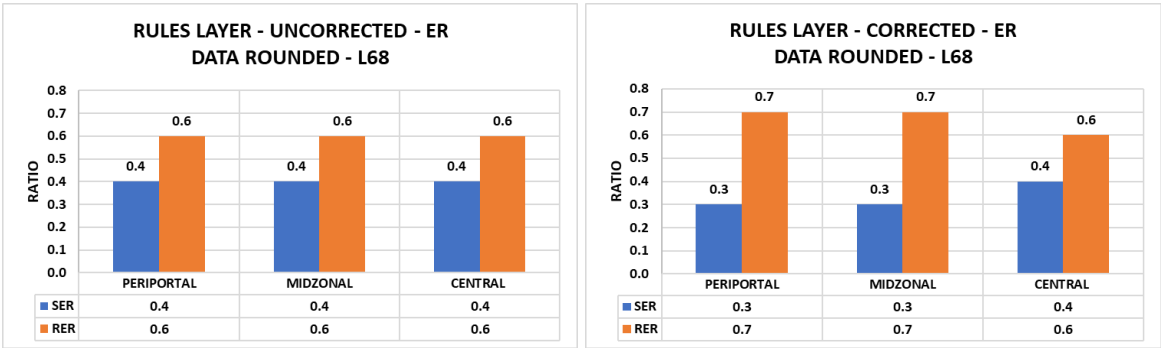
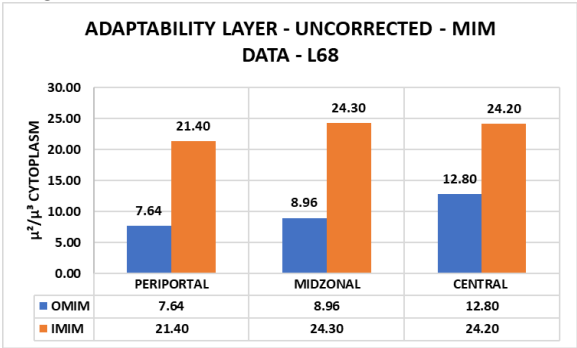


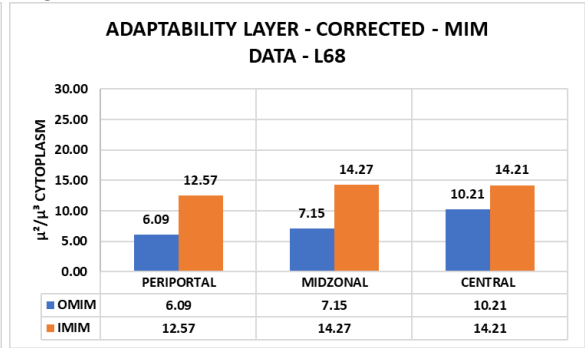
Figure 8.1 Comparisons of ratios calculated from uncorrected and corrected data revealed noteworthy differences in the results. Instead of similar proportions of SER:RER membranes (4:6, 4:6, 4:6) across the three lobular zones, the corrected data found a distinctly different pattern (3:7, 3:7, 4:6). Moreover, the corrected data in the adaptability layer detected considerably reduced amounts of membrane surface areas with larger differences.

The mitochondrial membranes showed a similar pattern (Figure 8.2). Uncorrected data gave one result (3:7, 3:7, 3:7) whereas corrected results another (3:7, 3:7, 4:6).

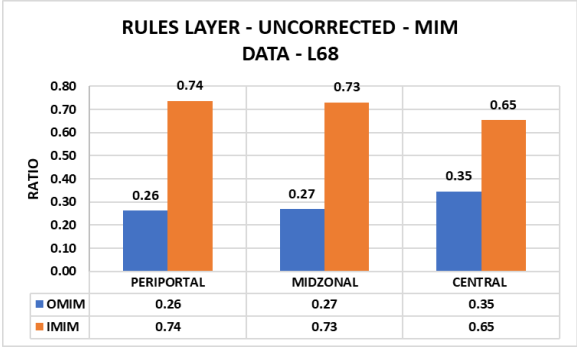
Original Data (Uncorrected)



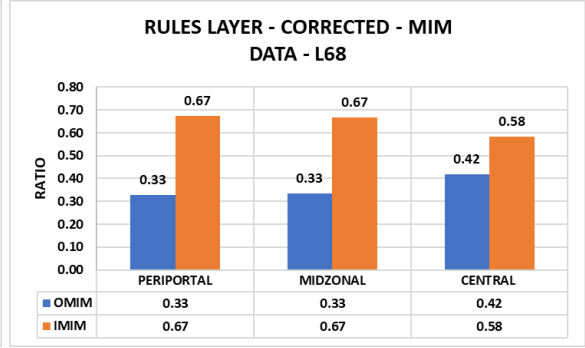
Original Data (Corrected)



Rules Layer (Uncorrected)



Rules Layer (Corrected)



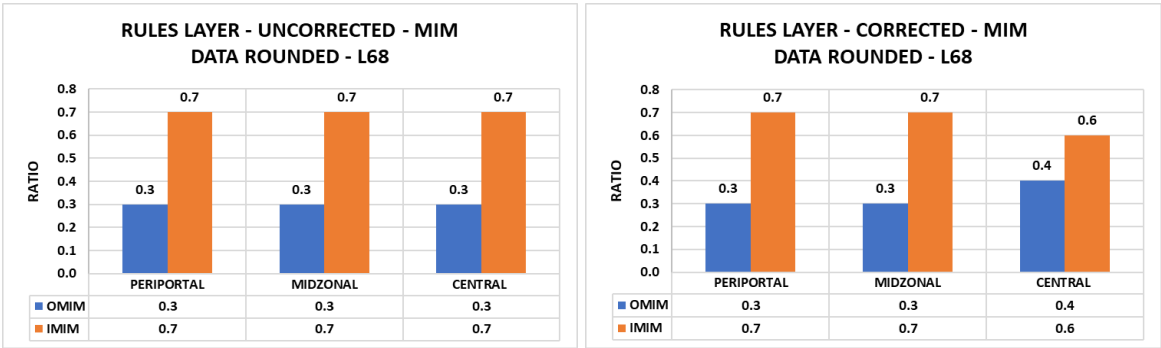


Figure 8.2 In the adaptability layer the mitochondrial membranes produced the expected differences between the corrected and uncorrected results, but the corrected data detected a difference in the ratio of the corrected central hepatocytes.

Using the rules layer, Figure 8.3 summarizes the structural properties of the lobular hepatocytes.

Rules Layer (Corrected for Section Thickness and Compression)

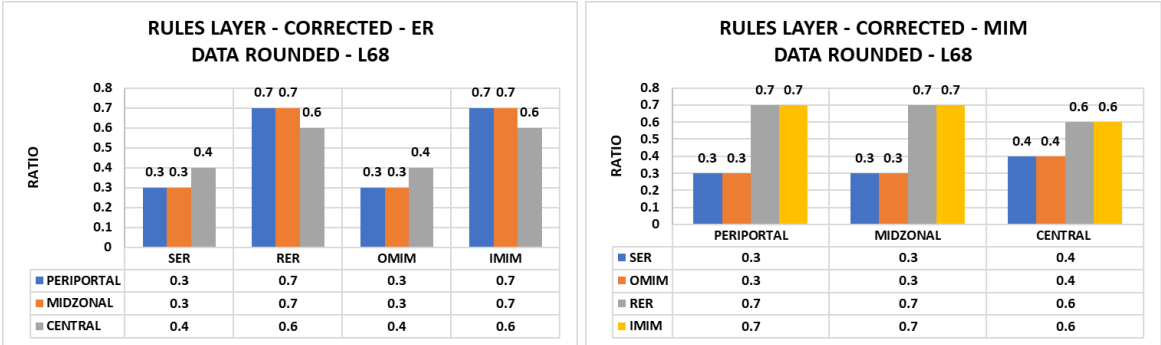


Figure 8.3 Combining the results summarizes the update. The periportal and midzonal hepatocytes use the same membrane ratio rules (0.3:0.7), while the central cells apply a different one (0.4:0.6). In addition to rules existing within the ER (SER:RER) and MI (OMIM:IMIM), rules also exist between membrane compartments (SER:OMIM and RER:IMIM). The SER and OMIM share one set of rules (0.3:0.3) while the RER and IMIM share a different one (0.4:0.6). This pattern of a quantitative linkage between ER and mitochondrial subcompartments appears often but not always.

Given the variability of the structural and functional events occurring across the liver lobule, how do the hepatocytes manage the complexity? Do they preserve order by applying basic design principles? Apparently yes. If we rearrange the organelles, they appear to be using a design strategy based – once again - on mirror image symmetry (Figure 8.4). Complexity embedded in complexity becomes patterns embedded in patterns, which extend in both directions hierarchically.

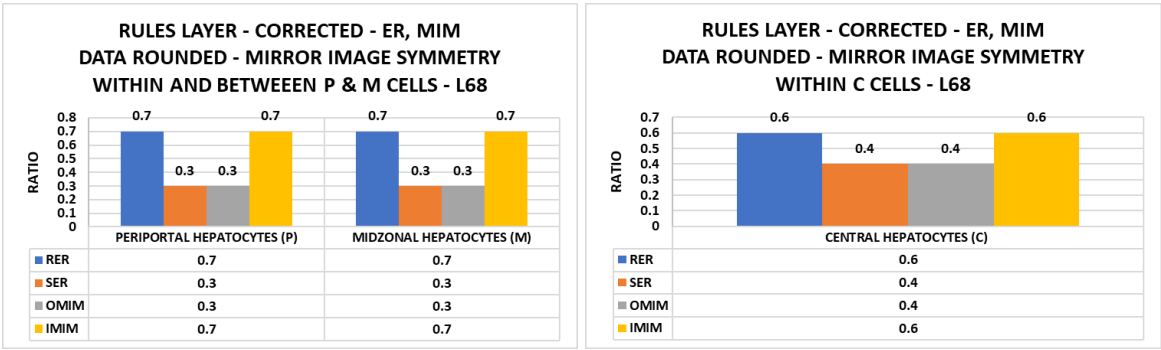


Figure 8.4 Lobular hepatocytes displaying mirror image symmetry suggest a minimalistic approach to design. Notice the pairing of RER (production) with OMIM (energy supply) and SER with OMIM (consistency and physical connectivity between SER and OMIM) .

Comment: Since the periportal (portal) and midzonal hepatocytes share the same ratios (rules) might this suggest that the liver lobule includes just two cell types – portal and central? How might one answer such a question? One could calculate enzyme densities for all three cell types – in control and experimental settings – to determine if the portal and midzonal cells give similar or different values.

8.1.2 Case Study 2: Lobular Cells in Development (Phenobarbital) - KKAOW84

Source: Kanai K., Kanamura S., Asada-Kubota M., Watanabe J., Oka M. (1984) Quantitative analysis of smooth endoplasmic reticulum proliferation in hepatocytes of early postnatal and adult mice treated with phenobarbital. *Gastroenterology* 87: 1131-37.

Topic: Comparison of ER membranes in the periportal and central hepatocytes during early development and exposure to phenobarbital.

Update: Apply corrections, report results in adaptability and rules layers and analyze the results.

Dataset: Morphology: ER (RER, SER).

The authors of this study (Kanai et al., 1984; KKAOW84) gave the hepatocytes two problems to solve at the same time – undergo development and respond to the drug (phenobarbital). By updating the study, we can use the membrane data to view the patterns associated with two subgroups of lobular hepatocytes, namely periportal (P) and periventricular (C). In the original publication, P stands for periportal (or portal) and V for periventricular (or central). The terminology used by the primer includes P for portal hepatocytes and C for central hepatocytes. Figure 8.5 includes the original data uncorrected and corrected for the section related biases.

Original Data (Corrected and Uncorrected)

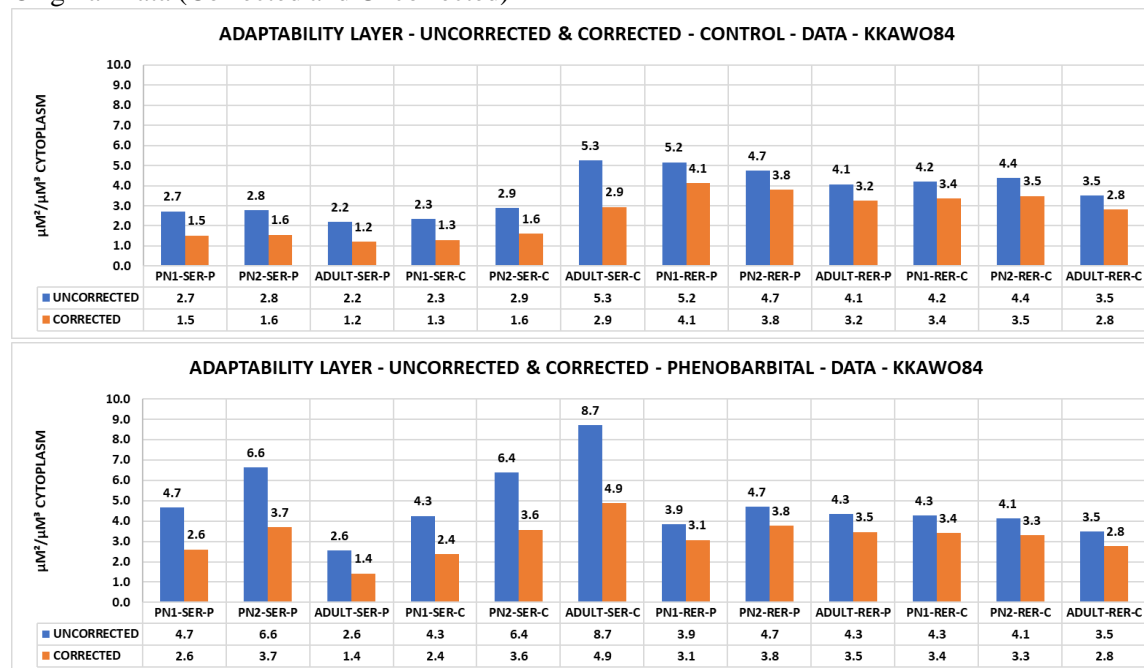


Figure 8.5 The two figures above show results before and after applying corrections for section thickness and compression.

When plotted as curves for both layers (adaptability and rules), the differences between the two cell phenotypes (portal and central) become apparent (Figure 8.6). At postnatal days 1 (PN1) and 2 (PN2), the ratios of RER and SER in the portal and central cells displayed similar patterns. In the phenobarbital (PB) treated animals, however, the ratios for PN1 and PN2 differed for the central hepatocytes, a pattern that persisted in the adult cells. The RER and SER of both portal and central cells responded similarly to PB. In the adult animals, the data suggest that the response to PB occurs principally in the SER of the central hepatocytes. In effect, the update duplicates the findings of the original study. Without the

enzyme density data, however, the biochemical contribution to the membrane changes remains an open question.

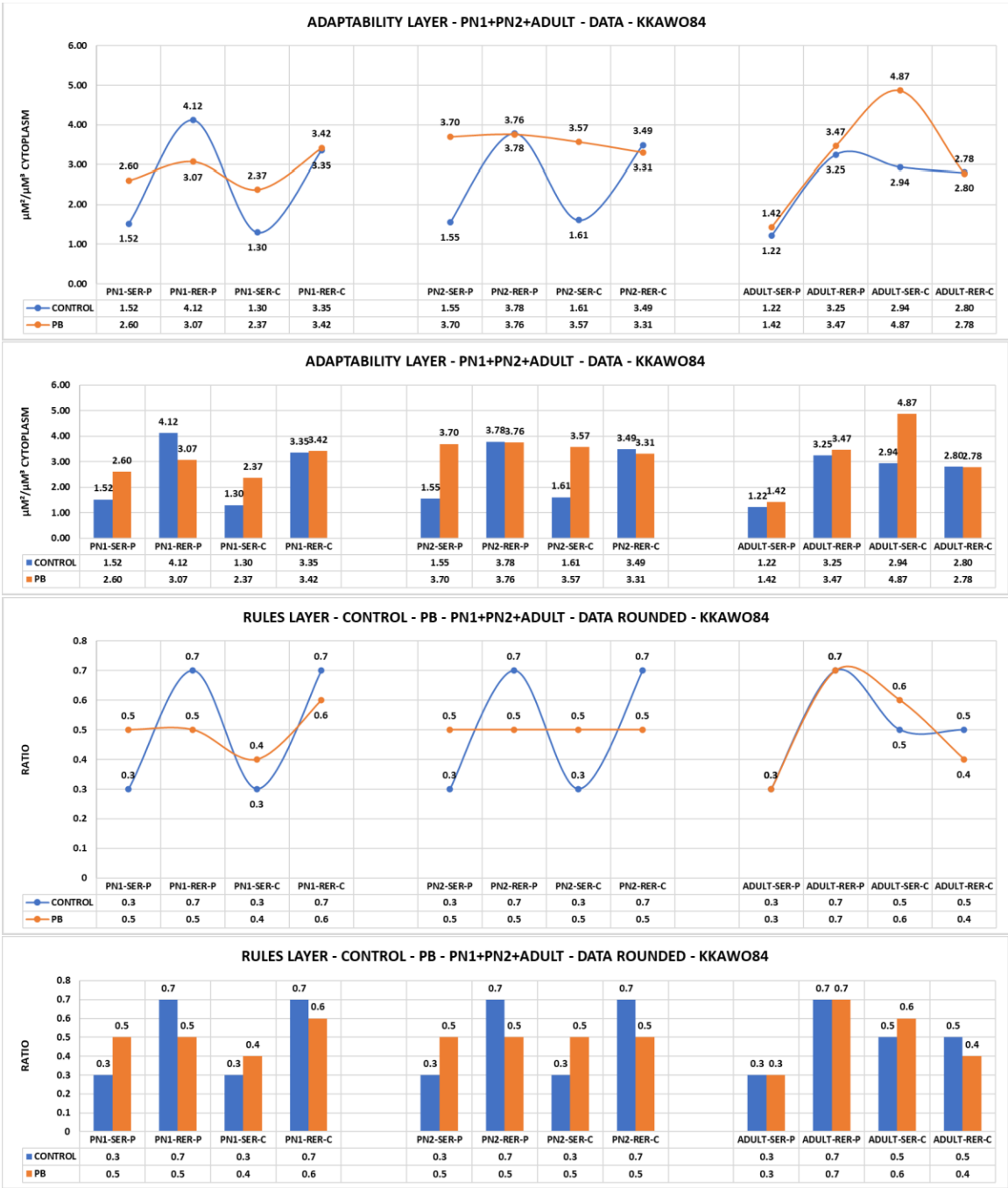


Figure 8.6 When plotted as curves for both layers (adaptability and rules), the differences between the two cell phenotypes (portal and central) become easier to compare. At postnatal days 1 and 2, the amounts of control RER and SER in the portal and central cells were similar. In the phenobarbital (PB) treated animals, a brief difference appeared in the central cells at PN1, which disappeared at PN2. In the adult animals, the data suggest that the response to PB occurred principally in the central hepatocytes. In short, the update duplicated the findings of the original study. Without the enzyme density data, however, the biochemical contribution to the changes in the membranes remains unknown.

Figures 8.7 and 8.8 summarize the results.

Rules Layer (Updated Data)

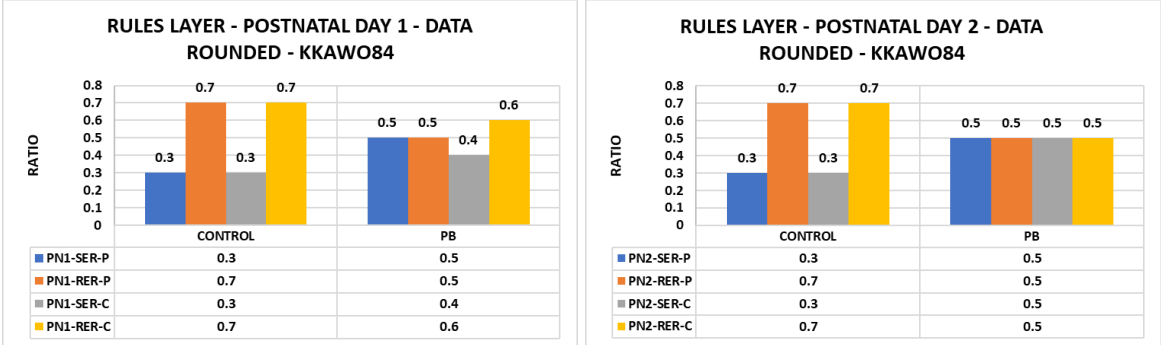


Figure 8.7 At postnatal days 1(PN1) and 2(PN2), the patterns of the ratios persisted for the controls, but symmetry didn't appear in phenobarbital (PB) treated animals until day two.

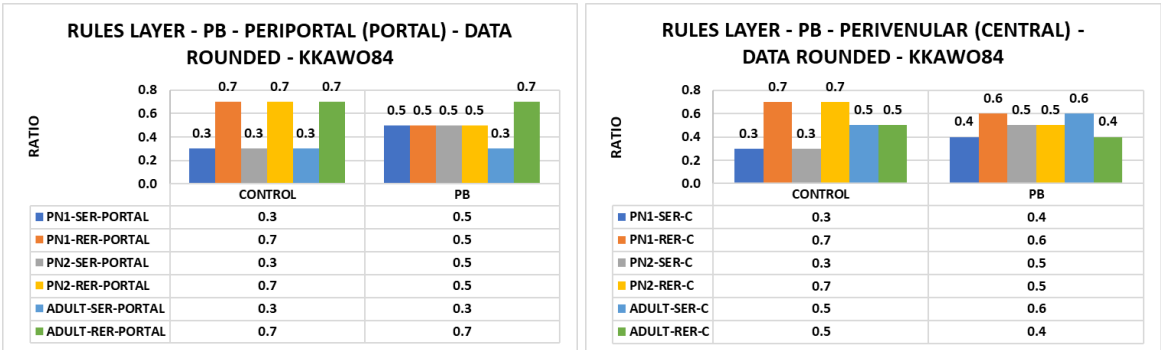


Figure 8.8 By including the adult data, the central hepatocytes treated with PB displayed a pattern consistent with mirror image symmetry. In this case the symmetry appeared as the result of the RER and SER switching ratios in the adult.

Summary: (1) Early development (PN days 1, 2) in the RER and SER showed similarities that turned into differences in the adult. (2) Phenobarbital exerted its greatest effect on the SER in the central hepatocytes of adults. (3) When combined, the PB dataset displayed mirror image symmetry marked by a single ratio switch.

8.1.3 Case Study 3: Isolated Lobular Hepatocytes (\pm Phenobarbital) – WDMPP75

Source: Wanson, J. C., Drochmans P., May C., Penasse W., Popowski A. (1975) Isolation of centrolobular and perilobular hepatocytes after phenobarbital treatment. *J Cell Biol* 66: 23-41.

Topic: Comparisons of biochemical constituents from hepatocytes isolated from livers exposed to phenobarbital.

Update: Apply corrections, report results in adaptability and rules layers, analyze patterns, and report results.

Dataset: Biochemistry: Protein, DNA, RNA, Glycogen, and G6PASE.

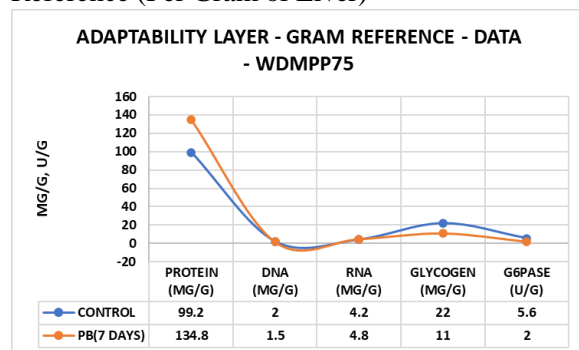
Wanson et al., (1975, WDMPP75), isolated hepatocytes physically into portal and central cells with the goal of characterizing the responses of each cell type to phenobarbital (PB) treatment. In addition, the authors included a biochemical analysis of the original collection of isolated hepatocytes before separating them into two cell types. Given the range of the published data, the update of this study offers an opportunity to explore answers to basic questions about our experimental methods.

When treated with phenobarbital, for example, hepatocytes responded by generating a new type of ER designed specifically to metabolize the drug. Since exposure to phenobarbital increased hepatocytic volumes, fewer of them fit into a gram or cm^3 of liver. Consequently, the data and the data reference become joined together as a concentration (data/reference) wherein both numerator (response to the drug) and the denominator (the number of cells filling a standard weight or volume) changed simultaneously. The consequence? Trying to detect changes and reproduce results correctly without the benefit of a constant reference creates a high-risk setting for an experiment.

The solution? By switching from relative concentrations (per gram) to absolute values (per liver), we can avoid the ambiguity and disorder created by the unstable references. Although a simple fix to a widespread problem, it's rarely applied to morphological and biochemical results. The point? Understanding the basic principles of designing experiments in biology begins by understanding two sets of interacting changes - how living systems change and how our methods change what we think we measure. Using the updated results of this study, we will consider solutions to the problems created by our methods. When plotted, for example, the gram and liver data types first appear to be detecting the changes similarly (Figure 8.9).

Original Data

Reference (Per Gram of Liver)



Reference (Per Liver)

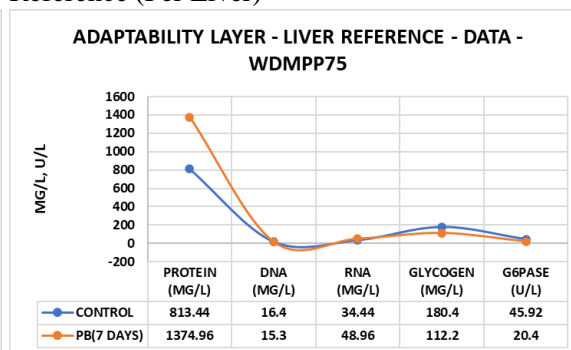


Figure 8.9 We have five parts collected from the liver including PROTEIN, DNA, RNA, GLYCOGEN, and G6PASE – related to a gram of liver and the liver. The update considers what happens to the same changes when related to different references.

If we express the results relative to the liver, we get as close to the correct results as our current methods allow by avoiding the cell packing error that occurs in response to the phenobarbital treatment (Figures 8.10 and 8.11).

We have two ways to observe (and correct) the effects of losing hepatocytes from a gram of liver.

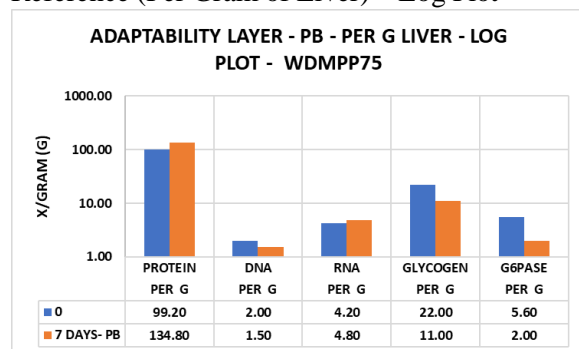
Method 1 (using liver weights): The phenobarbital treatment increased the liver weight by 25% presumably because the hepatocytes increased their average weight (or volume) by about 25%; control liver weighed 8.2 grams (100%) and the PB treated 10.2 grams (125% ↑). This means that we would expect the gram of liver reference to underestimate the true (liver) values by about 25%.

Method 2 (using DNA assays): Figure 8.10 shows that the PB treated hepatocytes lost 25% of the DNA per gram of liver (Control = 2, PB = 1.5), whereas the same DNA per liver remained essentially the same (Control = 16, PB = 15). This means that the gram of liver reference underestimated the true (liver) value by 25%. Both methods led to the same result.

The point? When publications compare results related to a gram of liver (or to mg of protein), the authors assumed that the data reference remained constant (contained the same number of hepatocytes). By not checking the results by comparing them to the same data related to the liver, they may have reduced the reliability of their results. When evaluating changes with the stereology literature database, the same data - expressed as concentrations or absolute values - agreed only about 50% of the time (Bolender 2019).

The following figures compare control to PB treated animals when both the parts and references change. We know that a gram of data coming from PB treated animals has about 25% fewer hepatocytes, but that the PB has changed the absolute and relative amounts of the parts.

Reference (Per Gram of Liver) – Log Plot



Reference (Per Liver) – Log Plot

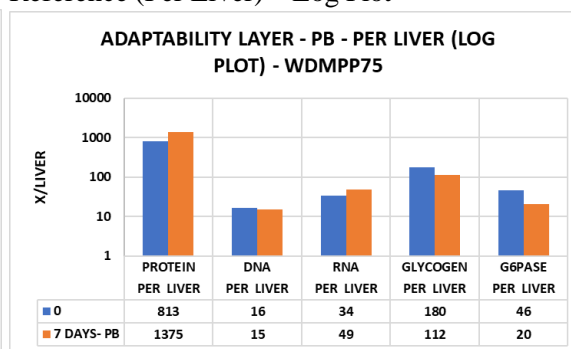
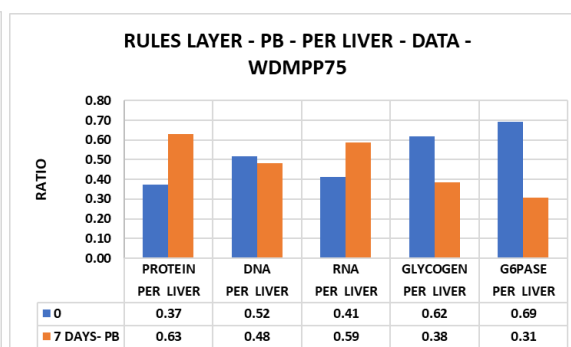
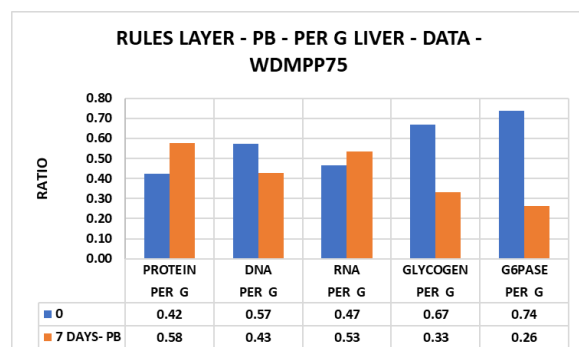


Figure 8.10 When treated with phenobarbital, hepatocytes respond by making new ER membranes designed specifically to neutralize the drug. The log plot accommodates the range of the data.



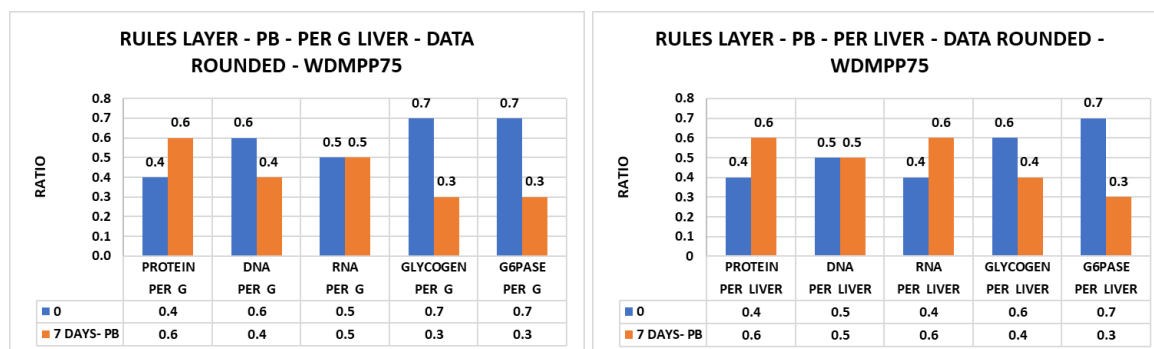
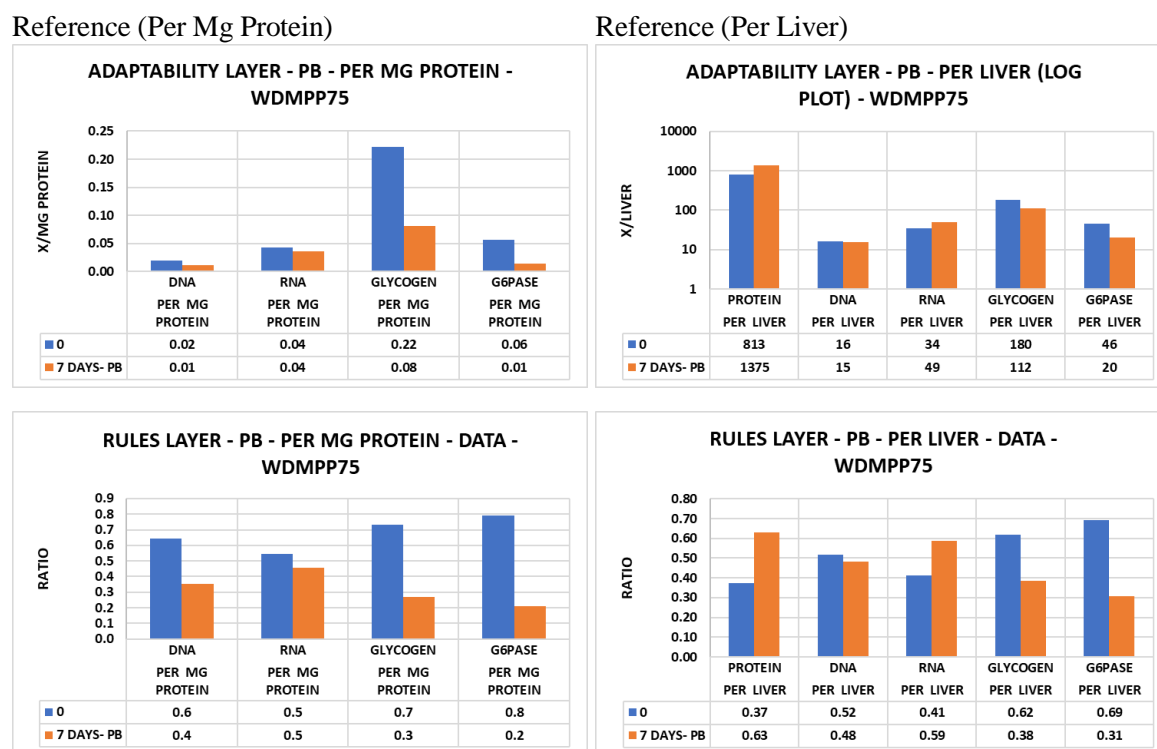


Figure 8.11 The rounded data plots interpret the results by comparing data ratios (gram vs liver). (1) G6PASE ratio was the same for both the gram and the liver. (2) Protein per gram and per liver remained the same. (3) Everything else differed.

If we relate the parts to a mg protein, we can compare the same changes related to protein and to the liver (Figure 8.12). When expressed as ratios, none of the changes related to a mg of protein resemble those related to the liver. The protein content of the liver and the average hepatocytes increased by 69% but the mg protein per gram increased by just 36%, which differ by 33% - a value somewhat larger than the 25% lost to the cell packing problem. Note that experiment used both cell isolation and centrifugation techniques.



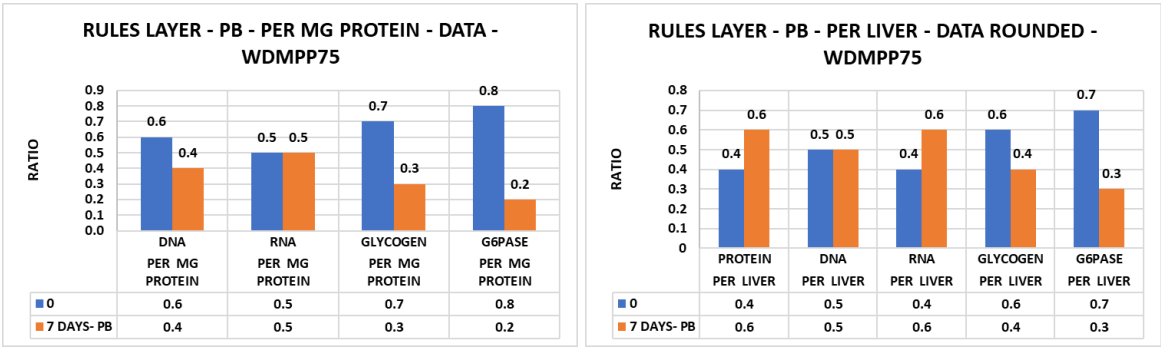


Figure 8.12 Compared to the liver, the mg protein reference detected notably different changes in both the adaptability and rules layers.

If we look at the changes detected by three data references shown above, striking differences appear in the amount of change detected (Figure 8.13).

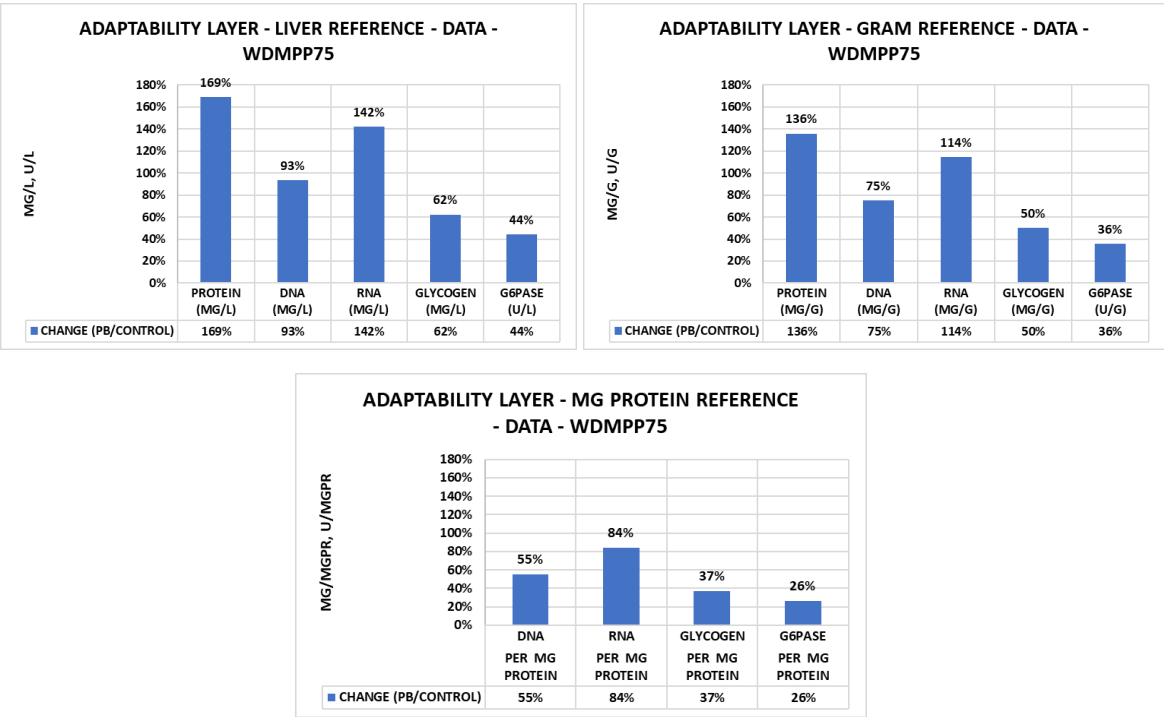


Figure 8.13 Changes in the same data related to different references produced notably different results. The data references most often published (g and mg protein) carry the most bias. To detect a change in a part, use the liver as a reference.

Comment: Different data references produce different results, which ultimately lead to different interpretations. When using the biology literature to construct large information models, pursuing a first principles approach to detecting biological changes requires an understanding of how cells change by rule. Unfortunately, the rules often become buried under the methods.

8.1.4 Case Study 4: Effects of Aging on Central and Portal Hepatocytes – SMJ78

Source: Update applied to the original data from Schmucker, D.I., Mooney, J.S., Jones, A.L. (1978) Stereological analysis of hepatic fine in the Fischer 344 rat - Influence of sublobular location and animal age. J Cell Biol 78: 319-337.

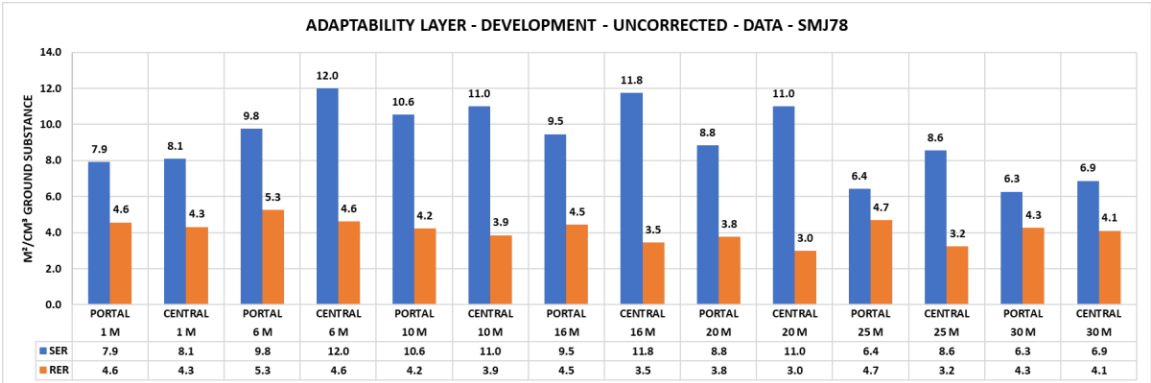
Topic: Morphological changes in lobular hepatocytes with aging – 1 to 30 months.

Update: Apply corrections, expand data, report results in adaptability and rules layers, normalize data, and analyze the results.

Dataset: Morphology: ER (RER, SER).

Aging in hepatocytes occurs as a progression of complex changes involving structural, functional, and lobular events. The update of this study (Schmucker et al., 1978; SMJ78), which begins by expanding the corrected dataset, uses normalized data to look for the changes (1) in the RER and SER of portal and central hepatocytes and (2) in the relationship of the RER to SER. Figure 8.14 illustrates the datasets before and after correcting the original estimates. The substantial differences between the two datasets emphasizes the importance of applying corrections to stereological estimates for membrane surface areas.

Original Data (Uncorrected)



Original Data (Corrected)

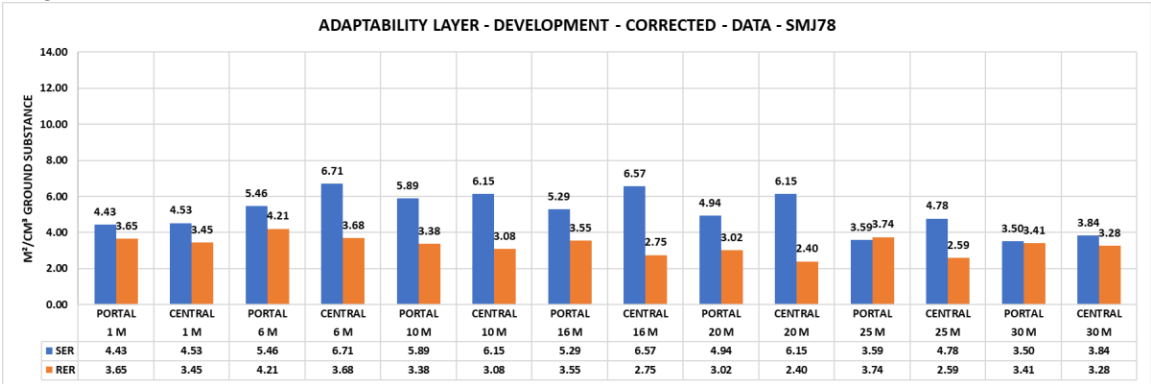
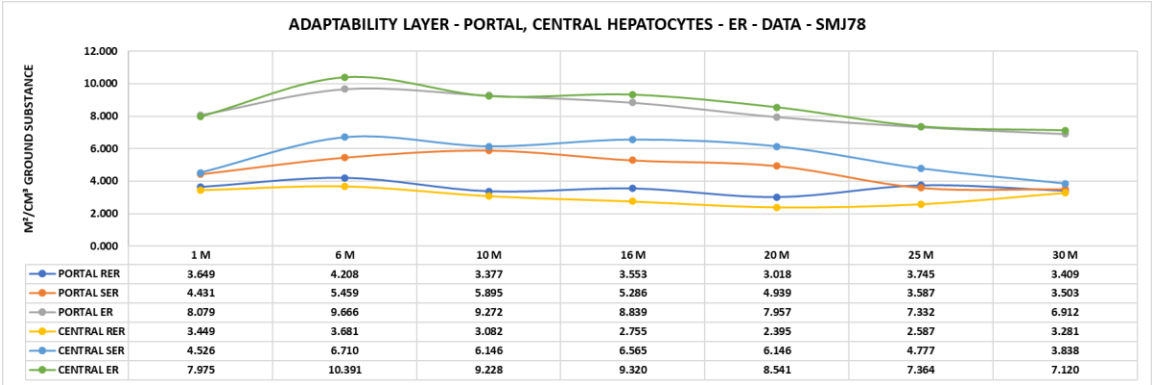


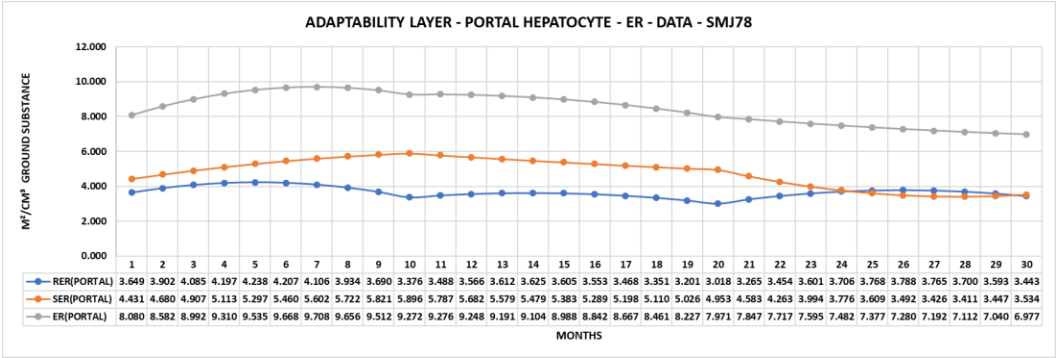
Figure 8.14 The results demonstrate the importance of applying corrections for section thickness and compression when estimating membrane surface areas stereologically.

Next, we expand the original portal and central data (Figure 8.15).

Original Data



Expanded Data (Portal)



Expanded Data (Central)

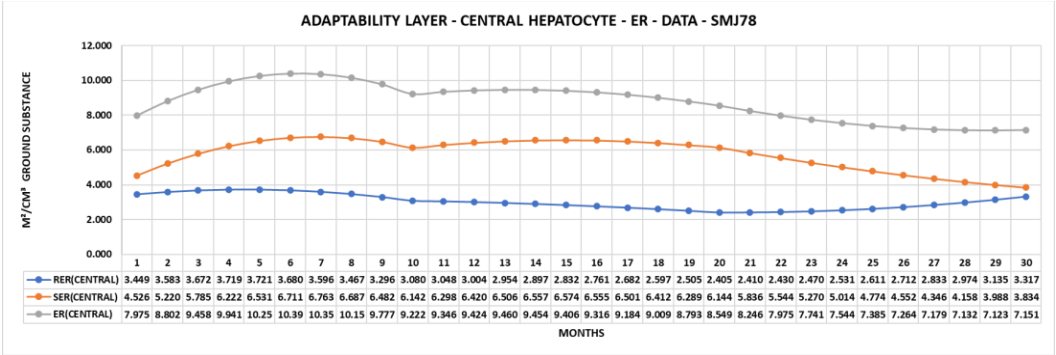


Figure 8.15 Preparing the original data for analysis.

When normalized (Figure 8.16), the similarities and differences between the changes in RER and SER membranes of portal and central hepatocytes become apparent. Notice that using the routines available in EXCEL, fitting the data to curves required multiple regressions to generate curves with $R^2 = 1$.

Normalized Data (NORM1)

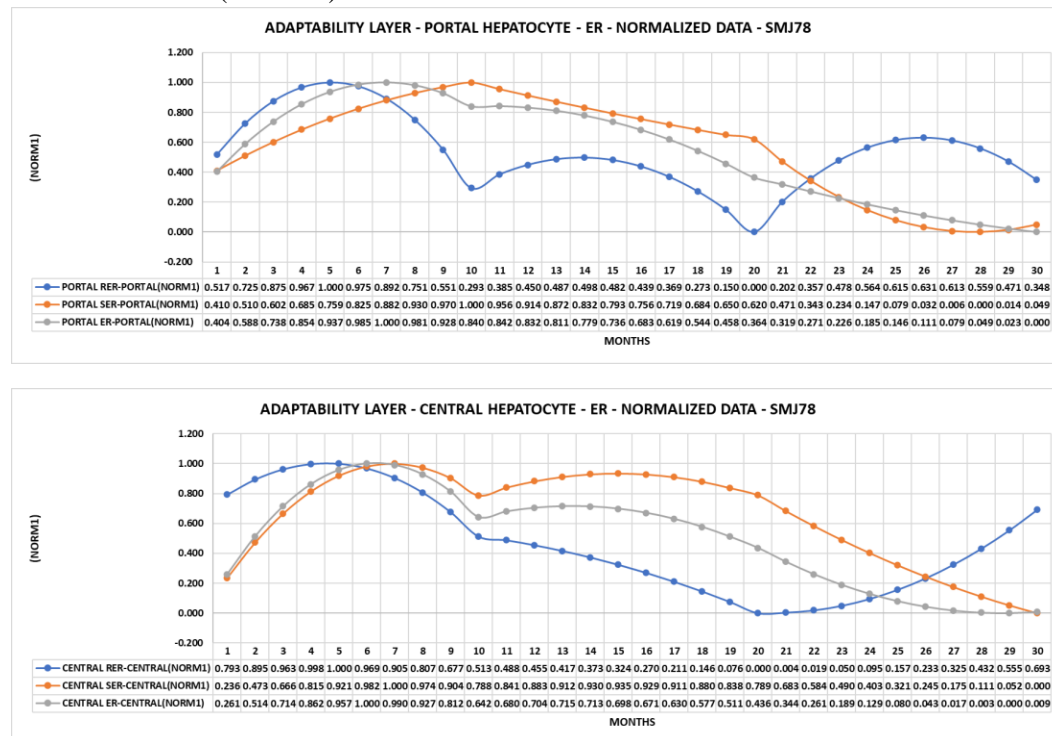
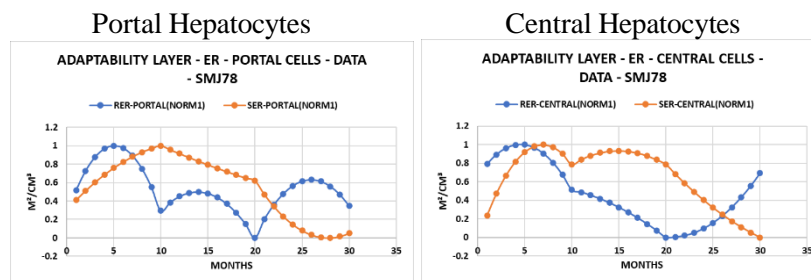


Figure 8.16 Normalized changes in the ER membranes of portal and central hepatocytes displayed similarities and differences between the three membrane compartments.

Starting with the normalized data, we can convert them into ratios and then use the resulting curves to look for patterns of similarity in the rules layer. The patterns show how the relationships of RER to SER changed during the aging process. Since such information quantifies the aging process, the approach might prove useful when testing the effectiveness of treatments designed to mitigate or reverse the effects of aging.

Plotting the ratios of the RER and SER surface areas within portal and central hepatocytes detected largely heterogeneous relationships (Figure 8.17). In effect, intracellular heterogeneity appears to be largely a property of the RER to SER relationship in both portal and central cells. Similarities in the changes of the data pair ratios would appear as superimposed curves – within or across the figures.



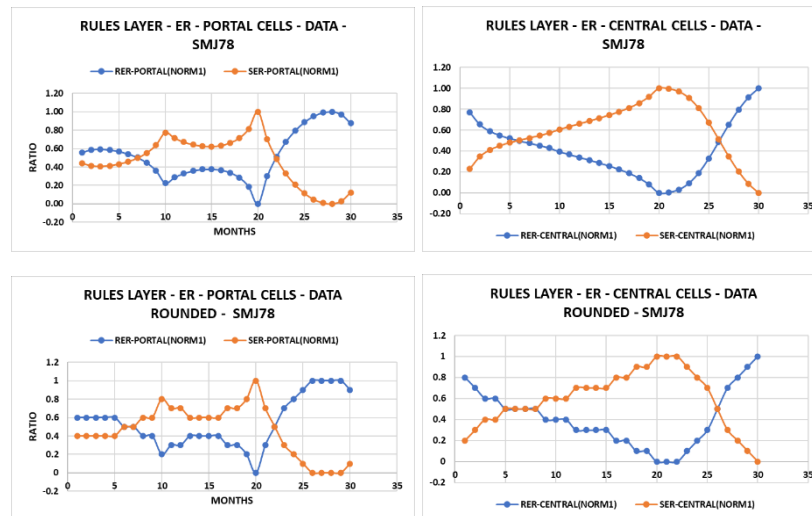


Figure 8.17 Plotting the ratios of the RER and SER surface areas within portal and central hepatocytes detected largely heterogeneous relationships. In effect, intracellular heterogeneity appears to be largely a property of the relationship of RER to SER in both portal and central cells. Similarities appear as overlapping curves.

When combined (Figure 8.18), the data rounded plots of the rules layer (first seen in Figure 8.16) suggest that the RER and SER membranes in portal and central hepatocytes age differently and that the early orderliness gives way to a declining order in old age. Combining the changes in portal and central hepatocytes produced the pattern shown in Figure 8.18).

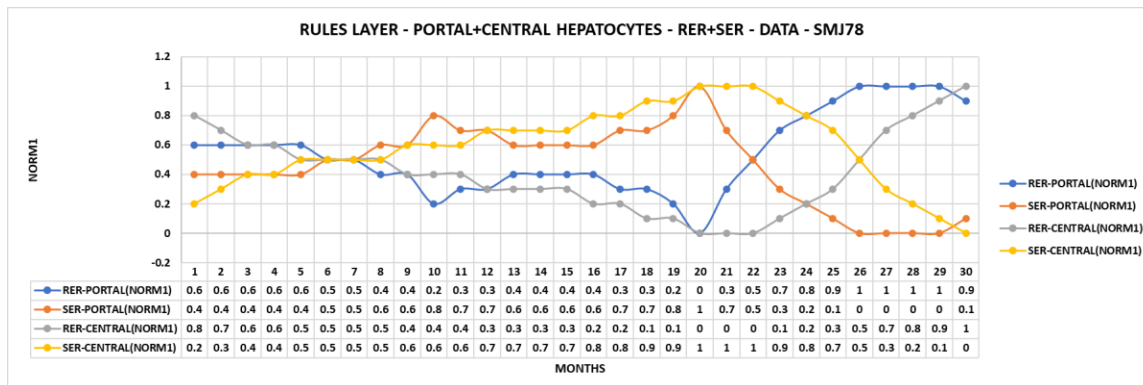


Figure 8.18 Pattern summarizing the changes in portal and central hepatocytes as they age.

The intercellular distribution of the ER in portal versus central hepatocytes, however, tells quite a different story (Figure 8.19). Until the age of 23 months, the relative amounts of ER across the liver lobule remained largely the same. At month twenty-three, however, the ER of the two cell types became abruptly uncoupled: ER began to increase in portal cells and decrease in central cells. In effect, the ratio data seem to be telling us that the hepatocytes have a “switch” at month twenty-three that they use to trigger and accelerate the aging process. If true, then figuring out how to inhibit the switch might serve as an experiential model for delaying the onset of aging. A key point not to overlooked is that the ratios tell us exactly where and when to look for the supposed switch. Since the ER of the central hepatocytes

decreased, one would expect the ratio of the mitochondrial membranes to parallel the increases and decreases of the ER. Was the switch applied to the ER, mitochondria, or both?

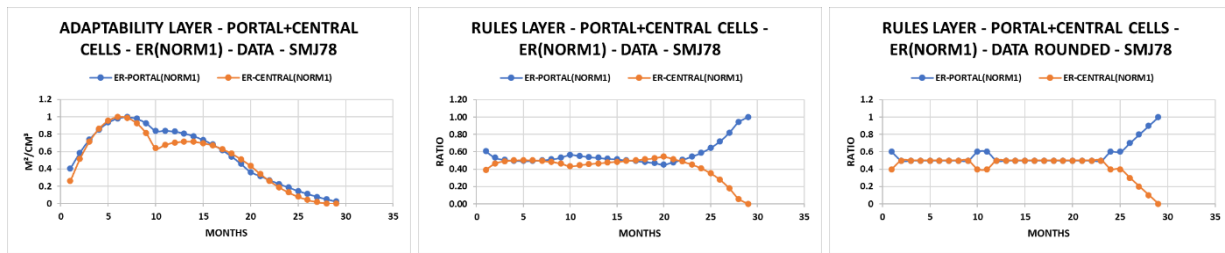


Figure 8.19 Changes in the relationship of ER surface area in portal and central hepatocytes occurred abruptly at 23 months.

By comparing the same membrane in the portal and central hepatocytes, the ratio plots in Figure 8.20 identified parallel changes for the RER and SER membranes (0.5:0.5) until about month fifteen but then the membranes diverged in opposite directions. After day fifteen, the RER increased in portal cells but decreased in central cells; the SER did the opposite. To explain what the hepatocytes were doing, however, we'd need access to the marker enzyme data for the ER and mitochondria. Without the structure-function data, one cannot explain what happened.

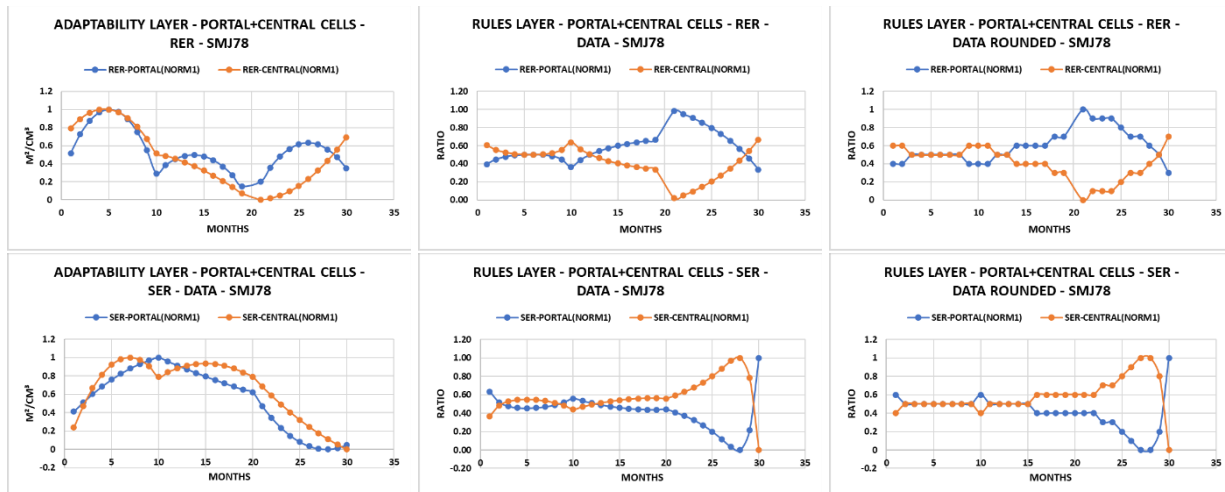


Figure 8.20 Comparing changes in RER of portal and central cells and those of the SER produced two different patterns, thereby identifying quantitatively two distinct populations of hepatocytes. Note the overlapping months showing patterns of similarity.

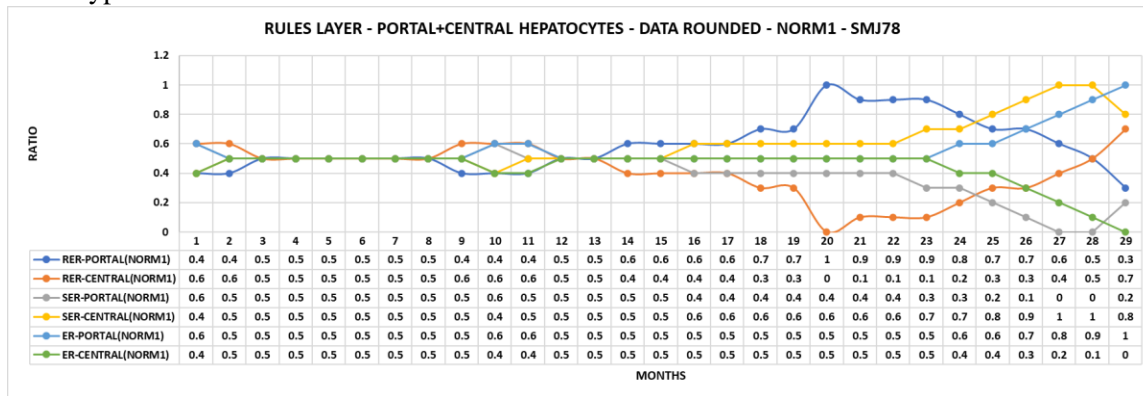
When combined, we can see when the hepatocytes control the changes to RER and SER compartments similarly and differently at the portal and central locations. The point to take from the Figure 8.21 is that the RER and SER membranes in portal and central hepatocytes can be both similar and different. It depends entirely on when and where we look.

The question arises as to how to manage the complexity of the portal and central hepatocytes in terms of their structure and function. The updated results of this study suggest that both homogeneity and heterogeneity characterize the changes in ER membranes and subcompartments (RER and SER) - depending on the timing of an experiment. How the hepatocytes populate these membranes with enzymes to create the all-important enzyme recipes remains unknown. What we do know, however, is that the ER of the portal and central cells change in unison much of the time for the first 23 months. While the

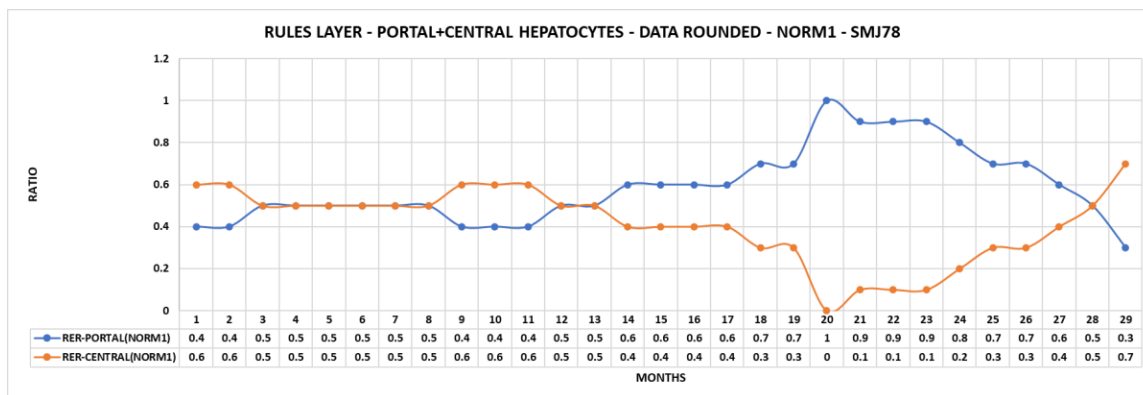
complexity of a cell membrane can be constant (ER) and variable (RER, SER) at the same time structurally, what happens to the biochemical complexity of the membrane? Detecting and explaining a cell change is always a two-part question involving quantitative relationships of structure to function.

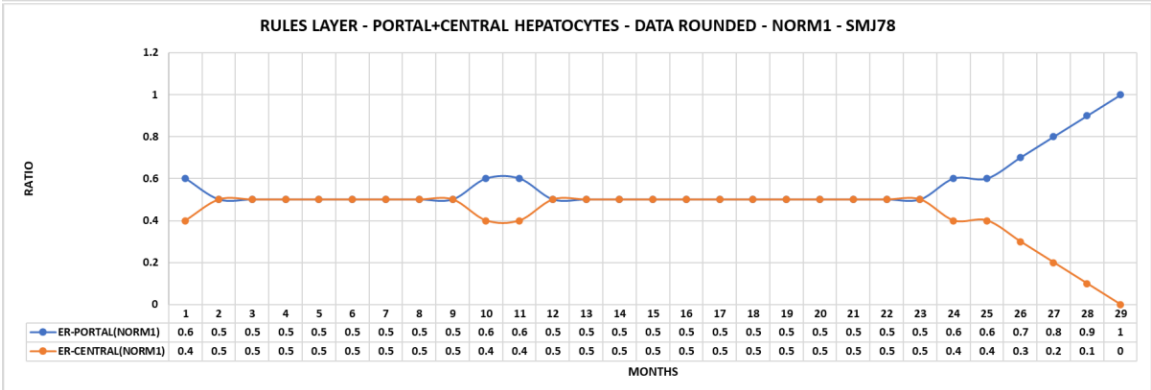
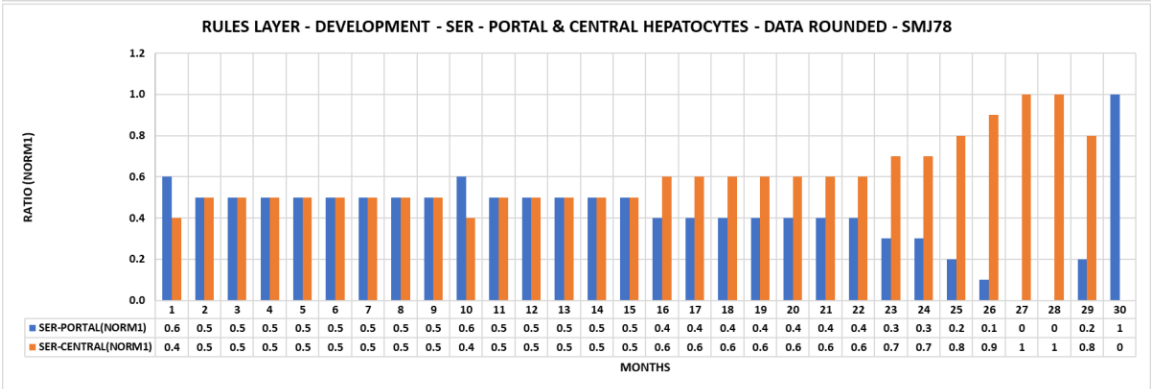
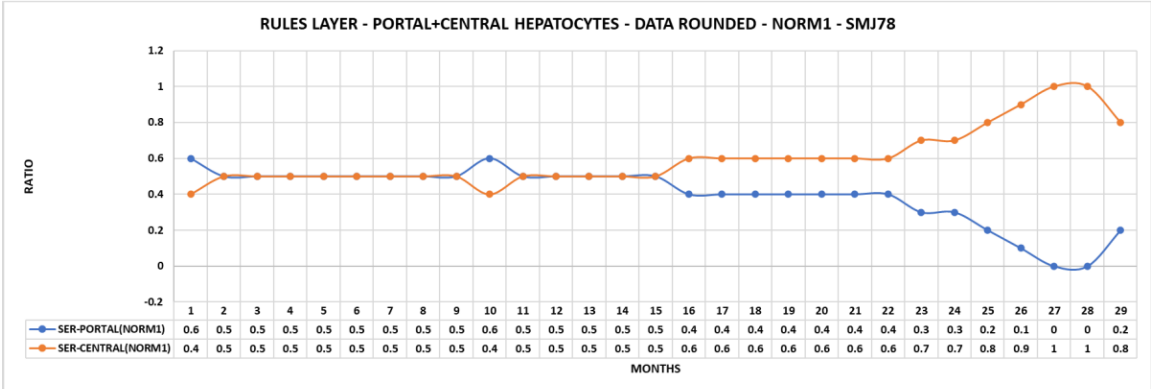
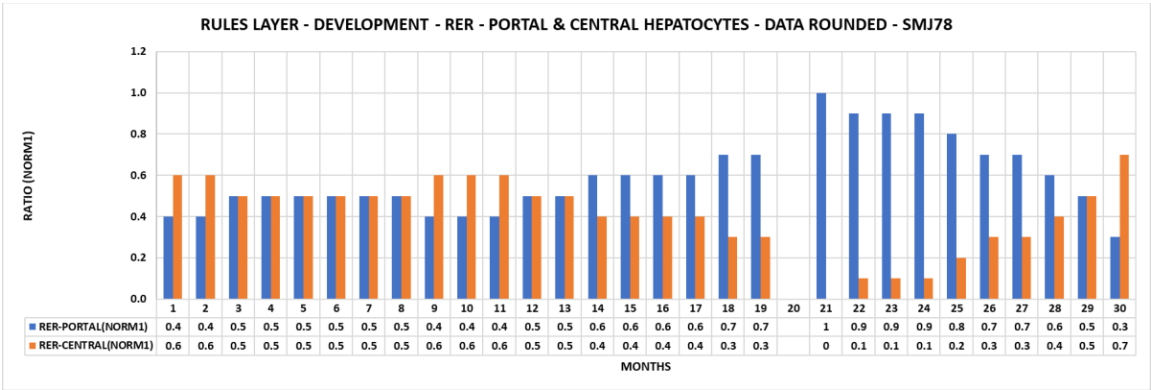
Figure 8.21 summarizes the aging of the ER. Although hepatocytes apply multiple levels of control on the RER and SER membranes across the aging liver lobule, the relationship of the ER membranes in portal and central cells stays largely constant until the end of the 23rd month. Notice the changes in the relationship between the portal and central cells and their RER. Early in life they contribute equal amounts (0.5:0.5), but at month nine the central cells contribute more RER (0.4:0.6). At month fourteen the cells switch roles, and the portal cells take lead (0.6:0.4). But at month twenty, the portal cells contribute practically all the RER to the lobule. After that, the central cells slowly come back and once again contribute RER to the lobule. Since one can attempt to use simultaneous equations to calculate enzyme densities for the ER in the portal and central cells and in turn for the RER and SER in each cell type, the liver should provide a wealth of new information about the basic mechanics of aging.

Phenotype Reconstituted from the Data Pairs



When we take the lobular phenotype apart (reverse engineer it), we can see how the individual parts changed.





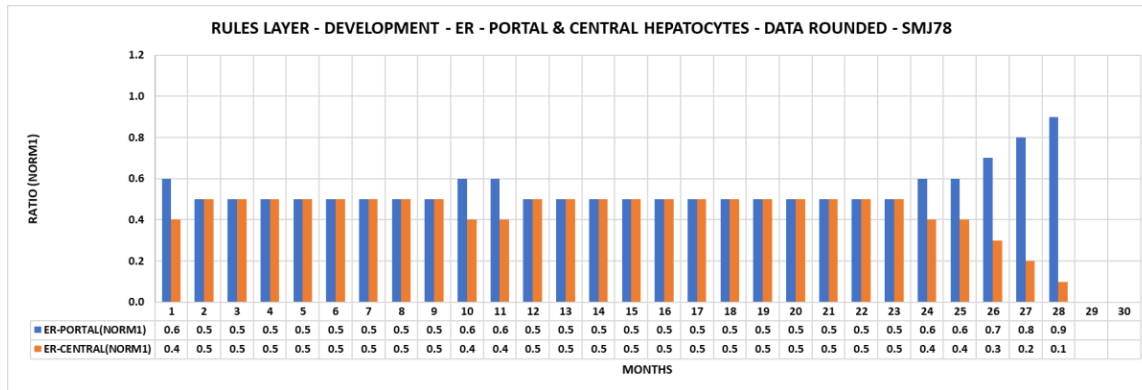


Figure 8.21 Unfolding a phenotype unfolds a change because one is the product of the other. As a problem-solving event, change defines the solution, wherein the solution is the cell. In development, the solution to youth is aging. Changing this solution appears to begin by finding out what happened at 24 months.

Detecting Complex Changes in Portal and Central Hepatocytes: Armed with a better understanding of the aging process across the liver lobule morphologically, how do we associate the biochemical activities of the ER marker enzymes with the ER surface areas in portal and central hepatocytes? In turn, how does one detect the enzyme densities of the ER in the portal and central hepatocytes as they change over time? The following model might prove useful when attempting to answer such questions. Although the model has not been tested empirically, understanding the basics of a cell change allows a researcher to leverage the mathematic logic of cells to assemble and test experimental models. It becomes an exercise in turning theory into practice, which a first principles approach encourages.

8.2 Lobular Model for Portal and Central Hepatocytes

Using data coming from two animals, one can set up pairs of linear equations (of the type, $AX + BY = C$), and then solve them simultaneously. Step 1 identifies the enzyme densities (ED) of the ER in portal and central cells from the ER surface areas and a marker enzyme activity (U/G). Step 2 (optional) looks for differences in the enzyme densities of the RER and ER in portal and central cells. It checks to see if (1) the enzyme densities of RER and SER are the same in portal and central hepatocytes and (2) if aging is associated with a loss of biochemical homogeneity.

When plotted, for example, the intersection of the two linear curves shown below gives the enzyme densities (EDs) for the ER membranes in the portal and central hepatocytes:

Step 1: Find the EDs (U/ER) of the ER membranes in portal and central cells.

Animal 1: $S_{ER(PORTAL)} \cdot ED + S_{ER(CENTRAL)} \cdot ED = U/G$

Animal 2: $S_{ER(PORTAL)} \cdot ED + S_{ER(CENTRAL)} \cdot ED = U/G$.

Data in: ER membrane surface areas (M^2/G), U(ER marker enzyme)/G

Data out: ED (Portal ER), ED (Central ER)

Calculate: U/G for Portal and for Central Hepatocytes ($U = ED \cdot S$).

Step 2: Find the EDs (U/RER, U/SER) of the RER and SER membranes in both the portal and central hepatocytes.

Using the enzyme density of portal hepatocytes with their ER surface area, for example, one can calculate the amount of enzyme activity associated with the portal hepatocytes ($U = ED \cdot S$). If we make this estimate for two animals, then we can write a new pair of equations for the RER and SER in portal and central cells and solve them for the EDs of their respective RER and SER membranes.

Plot or solve the following pairs of linear equations:

ANIMAL 1

$$[RER_{(PORTAL)} \cdot ED_{RER(PORTAL)}] + [SER_{(PORTAL)} \cdot ED_{SER(PORTAL)}] = U/G_{(PORTAL \text{ HEPATOCYTES})}$$

$$[RER_{(CENTRAL)} \cdot ED_{RER(CENTRAL)}] + [SER_{(CENTRAL)} \cdot ED_{SER(CENTRAL)}] = U/G_{(CENTRAL \text{ HEPATOCYTES})}$$

ANIMAL 2

$$[RER_{(PORTAL)} \cdot ED_{RER(PORTAL)}] + [SER_{(PORTAL)} \cdot ED_{SER(PORTAL)}] = U/G_{(PORTAL \text{ HEPATOCYTES})}$$

$$[RER_{(CENTRAL)} \cdot ED_{RER(CENTRAL)}] + [SER_{(CENTRAL)} \cdot ED_{SER(CENTRAL)}] = U/G_{(CENTRAL \text{ HEPATOCYTES})}$$

$$\text{ANIMAL 1: } [RER_{(PORTAL)} \cdot ED_{RER(PORTAL)}] + [SER_{(PORTAL)} \cdot ED_{SER(PORTAL)}] = U/G_{(PORTAL \text{ HEPATOCYTES})}$$

$$\text{ANIMAL 2: } [RER_{(PORTAL)} \cdot ED_{RER(PORTAL)}] + [SER_{(PORTAL)} \cdot ED_{SER(PORTAL)}] = U/G_{(PORTAL \text{ HEPATOCYTES})}$$

$$\text{ANIMAL 1: } [RER_{(CENTRAL)} \cdot ED_{RER(CENTRAL)}] + [SER_{(CENTRAL)} \cdot ED_{SER(CENTRAL)}] = U/G_{(CENTRAL \text{ HEPATOCYTES})}$$

$$\text{ANIMAL 2: } [RER_{(CENTRAL)} \cdot ED_{RER(CENTRAL)}] + [SER_{(CENTRAL)} \cdot ED_{SER(CENTRAL)}] = U/G_{(CENTRAL \text{ HEPATOCYTES})}$$

As a minimum, data from three animals taken two at a time would be a reasonable sample size to start.

Calculation Example: Recall the familiar solution. Using the pair of equations below, find the unknown values x and y.

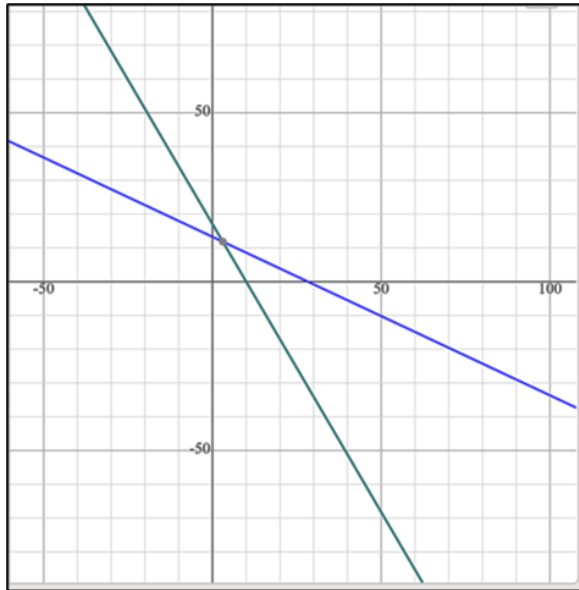
$$5.8x + 3.4y = 58 \text{ (red line)}$$

$$2.3x + 4.9y = 65 \text{ (blue line)}$$

$$X = 3.067$$

$$Y = 11.825$$

Solving these equations simultaneously gives $x = 3.06796$ and $y = 11.82524$. A graphical solution also exists, wherein the X and Y values occur at the intersection of the curves.



Summary: The lobular model described above is designed to detect differences in the ER membranes of portal and central hepatocytes. It has not been tested experimentally. Step 1 should work and provide new insights into the ways portal and central hepatocytes change. Step 2 checks for biochemical homogeneity across the liver lobule (if such information is needed). If step 1 fails to give the expected results, then there may be a stereological sampling problem. Sampling hepatocytes with vertical sections might solve the problem (Baddeley et al., (1986). For a worked example, see Bolender et al., (1993).

8.3 COMMENT

By copying biology's approaches to change, we become invited to study biology as a complex system based on the mathematics of first principles, which cells execute within a vast information processing network - optimized for success. We have at least two choices: (1) we can strive to detect "changes" by demonstrating significant differences between two data points or (2) we can figure out how to interface with biology's information networks to discover how living systems solve problems in our world of classical mechanics by borrowing heavily from the weirdness of quantum mechanics (or something else). Since cells are already known to be extraordinarily clever at applied physics, copying their approaches to solve our experimental problems becomes an exercise in learning how to play by the rules.

An assumption is an untested guess, a theory is a partially tested assumption, but rules and first principles become highly reproducible because they are hard coded into the fabric of nature. Biology does rules and first principles because they have guaranteed its success and survival. The point? If we encountered an intelligent life form on another planet, our primary goal would be to communicate with it. The primer approaches hepatocytes as our resident "alien" and tries to communicate with them by reconstructing the biology of hepatocytes from eclectic parts scattered throughout the literature. When asked, for example, how they could change the RER and the SER without changing the ER, the cells responded with two wave functions 180° out of phase, wherein we could deduce the rates of change by drawing an analogy to

what we know as the mean value theorem of calculus (Chapter 4). However, it's not clear how hepatocytes could solve complex "calculus" problems without computational machinery. Perhaps the microtubules sitting next to the ER might hold the answer (Hameroff and Penrose 1996, Markus et al., 2009).

CHAPTER 9

PUBLISHED DATA → GLOBAL PHENOTYPES

SUMMARY

The Biomatrix Workshop (Morowitz and Smith, 1987) posed a fundamental question: “How do we organize all the published data of biology?” Although several good answers no doubt exist, they all depend on answering the same basic question: How do we derive biology from first principles empirically? Currently, the biology literature includes a vast collection of largely isolated data, over simplified, incomplete, and typically unreproducible. Since the basic structure of the literature adheres to a statistical model for change and data interpretation, the recent challenges to the correctness of our published results has understandably resulted in calls for a stricter adherence to statistical rules (Ioannidis et al., 2015). The primer also supports better statistical comes but argues for a more realistic approach to detecting and reproducing biological changes. It argues that one should first demonstrate the existence of a biological change before testing it statistically for a significant difference. By applying statistics to experimental results *assumed* to represent a biological change, we effectively removed biology from the test and triggered the present crisis of confidence in our published results. When put to the test, the assumption collapsed. If, however, we derive a change from first principles, then reproducibility can confirm a change. Such an approach puts biology back in the game.

The phenotype model explored by the primer operates with data pair ratios instead of the isolated data points we usually encounter in the adaptability layer. Since biology defines rules with ratios, the analysis of a complex change begins with data pair ratios and solves problems by forming subgroups of data pairs that change in ways that solve their part of a larger problem. The data pair ratio defines an effective construct because it allow us to reassemble (forward engineer) a change from its many parts and then take it apart (reverse engineer) to discover the underlying rules. The simplest explanation for the connectivity of the two parts producing the data pair ratios is classical entanglement – if we know the value of one ratio, we always know the other. The sum of all the changes produces the phenotype (the solution to the problems).

In nature, exceedingly small parts become connected by quantum entanglement. Living systems entangle parts to introduce rules and generate complex adaptive systems. For the primer, isolated data became data pairs, data pairs became subgroups, subgroups became a phenotype, and the phenotype became the construct of an information processing system capable of solving problems by changing. By all accounts, cells cannot reduce a biological change to a single experimental data point because it's a complex event.

Phenotypes take the eclectic data of the biological literature and organizes them into a single, coherent dataset capable of displaying new properties, outcomes, and insights. In practice, it applies a first principles approach to understanding the complexities of biological changes. The strategy of this approach consists almost entirely of copying biology as it changes. Biology doesn't change to pass a p test or an effect size, instead it changes to solve problems by physically becoming the solutions. Solutions to such problems appear as new recipes that redefine complex relationships of structure to function that create new emergent properties.

The point of the chapter is to provide examples of how we can update the heterogeneous data of many publications to generate the single, homogeneous datasets needed to reconstitute phenotypes in the process of solving problems by changing. Such an exercise offers the reader a glimpse of what our literature can do when we put a biological test of change before the statistical one.

Note the following properties of a biological change:

1. A cell change occurs as a transition from one phenotype to another.
2. Detecting and explaining a cell change includes forward and reverse engineering phenotypes empirically using a first principles approach.
3. A cell changes to become the solution.
4. A cell change represents a complex event involving alterations to many parts and connections.
5. To work, a cell change must be highly reproducible.

9.1 Developmental Phenotype

Using normalized data, reconstructing phenotypes from published data becomes a straightforward task. We'll assemble phenotypes from both simple (enzymes or membranes) and complex data types (enzyme density = enzyme/membrane) by duplicating biology's unique approach to change, which occurs as a complex problem-solving event. To apply the phenotype model to developing hepatocytes, we begin by aggregating data from several published studies: Herzfeld et al., (1973; HFG73), Herzfeld and Greengard (1969; HG69), Vinicor et al., (1976; VHCC76), Jamdar and Greengard (1970; JG70), Jamdar and Greengard (1969; JG69), and Ballard and Oliver (1963; BO63).

Primary Data: The phenotype model begins with the simplest changes occurring in the primary unit of biological complexity, which posits the existence of two entangled parts defined as a data pair ratio. If, for example, we started with fifty parts, taking fifty parts 2 at a time would give us 1,225 data pair ratios (without left-right duplicates). But when applied to 20 time points, the data pair ratios in play would become $(1,225 \cdot 20 = 24,500)$.

Primary Unit of Change: The two entangled parts (the data pair ratio) display a general pattern of change over time. When the new phenotype first begins to form, the data pair ratios follow a polynomial curve that eventually becomes linear. Data pair ratios allow us to apply two rule-based operations to the enzyme data: forward and reverse engineering. By starting with the published parts and using them to put hepatocytes back together, we can then take them apart to discover how they changed. In effect, the phenotype model tests the central premise of the Biomatrix (Morowitz and Smith, 1987), which supports the view that organizing the biological literature creates opportunities to reverse engineer our way to the first principles of biology. In this chapter, we'll explore the mechanics of aggregating the changes of several publications into a single phenotype. By illustrating the updating process, the reader – once familiar with the basic rules - will begin to recognize a change as a complex event operating within the framework of an advanced information processing system.

9.1.1 Developmental Phenotype - Enzymes(NORM1)

Figure 9.1 summarizes changes in a set of data pair ratios (enzyme:enzyme) that define a hepatocytic phenotype during early postnatal development. The figure shows that by merely aggregating the normalized data pairs, they became forward engineered into a developmental phenotype. In turn, reverse

engineering this phenotype reveals the strategy used by the entangled pairs of enzymes to change as they work their way toward the solution(s). Moreover, the functional patterns of the enzymes indirectly predict the time course of gene expression (enzyme name \rightarrow gene name) thereby creating a putative link between the first principles of gene expression and those responsible for the phenotypic changes.

Total Set (202 Parts)

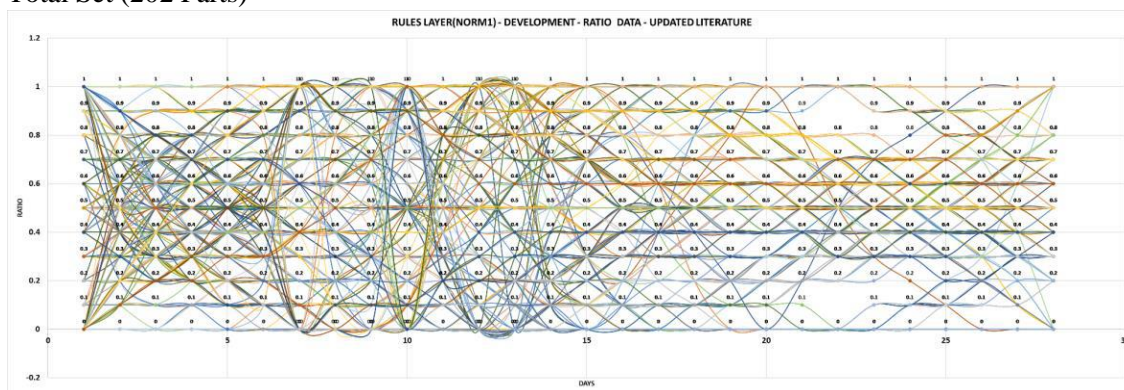
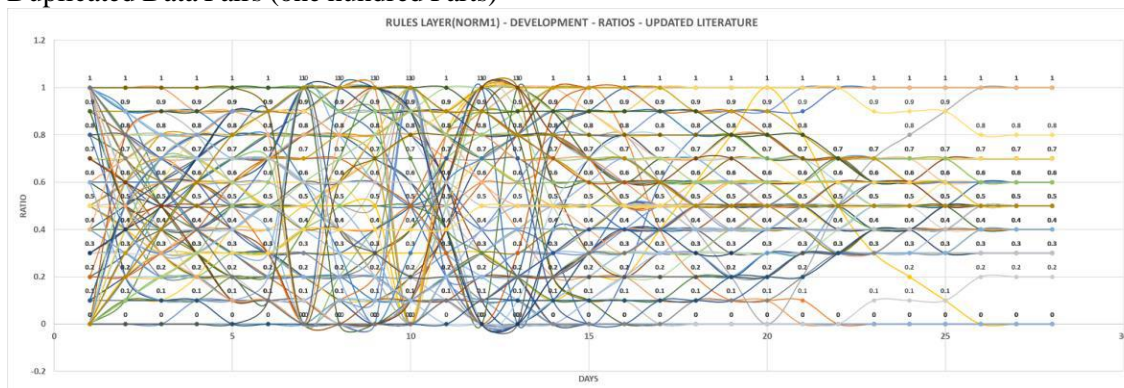


Figure 9.1 A developmental phenotype covering 28 days of early development reconstructed from changing enzymes expressed as data pair ratios.

Reverse Engineering Early Development: By selecting duplicated ratios with similar ratios at days 26, 27, 28, the normalized data pair ratios fall into subgroups (0.5:0.5, 0.4:0.6, 0.3:0.7, 0.2:0.8, 0.1:0.9, or 0.0:1.0). When a data pair maintains the same ratio for three consecutive days, we infer (posit) that a solution exists. Figure 9.2 shows the duplicated data pairs – collected from Figure 9.1 – needed to reverse engineer the phenotype. Reverse engineering starts with the first ratio rule (0.5:0.5) and continues to the last (0.0:1.0).

Duplicated Data Pairs (one hundred Parts)



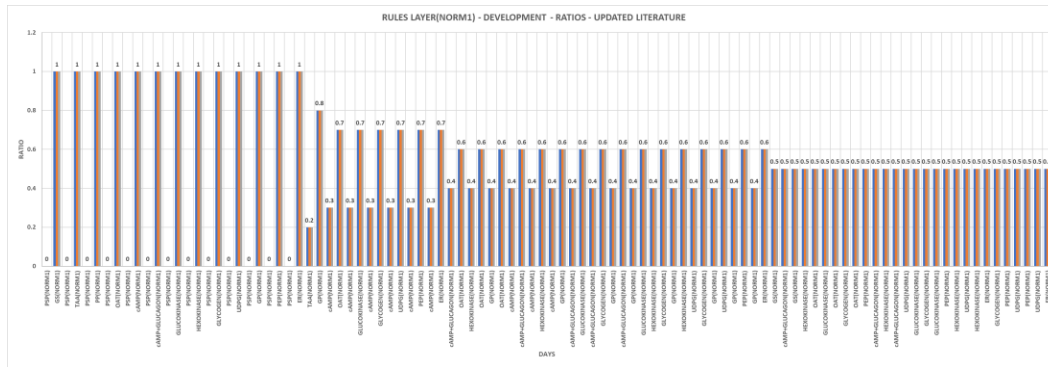


Figure 9.2 Duplicated data pairs sharing the same solution throughout the 28 days of development. The histogram identifies the ratios of the subgroups representing a solution at days 26-28. Each subgroup continues to unfold (reverse engineer) down to the original data pair ratios. [Histogram: bars with three colors (blue, red, and gray) represent the values on three consecutive days (26-28).]

Figure 9.3 shows the first subgroup (0.5:0.5). Following birth (day 0), the data pairs change as they work their way toward a shared goal (their part of the solution) of maintaining the same ratio (0.5:0.5) persistently (identified herein as three or more consecutive days). This represents the first of five subgroups collected by reverse engineering the phenotype. In turn, each subgroup unfolds back to a single data pair. This means that phenotypic changes can predict gene expression ranging from simple (a single data pair) to massively complex (all the data pairs).

Unfolded Set (28 Parts → 0.5:0.5)

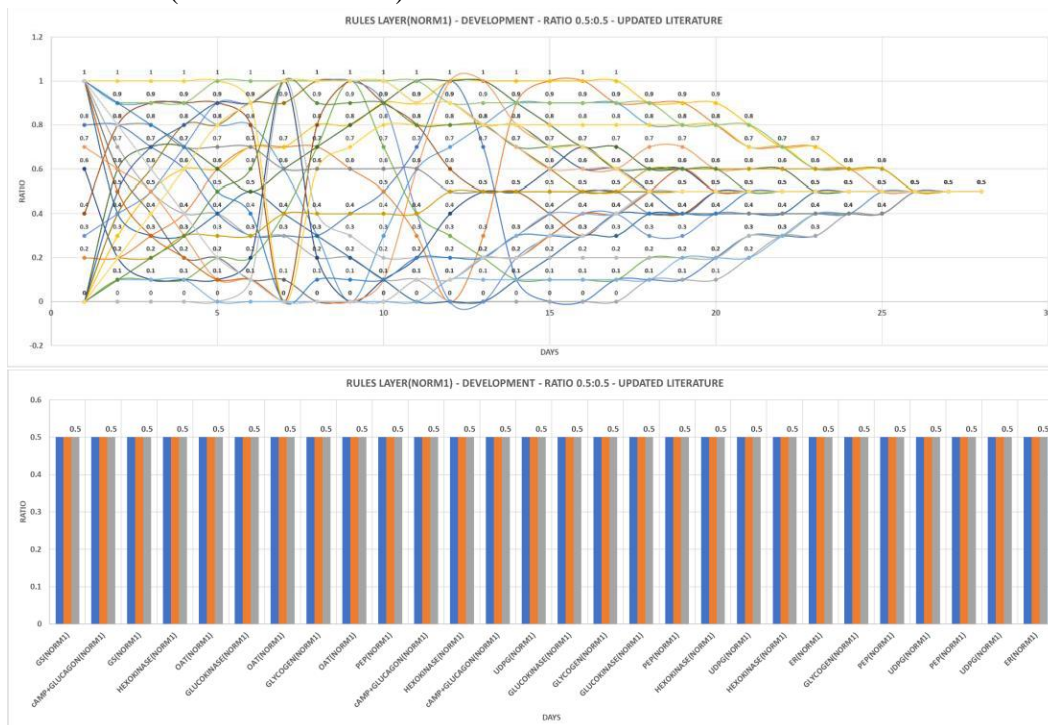
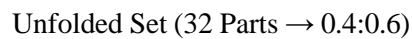


Figure 9.3 The first subgroup (0.5:0.5), indicates that both parts (enzymes) changed at the same rate. The reverse engineering continues until only a single data pair remains (not shown). The histogram displays the solutions of the data pairs between days 26 to 28. [Note the inclusion of the ER.] The first enzyme data pair to find the solution (0.5:0.5) appears at the far left of the histogram as two adjacent and named tricolor bars (GS, cAMP+Glu).



Unfolded Set (26 Parts → 0.0:1.0)

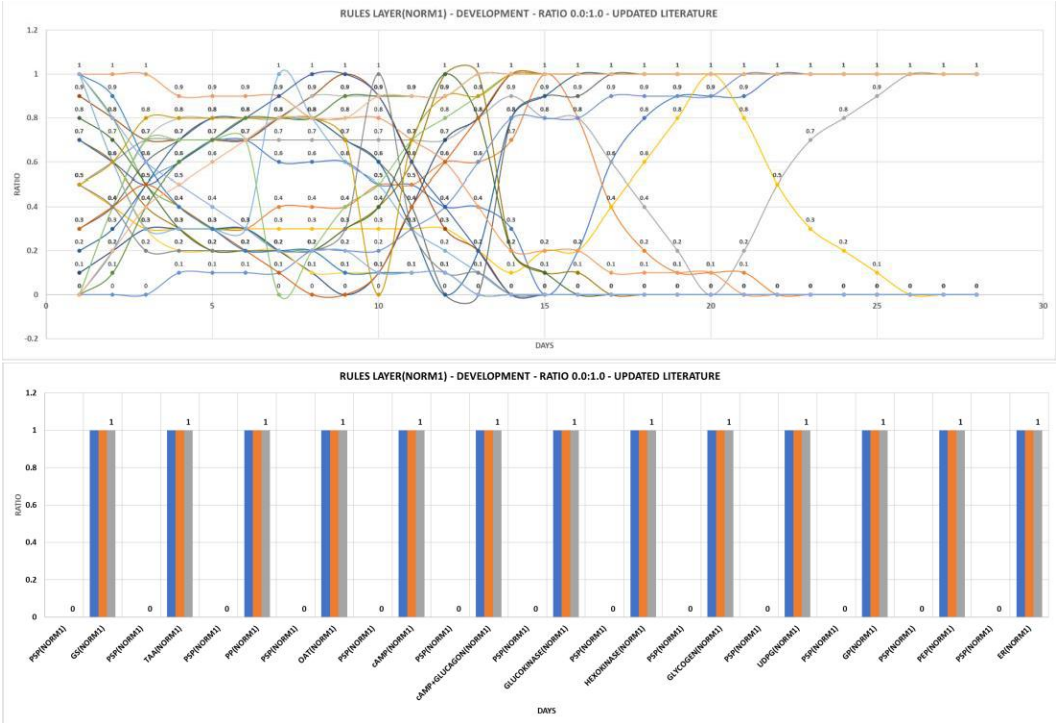


Figure 9.6 The final subgroup defines its solution as 0.0:0.1 accompanied by a histogram (days 26-28).

9.1.2 Developmental Phenotype - Enzyme Densities (ED-NORM2)

The enzyme densities (Figure 9.7), which define the cellular solution to the developmental problem with rule-based ratios, bundle changes in enzyme activities and membrane surface areas into relationships of structure to function. For hepatocytes, this intermediate solution defines a biochemical-morphological recipe for the ER membrane (e.g., days 26-28). During these three days, a best possible (optimal) solution presumably exists for all six ratio pairs: 0.5:0.5, 0.4:0.6, 0.3:0.7, 0.2:0.8, 0.1:0.9, and 0.0:1.0.

Complete Set (182 Parts)

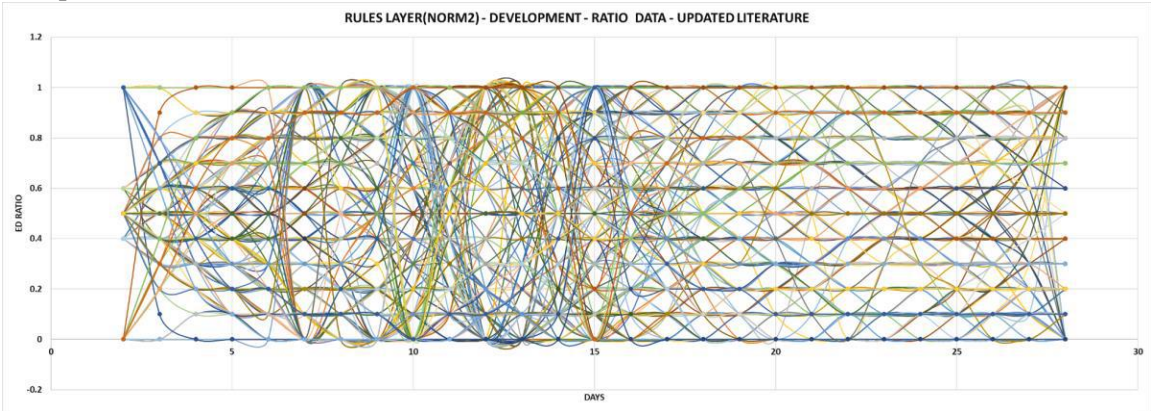


Figure 9.7 Starting with pairs of enzyme densities expressed in the rules layer, one can unfold (reverse engineer) the ED phenotype into subgroups using data pairs with duplicated ratios at day 26 to 28.

Duplicated data pair ratios, which identify patterns of connectivity between data pairs, define a biological change as a process that begins with many possible changes (magnitude and direction) but concludes with single outcomes for each of several subgroups. The data pair ratios allow us to put biology back together by rule (reconstitute the phenotype), which we can then take apart (reverse-engineer) to reveal the underlying subgroups responsible for the change. In the simplest of terms, a biological change consists of changes in data pair ratios occurring within multiple subgroups, which together define the change as a phenotype defined by a stable (persistent) recipe. In turn, recipes leads to emergent properties that currently we don't know how to identify or predict. In effect, a biological change represents the combined effects of multiple changes occurring simultaneously. Each subgroup solves its local problem (ED recipe) within the context of the larger developmental problem (Σ ED recipes).

If the phenotype expresses an optimal result, then where and how did the optimizations occur? Did it operate at the level of gene expression (RNA, protein synthesis) and/or did it occur in the cytoplasm with the membrane bound marker enzymes (arranged as pairs of “entangled” parts)?

Once again, we start with the total set of duplicated data pairs that unfold into their subgroups at days 26-28 (Figures 9.8 to 9.14). Recall that the histograms identify the duplicated data pairs for three consecutive days(blue, red, gray).

Duplicated Set (172 Parts)

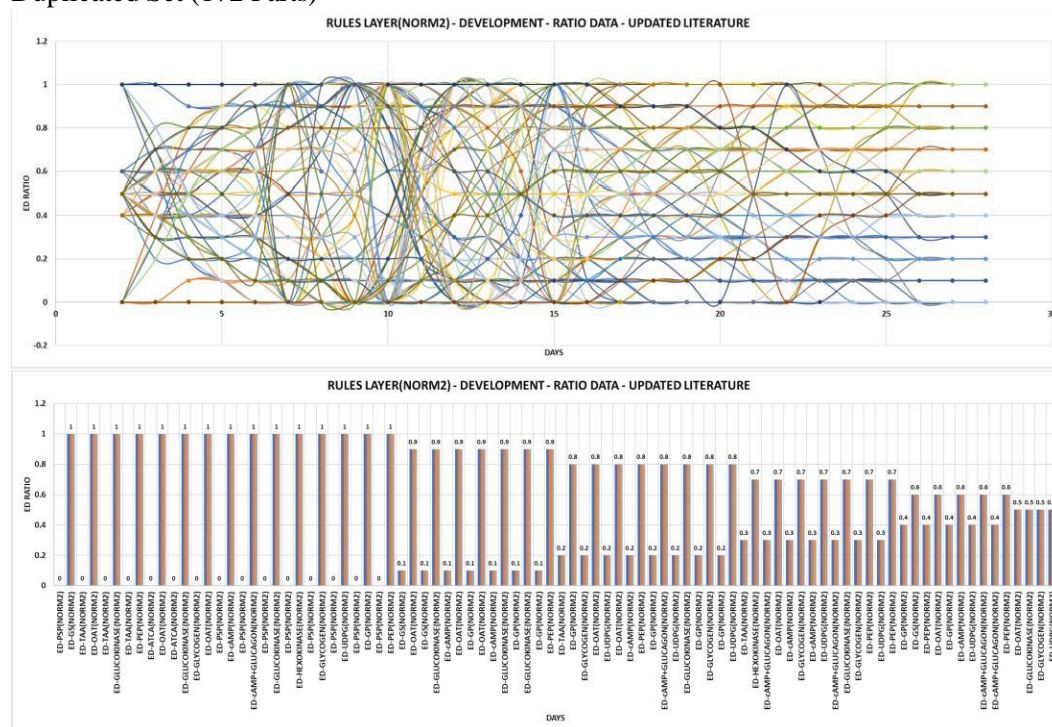


Figure 9.8 The graph shows changes expressed by the duplicated data pairs and the histogram the duplicated ratios at days 26-28.

By reverse engineering the ED phenotype (consisting of duplicated data pair ratios), we recover the specific subgroups one at a time (Figure 9.9).

Unfolded Set (4 Parts → Subgroup 0.5:0.5)

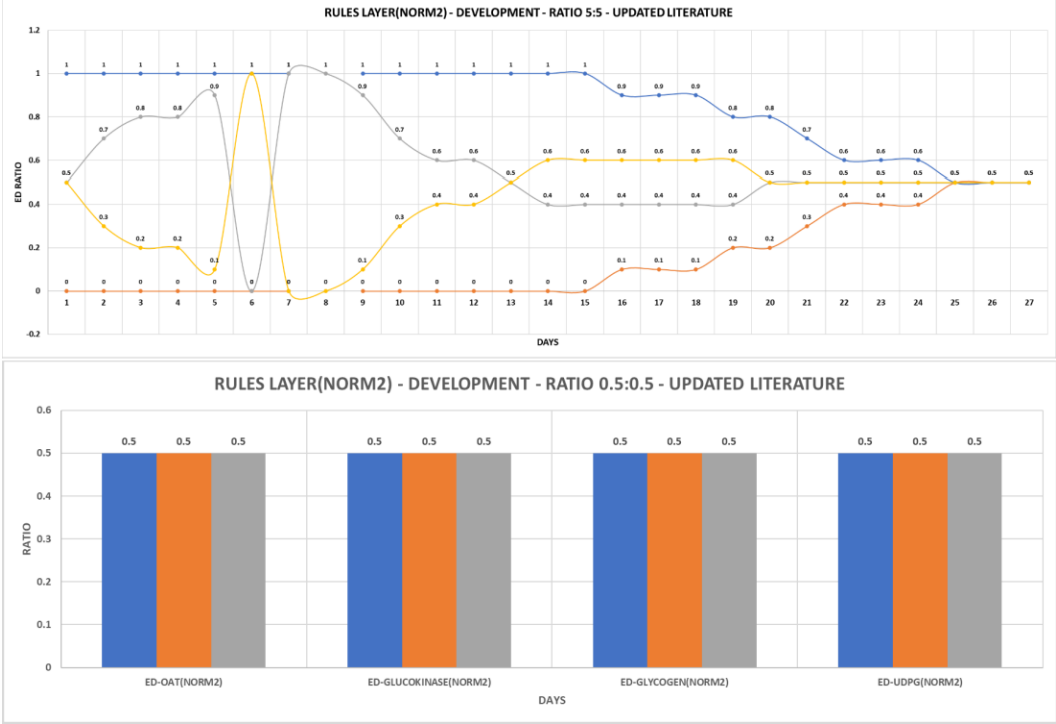


Figure 9.9 The first subgroup defines a new solution (0.5:0.5) accompanied by a histogram (days 26, 27, 28 – blue, red, gray).

Unfolded Set (10 Parts → Subgroup 0.4:0.6)

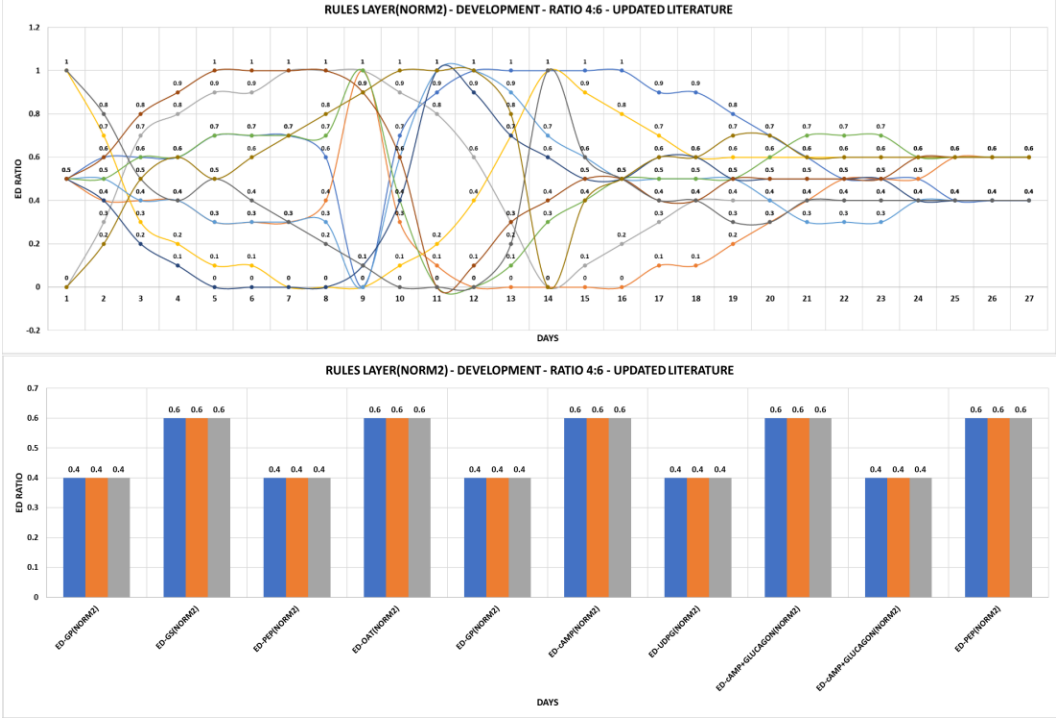


Figure 9.10 The second subgroup defines the solution (0.4:0.6) accompanied by a histogram (days 26-28).

Unfolded Set (14 Parts → Subgroup 0.3:0.7)

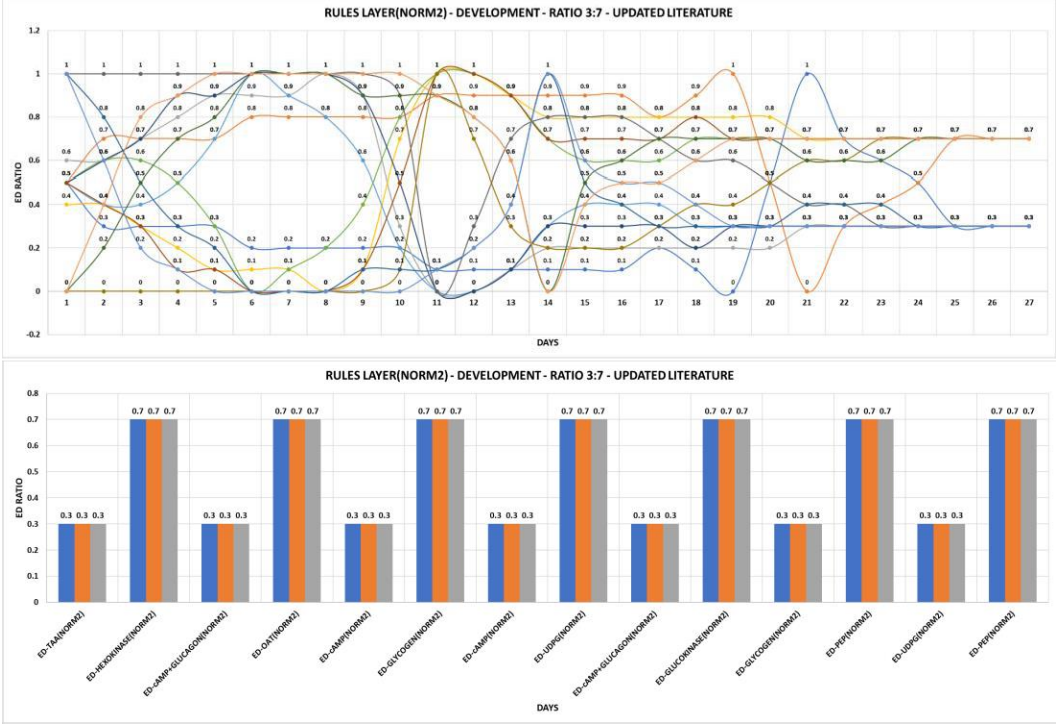


Figure 9.11 The third subgroup defines the solution (0.3:0.7) accompanied by a histogram (days 26-28).

Unfolded Set (16 Parts → Subgroup 0.2:0.8)

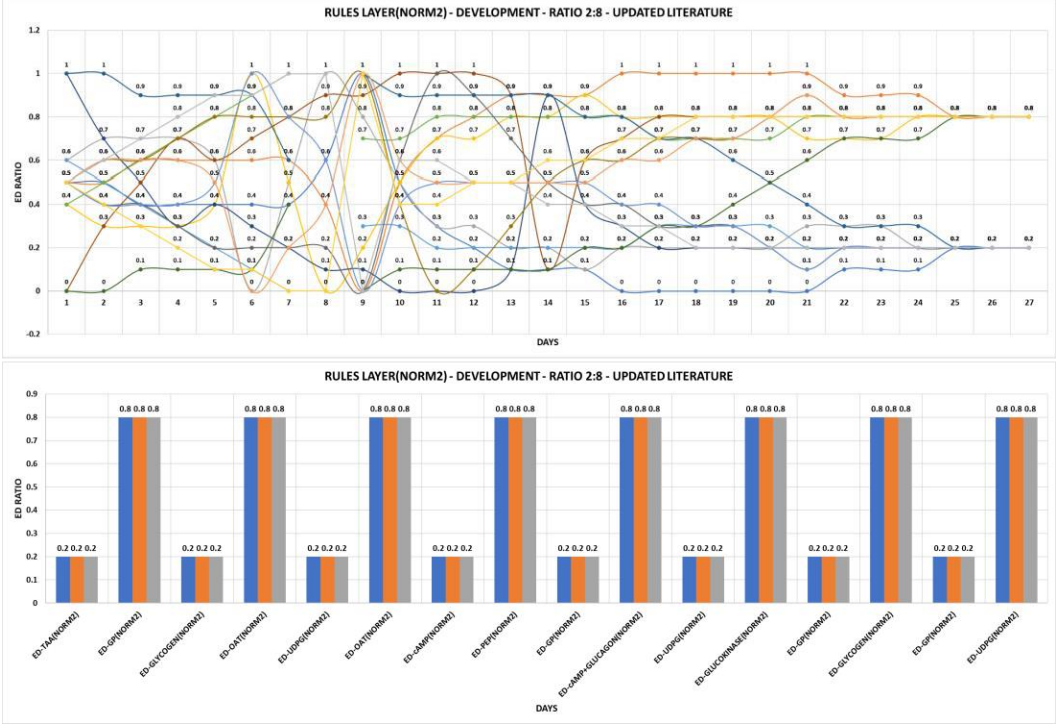


Figure 9.12 The fourth subgroup defines the solution (0.2:0.8) accompanied by a histogram (days 26-28).

Unfolded Set (14 Parts → Subgroup 0.1:0.9)

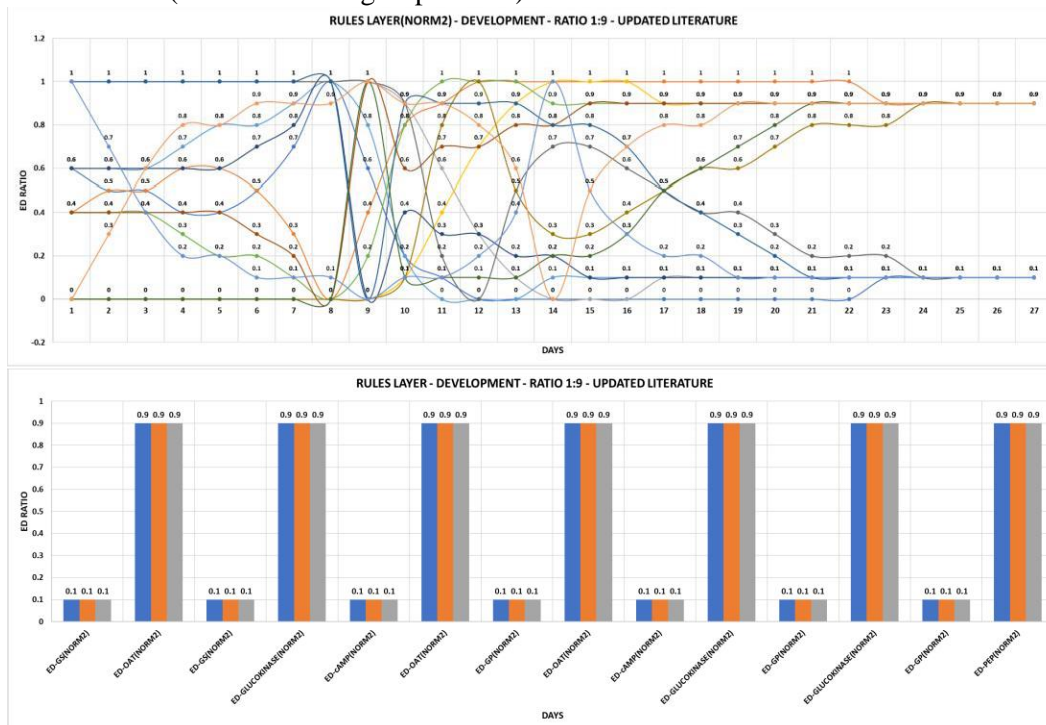


Figure 9.13 The fifth subgroup defines the solution (0.1:0.9) accompanied by a histogram (days 26-28).

Unfolded Set (30 Parts → Subgroup 0.0:1.0)

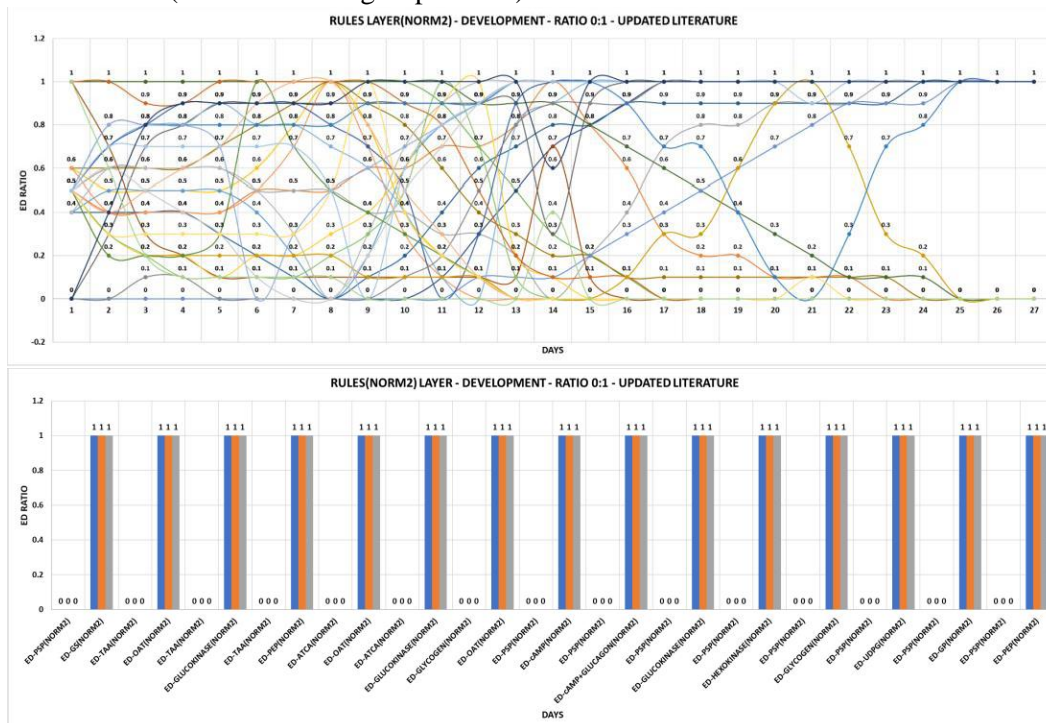


Figure 9.14 The sixth subgroup defines the solution (0.0:1.0) accompanied by a histogram (days 26-28).

Comments: Reverse engineering a phenotype reconstructed from changes in one part relative to another provides a strategy for (1) detecting and interpreting changes, (2) organizing the biological literature according to rules defined by and copied from biology, and (3) predicting genetic events. Moreover, we can unfold (reverse engineer) each dataset down to a single data pair.

As we begin to understand the basic mechanisms of a cell change, we'll receive direct feedback from the cells when we modify their genomes. Genetic engineering in hypercomplex systems invariably leads to unintended consequences. Our record with significant differences, reproducibility, and data references offers sobering evidence of the consequences that come from a willingness to substitute assumptions and simplified theory structures for the more rigorous experimental theories based on first principles.

9.2 Phenobarbital Phenotype

The exposure of the liver to phenobarbital has served as a major experimental model for studying drug induced changes in hepatocytic membranes and enzymes. Using data collected from four case studies we'll follow the changes involved in creating a phenobarbital phenotype (Valerino et al., 1974 - VVAJ74, Orrenius and Ericsson, 1966 - OE66, Menard et al., 1974 - MBH74, and Stäubli et al., 1969 - SHW69). Once again, changes that occurred within and between subgroups allowed hepatocytes to solve multiple problems simultaneously and thereby arrive at wide-ranging solutions (expressed as recipes).

9.2.1 Phenobarbital Phenotype - Enzymes(NORM1)

Using the phenotype model for change, we'll reverse engineer the changes in data pairs into subgroups based on the ratios of the final three time points (days 4.5 to 5).

Figure 9.15 summarizes the changes in the data pair ratios (enzyme:enzyme) as the hepatocytes generated a phenotype with a recipe capable of solving the problem created by the drug phenobarbital. In short, the hepatocytes recognized the problem and then changed their phenotypes to become the solution. The point? *Cells solve problems by changing into the solution.* This begins to explain why detecting cellular changes by demonstrating a significant difference between a control and experimental data points can detect differences but not cell changes. Meta-analyses detected the unfortunate mismatch between difference and change statistically (Ioannidis, 2005) whereas cells showed that change is a complex event.

Total Set (108 Parts; 54 Data Pairs)

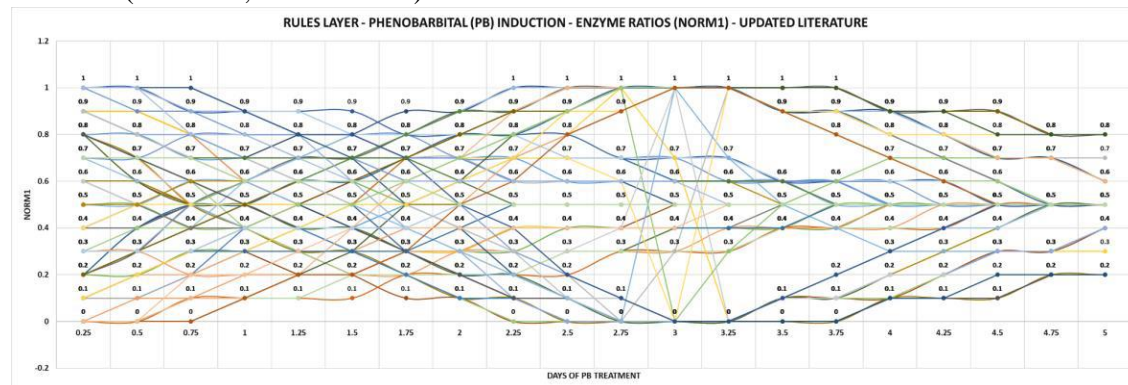


Figure 9.15 Set of original data pair ratios formed from pairs of hepatocytic enzymes.

Figure 9.16 identifies the duplicated data pair ratios (enzyme:enzyme) of the phenobarbital induction model.

Duplicated Data Pairs (66 Parts; 33 Data Pairs)

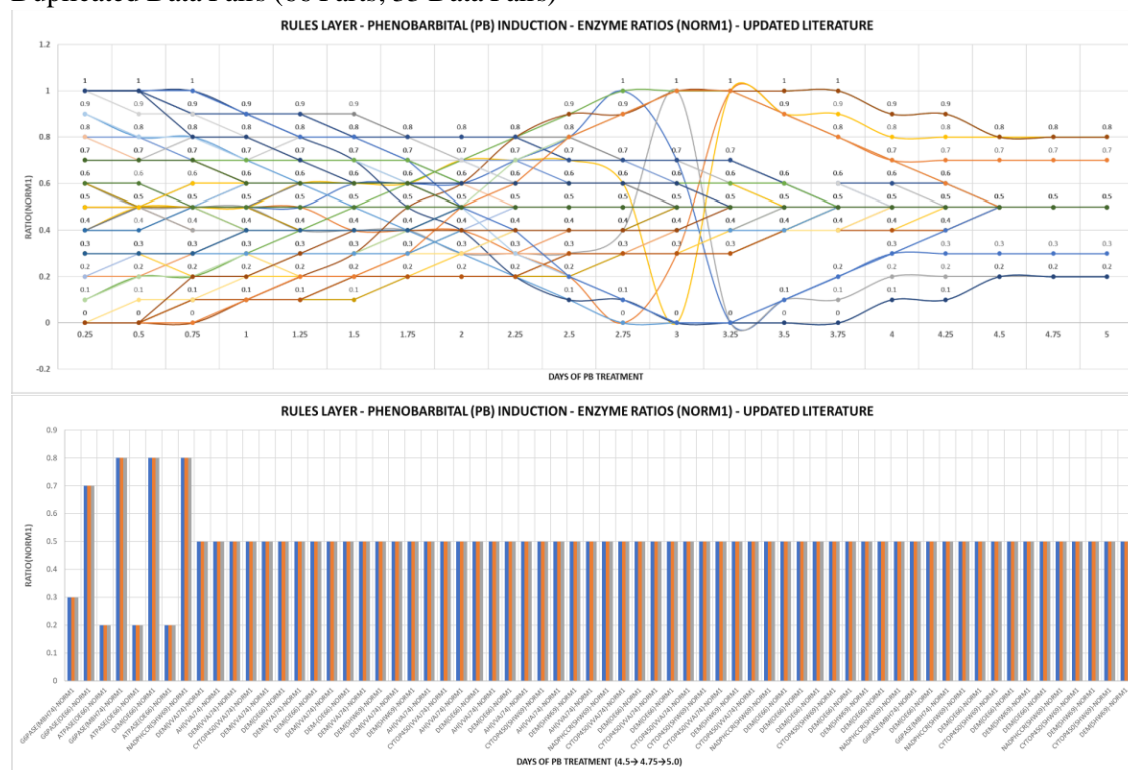


Figure 9.16 Normalized data pairs sharing duplicate ratios. The histogram, which identifies solutions to the PB problem at days 4.5 to 5, included three data pair ratios (0.5:0.5, 0.2:0.8, and 0.3:0.7).

The first cellular solution to the PB problem consisted of adjusting the ratios of the data pairs (enzyme:enzyme) such that they displayed – for three consecutive time points - the same ratio (0.5:0.5). This parsing of the dataset with the solution allowed us to collect enzyme pairs as subgroups based on their ability to share the same solution (data pair ratio). To provide connectivity between subgroups, each subgroup included data pairs with one part of a data pair in each of two subgroups. Such an arrangement suggests that optimization within groups also depends upon optimization between groups.

Figures 9.17 to 9.18 illustrate the process of reverse engineer the enzyme phenotype shown in 9.16 into subgroups. By including the source of each part in the data pairs, the histograms suggest that reverse engineering data pairs offers empirical evidence that the biology literature contains published data that are reproducible as complexities. With access to the raw data of the published studies, for example, it might have been possible to update most the PB literature. The point? Treating published research results as a renewable resource becomes a smart investment in our future. At a minimum, it prevents us from making unrecoverable mistakes.

Unfolded Set (52 Dat Pairs → Subgroup 0.5:0.5)

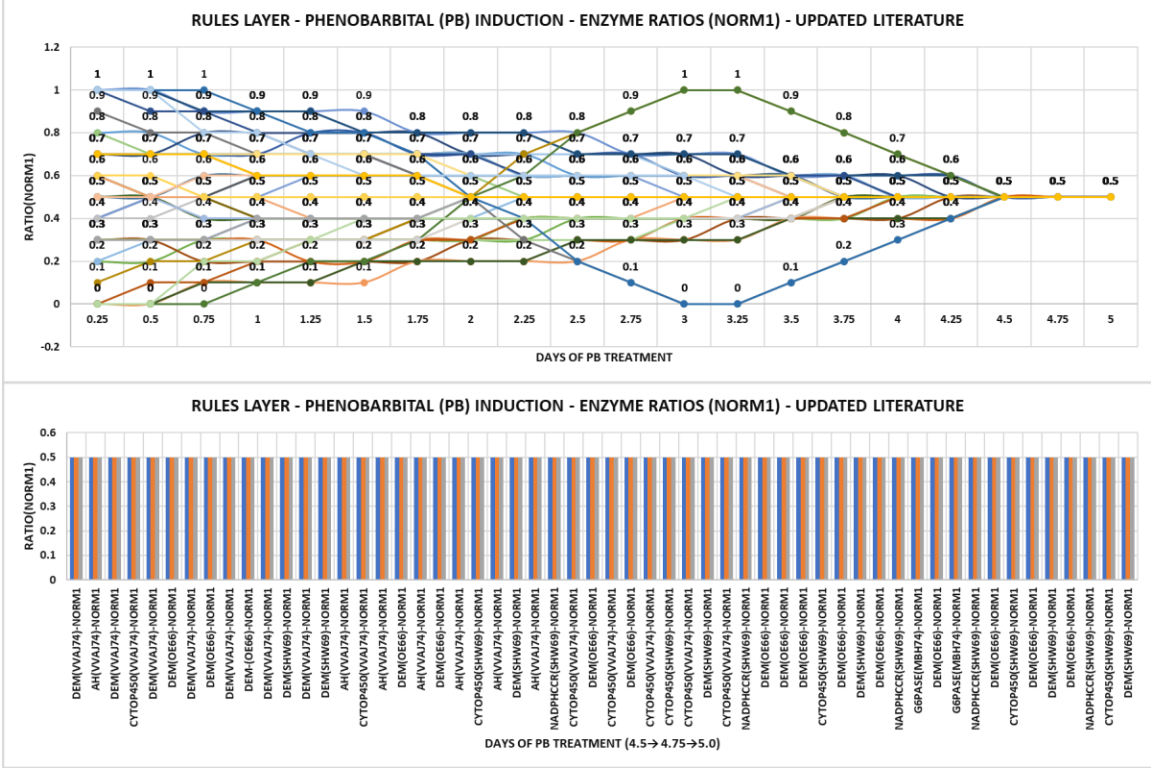
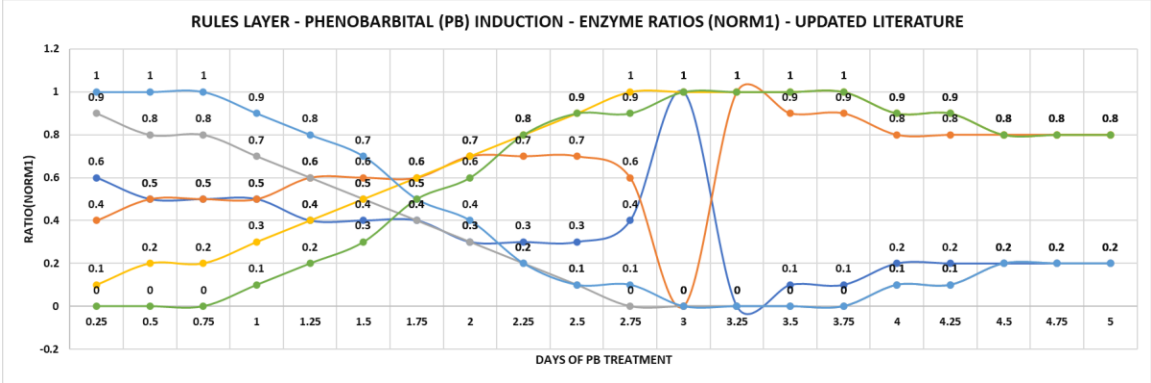


Figure 9.17 The first subgroup of data pair ratios defines a solution (0.5:0.5) accompanied by a histogram of the last three days (4.5-5).

Unfolded Set (33 Dat Pairs → Subgroup 0.2:0.8) [Just one pair for 3:7 (not shown)].



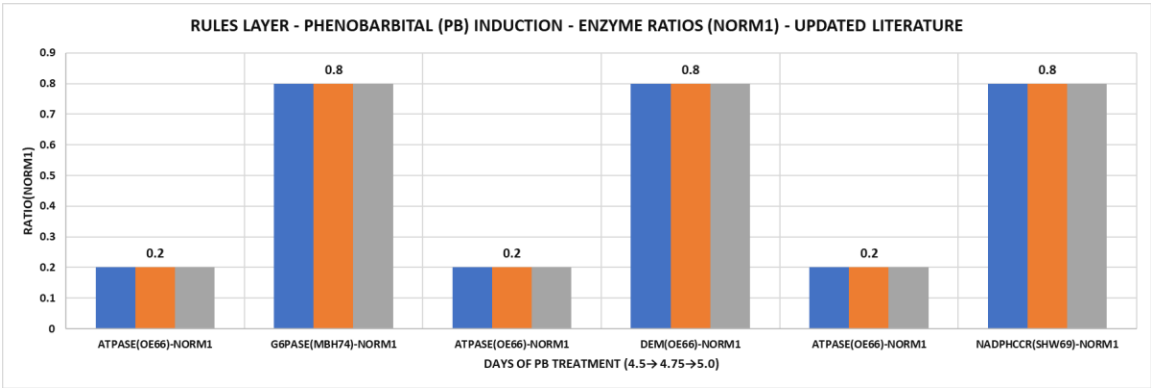


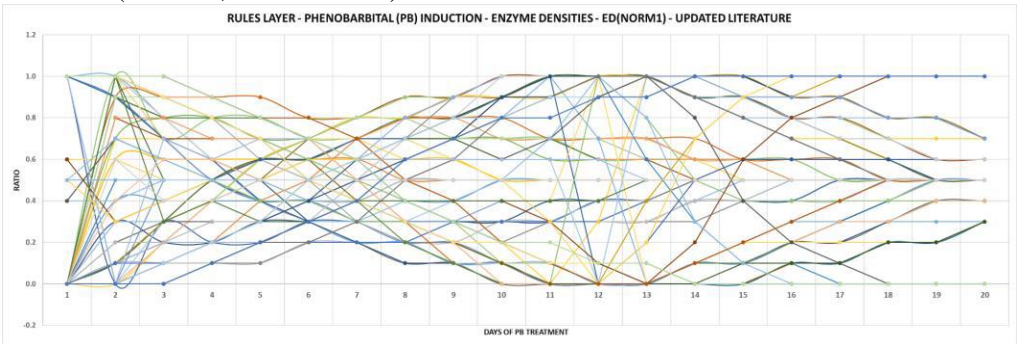
Figure 9.18 The last subgroup defines a solution (0.8:0.2) accompanied by a histogram of the last three days (4.5-5).

9.2.2 Phenobarbital Induction Phenotype – Enzyme Densities (ED-NORM2)

The phenobarbital phenotype - reconstructed from enzyme densities – combined changes in the relationships of structure to function occurring within and between subgroups into the higher-level solution (the PB recipe). Once reconstructed from the patterns of the rules layer, we can reverse engineer the phenotype into the detailed changes produced by the subgroups. Free from the limitations traditionally imposed by a hidden layer, reconstructing the cellular mechanisms responsible for the phenobarbital solution might lead to the biological algorithms for change or provide data suitable for testing with one of the newer optimizing routines of AI.

By reverse engineering the enzyme-membrane phenotype reconstructed from the ED-NORM2 data pair ratios, we can identify individual subgroups by defining a solution as identical data pair ratios spanning three consecutive time points (Figures 9.19 to 9.21).

Total Set (46 Parts; 23 Data Pairs)



Duplicated Set (172 Parts)

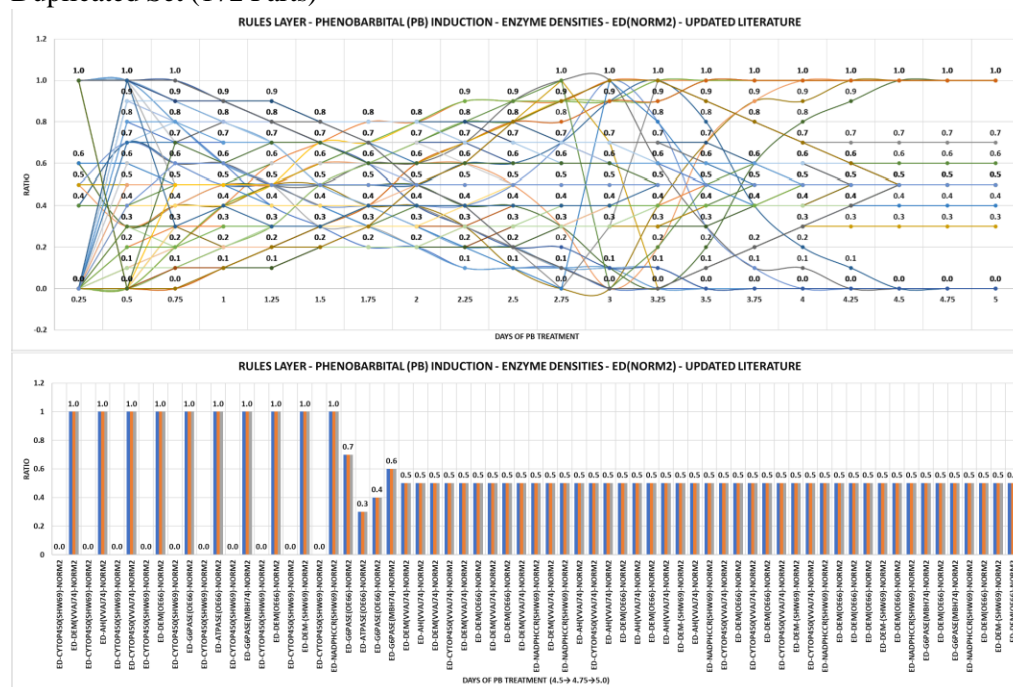


Figure 9.19 The histogram includes the data pair ratios for the last three days (4.5-5).

Unfolded Set (4 Parts → Subgroup 0.5:0.5)

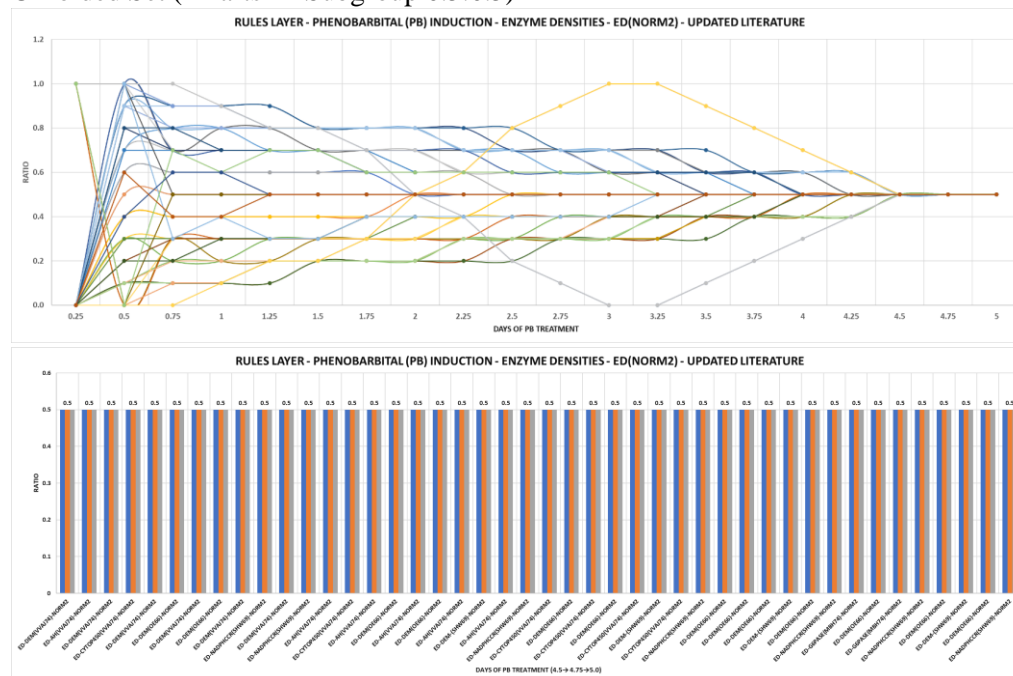


Figure 9.20 The first subgroup defines a solution (0.5:0.5) accompanied by a histogram of the last three days (4.5-5).

9.3 Comments

9.3.1 Interpreting Phenotypes

If the postulated entanglement of parts (enzymes and enzyme densities) supports the optimization of data pair ratios, then a principle of quantum mechanics becomes the likely candidate for the underlying mechanism of a biological change. In support of such a possibility, we would need additional proof - or at least reasoned speculation – to argue that the weird properties of quantum mechanics had somehow found their way into the classical mechanics of a biological change.

While photosynthesis, visual systems, and the optimizing behaviors of amoeba (Zhu, L. et.al., 2018) and slime molds (Farhad et al., 2023) offer evidence for the intersection of classical and quantum mechanics, the primer needed practical examples of tying classical data pair ratios to known properties of quantum mechanics. The phenotype model used forward and reverse engineering of published data to show that data pair ratios – the postulated classical analogue of quantum entanglement – were essential to detecting and explaining cell changes. Moreover, the model embraced the view that: (1) complexity (a change) arises from simplicity (two connected parts) (2) change embeds complexities in complexities (enzymes, membranes, and enzyme densities → data pair ratios → subgroups → solutions → recipes → emergent properties), and (3) optimization (best use of resources) defines the strategy and mechanism of a change. The recovery of hepatocytes from phenobarbital treatment, however, offered a most perplexing list of observations in Chapter 4.

1. Changes in two parts (RER, SER) defined two wave functions 180° out of phase (particle ↔ wave).
2. The hepatocytes applied a rule-based strategy designed to produce and deliver identical amounts of new ER membranes to its RER and SER subcompartments.
3. If we know the ratio of one part, we automatically know the ratio of the other part (fulfills the condition of entanglement).
4. If we know the rate of change of one part, we automatically know the rate of the other (property of entanglement?).
5. The rate of change calculations performed by the hepatocytes were analogous to integrating the polynomial wave equations using the mean value theorem of calculus (suggesting an entangled RER and SER combined with a calculation algorithm of unknown origin).

9.3.2 Cells as Information Processing Units

If the analogies listed above hold, then hepatocytes become prime candidates for exploring the computational capabilities of cells. Considering the need to manage the multilayered complexities of a biological change with phenotypes, one begins to recognize the advantages of viewing and interacting with biology as a data driven, information processing system. Under such a scenario, our present goal of detecting changes in the short term would logically shift from detecting changes with significant differences to identifying the algorithms, optimization, coding, and computational strategies currently available to changing cells but still foreign to us. To meet the current and future demands of the biological sciences, a data driven approach based on the rules and first principles of biology becomes an increasingly attractive option.

CHAPTER 10

CALCULATIONS - UPDATING PUBLICATIONS

The chapter includes examples of the calculations used to update published data using EXCEL worksheets and online calculators. Bear in mind that the methods used for the updating included (1) approximating routines (curve fitting), (2) applying approximate corrections (section thickness and compression), (3) sharing data across publications (mashups), and (4) working with normalized data (to aggregate changes and manage mixed data references). Although arguments exist in support of the workarounds, they represented temporary fixes needed to pursue the goals of the primer. By collecting and publishing the data required to detect and reproduce biological changes, we would need few workarounds.

Once familiar with the calculations, the reader can reproduce many of the curves displayed in the figures from the attached data tables. For example, when training AI models to detect biological changes, being able to compare results coming from simple (literature) and complex (primer) interpretations of the same published data may become helpful and rewarding.

10.1 Collect Data from Publication.

Perhaps the most challenging task of collecting published data consists of extracting numerical data from graphs. A general solution to the problem combines a measurement protocol with an equation that solves for the original data. For a worked example, see Chapter 7, Figure 7.1 (Bolender, 2019).

10.2 Morphological Corrections

- Correct surface area estimates (stereological) for section thickness and compression according to Weibel and Paumgartner (1978).
- For the primer, the correction factors the surface area estimates came from Blouin et al., (1977; BBW77). For example: $\text{RER (corrected)} = 5.91 \text{ m}^2/\text{g(uncorrected)} \cdot 0.7984 = 4.7185 \text{ m}^2/\text{g}$ (see Table 10.1).

Table 10.1 Corrections used by the update for section thickness and compression.

HEPATOCYTIC MEMBRANES	CORRECTIONS FOR SECTION THICKNESS AND COMPRESSION (USED TO UPDATE PUBLISHED DATA)
RER	0.7984
SER	0.5587
OMIM	0.7975
IMIM	0.5872

- c. Example: Figure 10.1 displays the original data before and after applying corrections for section thickness and compression to original data (Published data adapted from De la Iglesia, et al., (1975; DMF75).

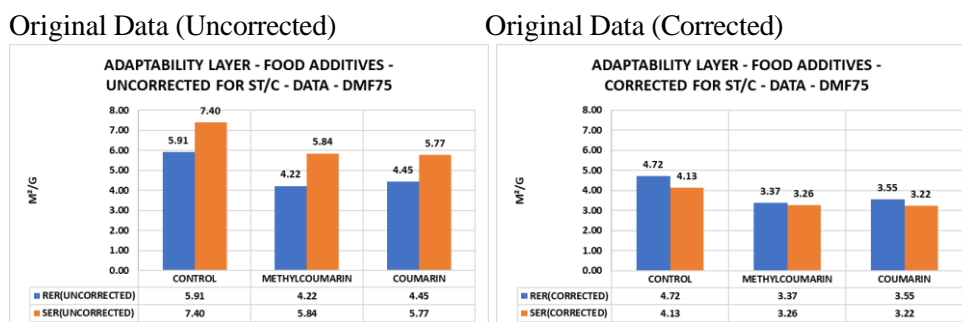


Figure 10.1 Corrections for section thickness and compression shown before (uncorrected) and after (corrected). Often both the absolute and relative amounts change.

10.3 Adaptability Layer

- a. The adaptability layer includes data copied from the original publication (Figure 10.2; Original data adapted from Greengard, O. et al., 1972 - GHK72).

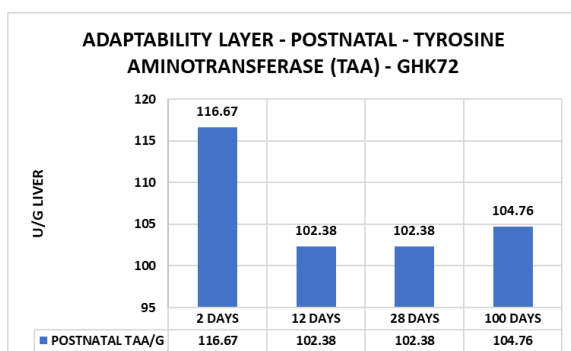


Figure 10.2 The adaptability layer includes, for example, the original biochemical data. By comparing results related (1) to a gram of liver and (2) to the liver one can determine if the number of cells contained in a gram of liver changed. The difference between the two estimates identifies the experimental errors associated with the gram of liver reference.

[Note: Unless labeled as uncorrected, stereological estimates for membrane surface areas include corrections for section thickness/compression. When detecting biochemical and morphological changes with concentrations, estimates require a correction for changes occurring in the number of cells filling a gram of liver. The calculation consists of multiplying units (U) per gram (U/G) or surface area (S) per gram (S/G) by the weight of the liver [(U/LIVER = U/G · W(LIVER); (S/LIVER = S/G · W(LIVER))]. Data related to the liver becomes a measure of total liver capacity. Detecting and interpreting a biological change requires data from both the hepatocytes and the livers.

10.4 Adaptability Layer (Expanded Data)

- a. Managing the complexity of biological changes within and across publications requires expanded and uniform datasets. Using regression analysis (EXCEL), one can fit the original data to polynomial equations and solve them for equally spaced time points (Figure 10.3). This expands the original data into a uniform dataset (Original data adapted from Greengard, O. et al., 1972).

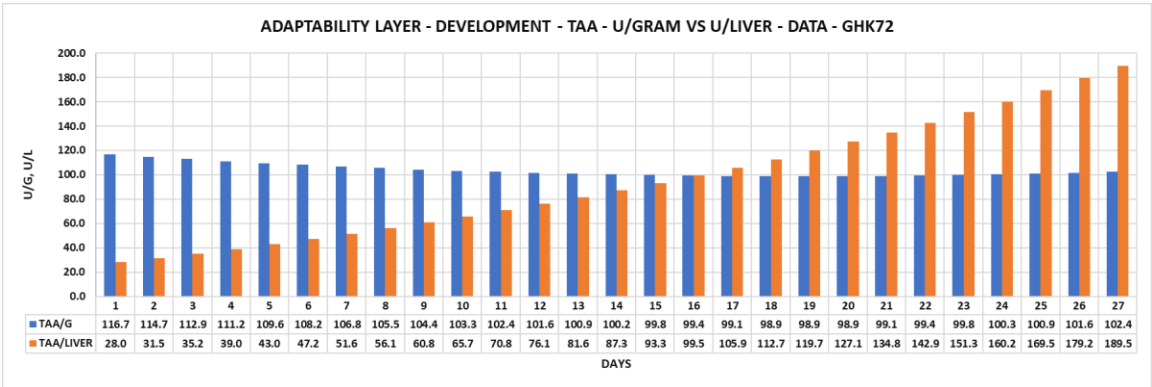


Figure 10.3 Regression analysis expands the original dataset to include any number of equally spaced time points (expressed as a polynomial equation with an $R^2 = 1$). The expanded dataset becomes the first step in integrating data within and across publications. Notice that (1) the gram of liver (G) detected a downward trend whereas the liver reported the correct increase. At day sixteen, however, both references gave the correct results because the liver weighed one gram. For this dataset, using a gram of liver reference to estimate changes in the enzyme activity would qualify as high-risk strategy because it detected the correct result only 3% (1/27) of the time. At 100% correct, the liver reference becomes the better choice.

- b. To expand the original data shown in Figure 10.4, one can fit the available data to a polynomial equation with an $R^2 = 1$ (in EXCEL) and then evaluate the equation repeatedly to generate the table of expanded data shown in Table 10.2. Online polynomial calculators speed the process (Original data adapted from Greengard, O. et al., 1972 – GHK72).

Original Data

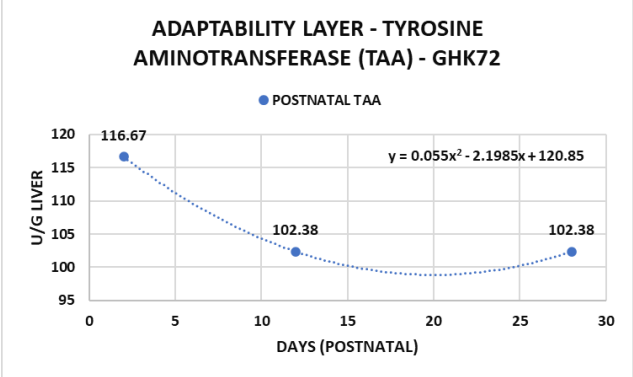


Table 10.2 Expanded data.

DAYS	TAA
2	116.673
3	114.750
4	112.906
5	111.233
6	109.639
7	108.156
8	106.782
9	105.519
10	104.365
11	103.322
12	102.388
13	101.565
14	100.851
15	100.248
16	99.754
17	99.371
18	99.097
19	98.934
20	98.880
21	98.937
22	99.103
23	99.380
24	99.766
25	100.263
26	100.869
27	101.586
28	102.412

Figure 10.4 Original data expanded by evaluating the polynomial equation for each day of the new dataset (see below).

- c. Check the equation used to generate the expanded data to see if it matches the original (it matches; see Figure 10.4). Caution! While the EXCEL plot in Figure 10.5 at the left gets the expected result (original and expanded equations match), the one at the right does not. Why? When using the “Line” plot at the right, the polynomial equation fails to match the “XY (Scatter)” plot of the original and expanded curves at the left. Why? The linear plot uses equally spaced bins for the data. The point? When curve fitting and expanding data use the XY(Scatter) plot.

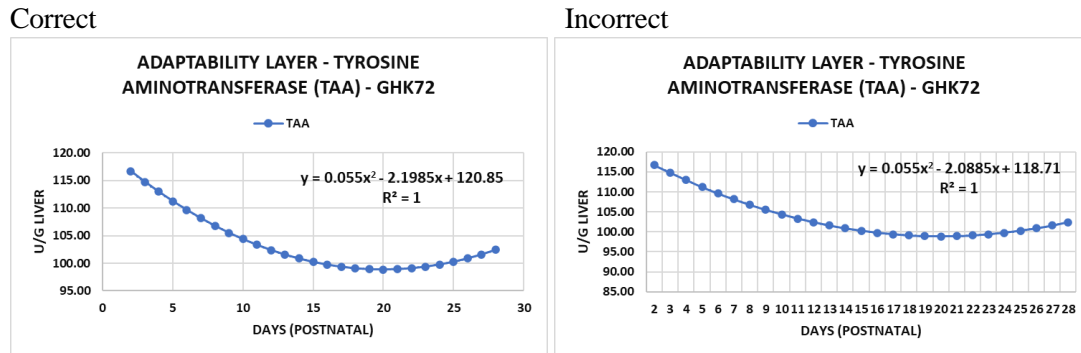


Figure 10.5 Different plots in EXCEL can produce different results.

10.5 Rules Layer

The expanded data of the adaptability layer become the ratios (i.e., data pair ratios) of the rules layer. The calculation consists of identifying two parts, summing then, and then calculating their relative amounts as a ratio. Results include three plots: (1) Adaptability Layer, (2) Rules Layer (Data), and (3) Rules Layer (Data Rounded). The rules layer has only twelve possible ratios: 0:0, 0.1:0.9, 0.2:0.8, 0.3:0.7, 0.4:0.6, 0.5:0.5, 0.6:0.4, 0.7:0.3, 0.8:0.2, 0.9:0.1, 1.0:0, 0:0, which usually reduce to four: 0.2:0.8, 0.3:0.7, 0.4:0.6, 0.5:0.5.

- a. Calculating Ratios in EXCEL (Original data adapted from Roessner et al., 1978 – RKSVB78). To round the data to a single digit, use the round function (=ROUND(number, num_digits)). Highlight the data, add a comma, space, type “1”, and press ENTER. Table 10.3 includes the data shown in Figure 10.6.

Table 10.3 Calculating ratios before and after applying Corrections for section errors related to stereological estimates.

Original Data	OMIM		IMIM		Ratios		OMIM	IMIM	OMIM	IMIM
	UNCORRECTED	CORRECTED	UNCORRECTED	CORRECTED	UNCORRECTED	CORRECTED				
	1.162	0.927	2.171	1.275	3.33	2.20	0.35	0.42	0.65	0.58
					0.3	0.4				

- b. Viewing the Results (Original data adapted from Roessner et al., 1978).

The adaptability layer displays the original data, whereas the rules layers form ratios from the original data as calculated or rounded to a single digit (Figure 10.6). Note that the mitochondrial surface areas uncorrected for section thickness and compression lead to an

incorrect result. Since our ability to test for reproducibility and reconstitute phenotypes depends on applying biological rules, applying corrections becomes essential. A ratio of OMIM-0.4:IMIM-0.6 (i.e., 4:6) means that four units of the outer mitochondrial membrane (OMIM) surface area accompany six units of the inner mitochondrial membrane (IMIM).

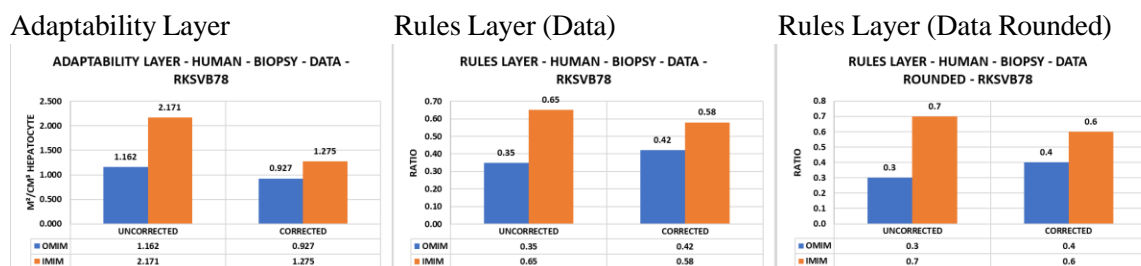


Figure 10.6 By translating data from the adaptability layer into ratios, they become the rules of the rules layer. Ratios, which represent the relative amounts of two parts, provide the underlying rule-based order that allow the data in the adaptability layer to be widely variable. In effect, the same data represent the application of a rule and the rule at the same time. The application of rule can have many values, but a rule just one. When the same data exist at the same time as a constant and a variable, a paradox exists. Curiously, paradoxes and symmetries seem to play a fundamental role in the logic used by cells when changing to solve problems. Notice in the figure that the uncorrected data offer a wealth of new information when corrected, which means that updating published results becomes a highly cost-effective approach to generating new research data from old.

10.6 Normalizing Data (NORM1)

- Properties:** Normalization expresses an original dataset (expanded) within the range of 0 to 1, where 0 represents the smallest value and one the largest. Normalization allows us to integrate published data within and across publications because it removes data units and data references but retains the changes as numerical values. In turn, the normalized changes become the data points that one can fit to curves that define linear or polynomial equations.
- Normalizing Equation:** $Y = (X - X_{\text{MIN}}) / (X_{\text{MAX}} - X_{\text{MIN}})$ (see Wikipedia)
- Normalizing Original Data (NORM1):** Normalizing data with EXCEL becomes an acquired skill. Proceed as described below (Table 10.4 and Figure 10.7).

Table 10.4 Normalizing Data (start (left) → finish (right)).

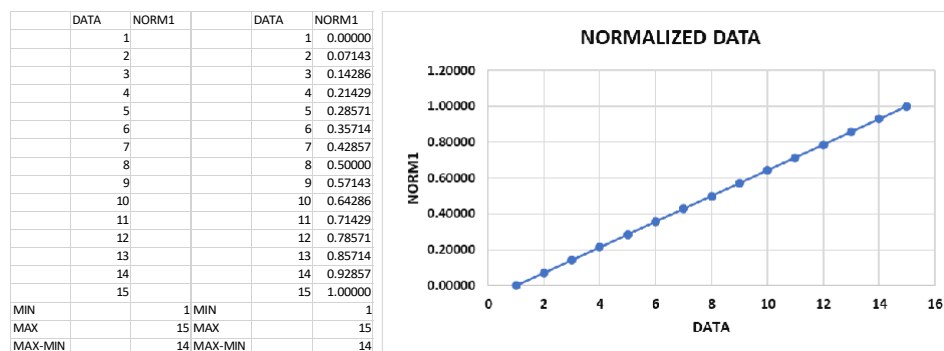


Figure 10.7 Normalizing data resets the values between zero (lowest value) and 1 (highest value).

Equation: $Y = (X - X_{\text{MIN}}) / (X_{\text{MAX}} - X_{\text{MIN}})$, where $X = 1 \dots 15$, $X_{\text{MIN}} = 1$, $X_{\text{MAX}} - X_{\text{MIN}} = 14$.

Procedure (Evaluate the equation.) Duplicate the empty practice table in EXCEL (Table 10.4).

1. Find the three values: First the minimum value (MIN): Click on the empty space (already filled with a 1).
2. Type: =min Double click on MIN Highlight the column (1 to 15) and press enter. 1 will appear in the rectangle.
3. Type: =max Double click on MAX Highlight the column (1 to 15) and press enter 15 will appear
4. MAX-MIN Subtract: $15 - 1$ 14 will appear
5. Calculate the first value: Put the cursor on the open space to the right of number 1.
6. Type: =(Click on 1 enter - (minus sign) click on MIN (the number 1) type) type / and click on MAX-MIN (the number 14)
7. The cursor sits at the end of the entry for row 1. Hold down the Fn key and press F4 (dollar signs - \$ - appear).
8. Place the cursor on the central value (a location). Hold down the Fn key and press F4 (more dollar signs - \$ - appear).
9. Press enter: a 0 should appear.
10. Apply the calculation to the remaining entries. Highlight the rectangle containing the zero. Put the cursor on the black square on the lower right-hand corner of the rectangle and pull it down to the bottom of the column (to item 15). The numbers should duplicate those in the Table 10.4 above. That's it.

- d. **Normalized Results:** The expanded datasets shown in Figure 10.8 plot changes in membrane surface areas from juvenile to old age in portal and central hepatocytes. But how do we use these data to explain the roles of the individual membrane compartments operating at two different lobular locations? (Original data adapted from Schmucker et al., 1978; SMJ78.)

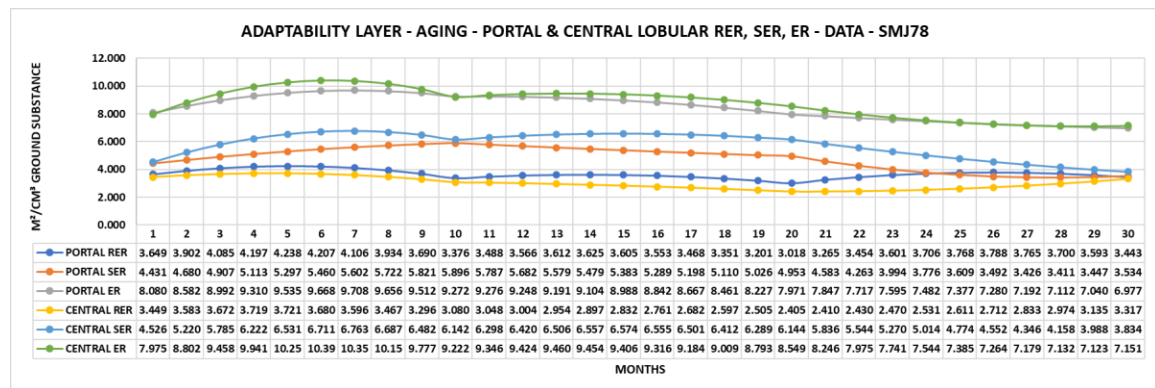


Figure 10.8 Original data corrected for section thickness and compression and expanded (Original data adapted from Schmucker et al., 1978; SMJ78).

Normalization puts all the data in the same setting (all the changes range from 0 to 1). Now the differences among the membrane compartments begin to appear - first in the portal hepatocytes and then in the central ones (Figure 10.9).

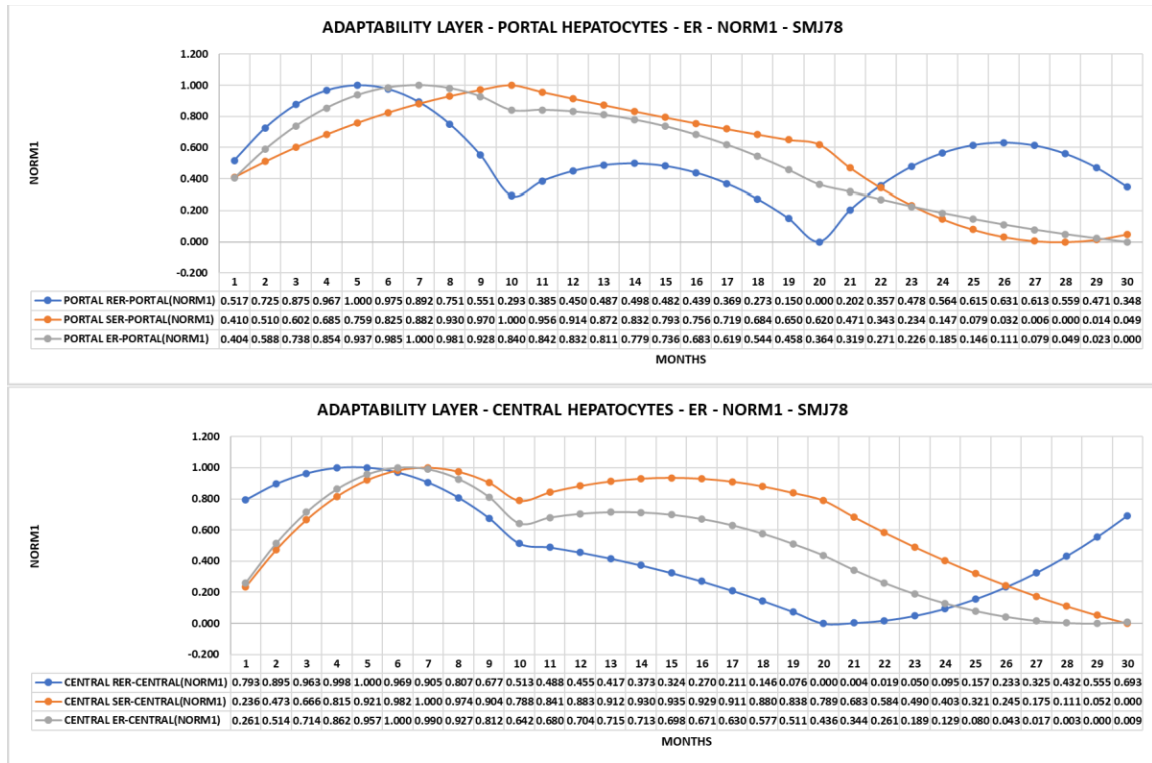


Figure 10.9 Results expressed after normalization (Original data adapted from Schmucker et al., 1978; SMJ78).

- e. **Generating Ratios from Normalized Data (NORM1):** Starting with the first two columns of normalized data in Table 10.5 (note that the values range from 0.000 and 1.000), sum the first two values (0.517, 0.410) and divide each value by the sum (0.927). This gives the ratio (0.56:0.44) (showing two digits to the right of the decimal point). Highlight the row and apply the calculations to the remaining rows. To obtain a single digit ratio (0.6:0.4), apply the round function (e.g., =ROUND(T91:U120, 1)) to all the rows in the table. (For sections e. to g., the original data were adapted from Schmucker et al., (1978; SMJ78).

Table 10.5 Generating ratios from expanded and normalized data.

MONTH	PORTAL	PORTAL	RER-PORT	SER-PORT	RER-PORT	SER-PORT
1	0.517	0.410	0.927	0.56	0.44	0.6
2	0.725	0.510	1.236	0.59	0.41	0.6
3	0.875	0.602	1.477	0.59	0.41	0.6
4	0.967	0.685	1.651	0.59	0.41	0.6
5	1.000	0.759	1.759	0.57	0.43	0.6
6	0.975	0.825	1.800	0.54	0.46	0.5
7	0.892	0.882	1.774	0.50	0.50	0.5
8	0.751	0.930	1.681	0.45	0.55	0.4
9	0.551	0.970	1.521	0.36	0.64	0.4
10	0.293	1.000	1.293	0.23	0.77	0.2
11	0.385	0.956	1.342	0.29	0.71	0.3
12	0.450	0.914	1.363	0.33	0.67	0.3
13	0.487	0.872	1.359	0.36	0.64	0.4
14	0.498	0.832	1.330	0.37	0.63	0.4
15	0.482	0.793	1.275	0.38	0.62	0.4
16	0.439	0.756	1.194	0.37	0.63	0.4
17	0.369	0.719	1.088	0.34	0.66	0.3
18	0.273	0.684	0.957	0.29	0.71	0.3
19	0.150	0.650	0.800	0.19	0.81	0.2
20	0.000	0.620	0.620	0.00	1.00	0
21	0.202	0.471	0.673	0.30	0.70	0.3
22	0.357	0.343	0.700	0.51	0.49	0.5
23	0.478	0.234	0.713	0.67	0.33	0.7
24	0.564	0.147	0.711	0.79	0.21	0.8
25	0.615	0.079	0.694	0.89	0.11	0.9
26	0.631	0.032	0.664	0.95	0.05	1
27	0.613	0.006	0.619	0.99	0.01	1
28	0.559	0.000	0.559	1.00	0.00	1
29	0.471	0.014	0.486	0.97	0.03	1
30	0.348	0.049	0.398	0.88	0.12	0.9

- f. **Generating Patterns from Ratios of Normalized Data (NORM1):** To view the changes in the relationships of two membrane parts, we use the ratios of the normalized data to reassemble a phenotype from the individual changes in data pair ratios (Figure 10.10). In turn, we can take the phenotype apart to see how the relationships of individual and subgroups of membrane pairs changed during development. The point? By reconstructing a phenotype from its parts (expressed as data pair ratios), we can then reverse engineer the process to follow the progress of a change occurring at multiple levels of complexity.

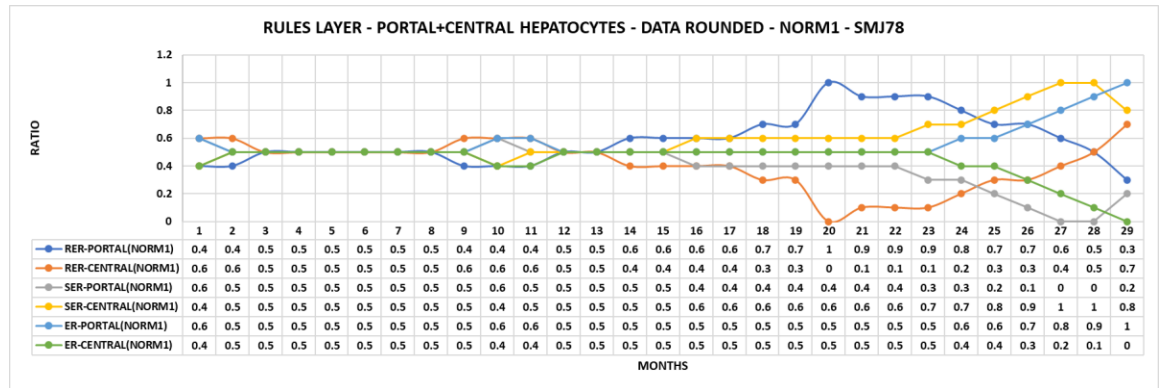
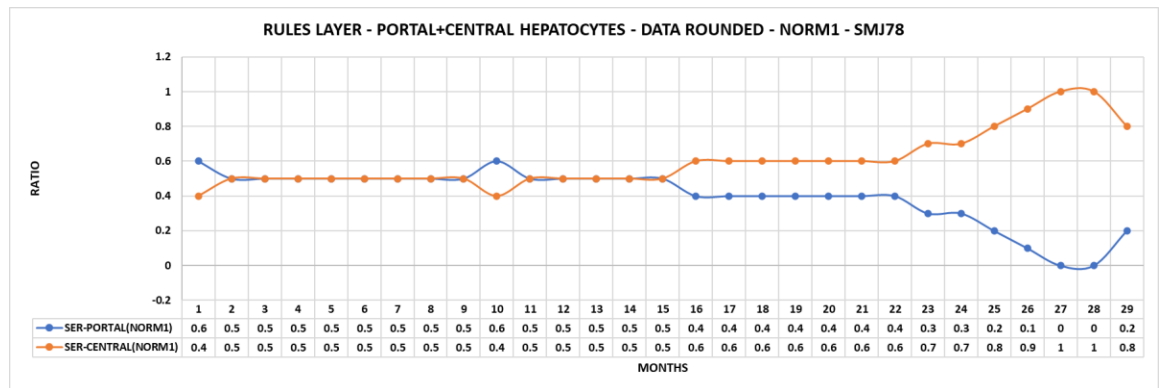


Figure 10.10 The plot follows changes in three data pair ratios (rules) over twenty-nine months. Are, for example, the ER membranes in portal and central hepatocytes the same or different? Yes, they are (month 5), no, they're not (month 27). The answer depends on when and where one chooses to look.

- g. **Reverse Engineering Changes in ER Membranes Across the Liver Lobule:** The question: Did the ER organelles of hepatocytes located in the portal and central regions of the liver lobule display both homogeneity ($= 0.5:0.5$) and heterogeneity ($\neq 0.5:0.5$)? Yes, but it depends on how, when, and where we look (Figure 10.11). Notice that at month 16 that the central SER changed from being the same as the portal SER at month 15 to more than its portal counterpart ($0.5:0.5 \rightarrow 0.6:0.4$), whereas the RER did the opposite. At month 14, the portal RER changed relatively more than the central RER ($0.5:0.5 \rightarrow 0.6:0.4$).



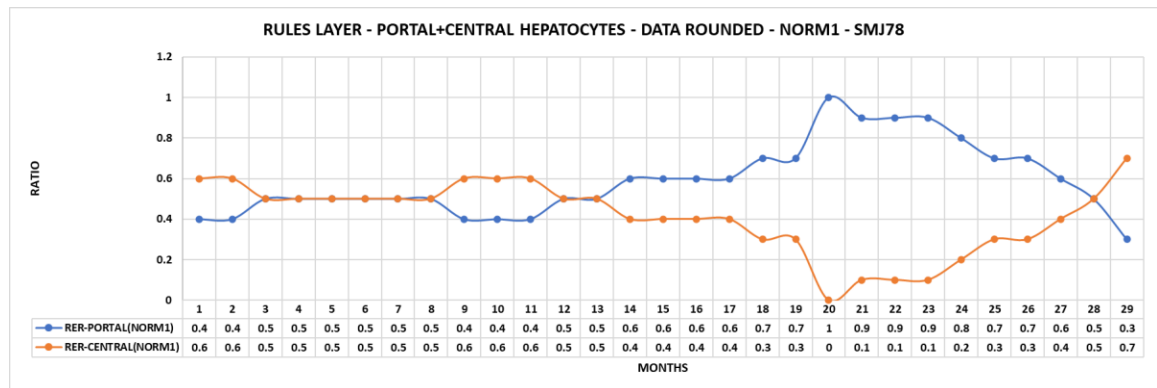


Figure 10.11 By reverse engineering down to the original data pairs, one can see how the relationship between the two parts changed over time.

Notice that most of the variability in the SER and RER compartments disappears when we recombine them into ER (Figure 10.12). The amount of ER surface area in the portal and central cells remained largely the same until it's time to become old at month twenty-four. Now the portal hepatocytes tried to compensate for the ER lost by the central cells. This suggests that aging triggers a severe loss of synthesizing capacity in central hepatocytes.

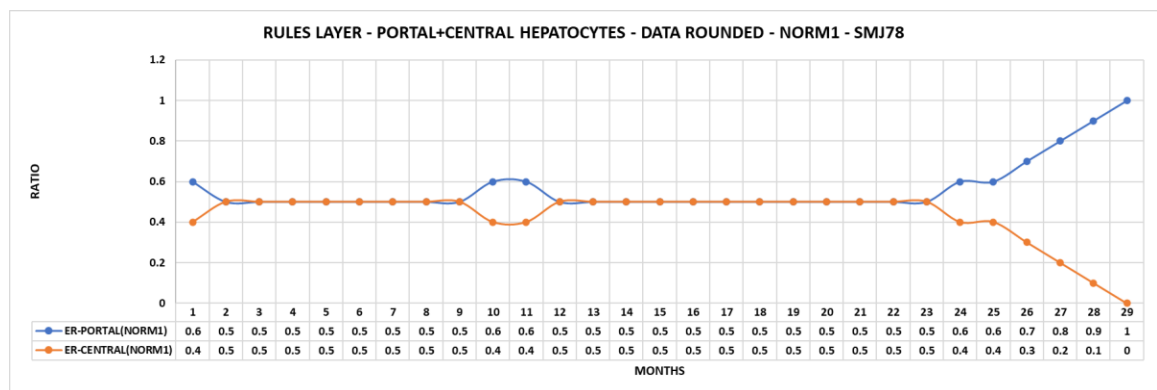


Figure 10.12 At twenty-three months, something happened to cause the ER surface area in the portal hepatocytes to increase but to decrease in the central hepatocytes. Apparently, the central hepatocytes were shutting down. If, for example, we found an ncRNA responsible for the event and blocked it, would the animal outlive its peers?

10.7 Enzyme Densities (ED)

When a cell changes, it redefines relationships of structure to function thereby changing its recipe and phenotype. Enzyme densities capture these complex events by relating enzyme activities to a standard unit of membrane surface area. However, one can calculate enzyme densities (1) from the original data (with $ED = U/S(NORM1)$ or without normalization ($ED = U/S$) and (2) from the original data normalized ($ED = [U(NORM1)/S(NORM1)]$ -normalized (NORM2). The first option applies locally to a given experiment ($ED = U/S$, ED -(NORM1), whereas the second one generalizes the results across publications (ED -NORM2).

- a. **ED (Original Data):** The original data (Table 10.6, Figure 10.13) - related to a gram of liver – supplies the data for the equation: $ED(\text{original data}) = (\text{units of enzyme activity/gram of liver})/(\text{m}^2 \text{ of membrane surface area/gram of liver})$ or simply $ED = (U/g)/(S/g)$.

Table 10.6 Calculating ED from Original Data

DAYS	DEM/G	CYT0P450/G	NADPHCCR/G	ER/G		ED-DEM	ED-CYT0P450	ED-NADPHCCR
0	1.769	9.30	0.0990	6.609		0.2676	1.4078	0.0150
0.25	4.267	11.24	0.0974	7.849		0.5436	1.4321	0.0124
0.5	5.595	12.94	0.0979	8.560		0.6536	1.5119	0.0114
0.75	6.269	14.48	0.0997	8.477		0.7395	1.7076	0.0118
1	6.504	15.89	0.1023	8.399		0.7744	1.8920	0.0122
1.25	6.881	17.23	0.1054	8.326		0.8265	2.0692	0.0127
1.5	7.260	18.52	0.1086	8.258		0.8791	2.2422	0.0132
1.75	7.640	19.77	0.1120	8.195		0.9323	2.4128	0.0137
2	8.022	21.01	0.1156	8.137		0.9859	2.5820	0.0142
2.25	8.406	22.23	0.1190	8.084		1.0398	2.7499	0.0147
2.5	8.792	23.43	0.1230	8.036		1.0940	2.9161	0.0153
2.75	9.179	24.62	0.1272	7.993		1.1483	3.0797	0.0159
3	9.568	25.77	0.1317	7.955		1.2027	3.2396	0.0166
3.25	9.959	26.89	0.1364	7.922		1.2571	3.3943	0.0172
3.5	10.351	27.97	0.1414	7.894		1.3112	3.5429	0.0179
3.75	10.746	29.00	0.1466	7.871		1.3651	3.6842	0.0186
4	11.142	29.98	0.1520	7.854		1.4187	3.8179	0.0194
4.25	11.540	30.93	0.1577	7.841		1.4718	3.9442	0.0201
4.5	11.939	31.83	0.1636	7.833		1.5242	4.0643	0.0209
4.75	12.341	32.73	0.1697	7.830		1.5760	4.1801	0.0217
5	12.744	33.64	0.1761	7.832		1.6271	4.2953	0.0225

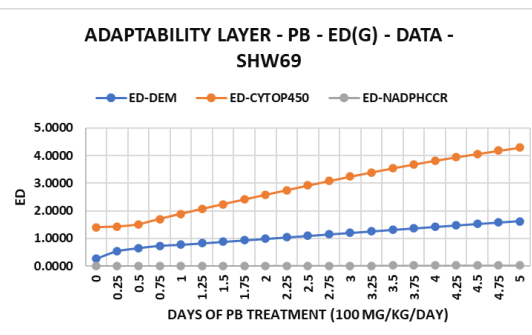


Figure 10.13 Calculating enzyme densities from original data.

The literature, however, creates a problem. Membrane surface area are often related to cm^3 of hepatocyte cytoplasm - not to the required gram of liver. Consequently, calculating the enzyme densities from the mixed data references becomes an illegal procedure (Figure 10.14). This reduced the problem to finding a solution to a paradox. How can the same mixed data references (S/cm^3 and U/g) produce both incorrect (Table 10.7, Figure 10.14) and correct (Figure 10.15) results when used to calculate the same enzyme densities?

The solution to the paradox depended on observing that changes in the same ER surface area related (1) to a cm^3 of hepatocyte cytoplasm and (2) to a gram of liver detected the same changes in the enzyme densities (by comparing polynomial curves and equations) when one normalized all the parts before making the ED calculation. [Worked examples included in the case studies (e.g., 5.3.10) showed this to be the case.]

Table 10.7 Calculating EDs from original data.

DAYS	GS	ER	ED-GS
2	3.727	4.080	0.9135
3	3.687	4.190	0.8800
4	3.650	4.300	0.8489
5	3.616	4.400	0.8219
6	3.586	4.490	0.7986
7	3.559	4.570	0.7787
8	3.535	4.650	0.7601
9	3.514	4.720	0.7444
10	3.496	4.780	0.7314
11	3.481	4.840	0.7193
12	3.470	4.900	0.7082
13	3.462	4.940	0.7008
14	3.457	4.990	0.6928
15	3.455	5.020	0.6883
16	3.457	5.040	0.6859
17	3.462	5.060	0.6841
18	3.470	5.070	0.6843
19	3.481	5.080	0.6852
20	3.495	5.070	0.6893
21	3.512	5.070	0.6928
22	3.533	5.060	0.6982
23	3.557	5.040	0.7058
24	3.584	5.000	0.7168
25	3.614	4.970	0.7272
26	3.648	4.930	0.7399
27	3.685	4.880	0.7550
28	3.725	4.830	0.7711

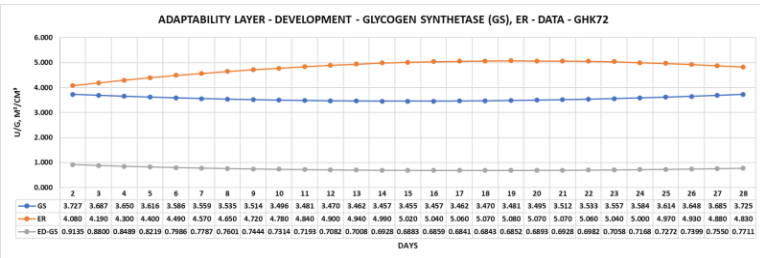


Figure 10.14 How then was the ED calculation calculated from the incompatible data references? The calculation was incorrect. If we multiply the gram of liver reference by 0.7 first (see Case Study 5.3.10), the U/G becomes U/cm³ and the calculation becomes possible. However, this workaround comes at an unknown price. Multiplying the gram by 0.7 assumes a liver density equal to 1.07 g/cm³ of liver and that the hepatocyte cytoplasm always accounts for \approx 70% of a gram of liver (see Case Studies for tests of the method). In a differentiating cell changing its volume in a liver also changing its volume, applying a single correction to data in the adaptability layer merely adds more uncertainty. Original data adapted from Greengard et al., (1972; GHK72)

- b. **ED (NORM1):** Since enzyme assays and membrane surface areas can display a wide range of values, using enzyme densities to calculate ratios in the rules layer can result in uninformative ratios (0:1 or 1:9) when values range from small to large. If this occurs, the structure-function ratios often fail to form useful patterns. Normalizing the original enzyme and membrane data before calculating the enzyme density usually solves the problem (Table 10.8, Figure 10.15.)

Table 10.8 Normalizing Data.

DAYS	ED-GS	ED-GS(NORM)
2	0.9135	1.0000
3	0.8800	0.8537
4	0.8489	0.7182
5	0.8219	0.6007
6	0.7986	0.4993
7	0.7787	0.4123
8	0.7601	0.3313
9	0.7444	0.2628
10	0.7314	0.2090
11	0.7193	0.1594
12	0.7082	0.1049
13	0.7008	0.0728
14	0.6928	0.0379
15	0.6883	0.0184
16	0.6859	0.0078
17	0.6841	0.0000
18	0.6843	0.0009
19	0.6852	0.0046
20	0.6893	0.0227
21	0.6928	0.0378
22	0.6982	0.0616
23	0.7058	0.0943
24	0.7168	0.1426
25	0.7272	0.1880
26	0.7399	0.2434
27	0.7550	0.3092
28	0.7711	0.3793
	0.6841	0.9135
		0.2294

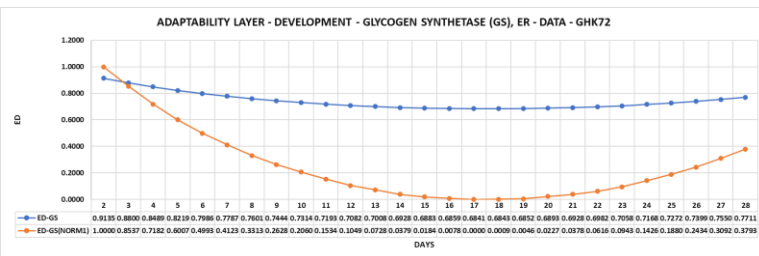


Figure 10.15 The min-max equation normalized the original data (blue line) the using the following: minimum value = 0.6841, maximum value = 0.9135, and max-min = 0.2294.

- c. **ED (NORM2):** Given the wide range of published values for the same enzymes or membranes, aggregating and evaluating data across the literature becomes largely an impossible task. Moreover, reproducing enzyme densities based on the original values in the adaptability layer would require duplicating exactly both the units of enzyme activity per gram as well as the

membrane surface area per gram. Normalization solved both data related problems by removing everything except the changes (Table 10.9, Figure 10.16).

Table 10.9 Calculating $ED(NORM2)$

DAYS	GS	GS(NORM1) ER	ER(NORM1)	GS(NORM1)/ER(NOR
2	3.727	1.0000	4.08	0.0000
3	3.587	0.9524	4.19	0.1100
4	3.650	0.7166	4.3	0.2200
5	3.616	0.5926	4.4	0.3200
6	3.586	0.4803	4.49	0.4100
7	3.559	0.3798	4.57	0.4900
8	3.535	0.2911	4.65	0.5700
9	3.514	0.2142	4.72	0.6400
10	3.496	0.1491	4.78	0.7000
11	3.481	0.0927	4.84	0.7600
12	3.470	0.0541	4.9	0.8200
13	3.462	0.0243	4.94	0.8600
14	3.457	0.0063	4.99	0.9100
15	3.455	0.0000	5.03	0.9400
16	3.457	0.0055	5.04	0.9600
17	3.462	0.0228	5.06	0.9800
18	3.470	0.0519	5.07	0.9900
19	3.481	0.0927	5.08	1.0000
20	3.495	0.1454	5.07	0.9900
21	3.512	0.2098	5.07	0.9900
22	3.533	0.2860	5.06	0.9800
23	3.557	0.3739	5.04	0.9600
24	3.584	0.4737	5	0.9200
25	3.614	0.5852	4.97	0.8900
26	3.648	0.7085	4.93	0.8500
27	3.685	0.8436	4.88	0.8000
28	3.725	0.9904	4.83	0.7500
		3.455	4.880	0
		3.727	5.080	1
		0.272	1.000	

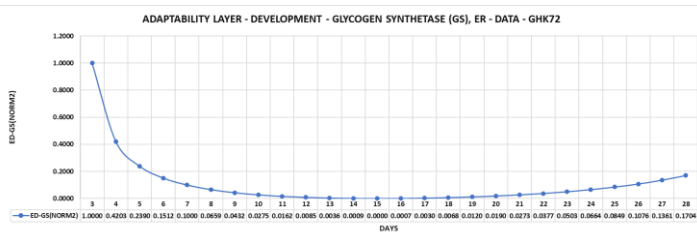


Figure 10.16 In this example, the $ED-GS(NORM2)$ used mixed data references (U/G vs M^2/CM^3). Normalizing each part separately ($NORM1$) removed the data units and references to allow the ED calculation [$ED = GS(NORM1)/ER(NORM1)$]. A second normalization ($NORM2$) recovered the $0 \rightarrow 1$ range. Notice that dividing numbers by zero ($\#DIV/0!$) reduces the number of data points.

10.8 Phenotypes

Biology approaches change as a problem-solving event wherein one cell phenotype transforms into another. The process, which occurs over time, involves multiple levels of complexity. A change starts with pairs of parts (data pairs) that form subgroups to work on different parts of the problem until a partial or full solution appears. In effect, a change progresses from detecting a problem with the current phenotype to the resolution of the problem defined by a newly configured phenotype. With everything properly adjusted (presumably optimized), the widely ranging ratios of multiple data pairs become synchronized to single or multiple ratios. Such a solution allows the entire dataset (a set of subgroups) to act as a single, cohesive unit focused on solving a specific problem, while at same time fulfilling its many housekeeping duties. More complex changes, such as those seen during development, display temporary solutions that continually update during differentiation.

- Aggregated Results from Multiple Publications (Global Phenotype):** Aggregating enzyme densities ($ED-NORM2$) across publications generates (forward engineers) a global phenotype. Taking a phenotype apart (reverse engineering) allows us to follow the formation of multiple subgroups each of which solves its subproblem. Using three consecutive days with the same ratio to indicate a solution, one can identify the individual data pairs belonging to a given subgroup within the phenotype. In turn, the reverse engineering continues with the subgroups down to individual data pairs. For further details and references, see Case Studies (Chapters 5-8).

Table 10.10 applies color coding to pairs of enzyme densities (data pair ratios) to identify the subgroups (three consecutive days with the same ratio). When plotted, the subgroups become the phenobarbital phenotype displayed in Figure 10.17.

Table 10.10 Table used to plot the changes in the enzyme densities of the data pairs and the highlight the values of the last three days of the dataset.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

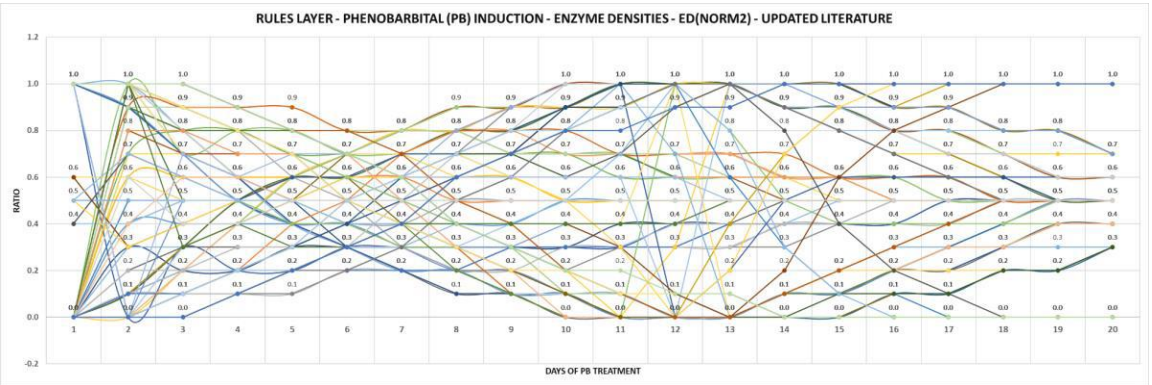


Figure 10.17 The plot chronicles the progress of the data pairs ratios (pairs of enzyme densities) as the hepatocytes find the many solutions needed produce an enzyme-membrane recipe that solves its phenobarbital problem. The fact that local solutions occur at different times adds substantially to the complexity of a biological change and explains why reverse engineering the changes became a key part of the data analysis.

b. Multiple Publications Contributing to the Solution: Multiple data pairs repeatedly changed their ratios but eventually synchronized to a single ratio (e.g., 0.5:0.5), as shown in Figure 10.18. When the data pair ratios became persistent, they defined the subgroup. In turn, the subgroups defined the phenobarbital phenotype.

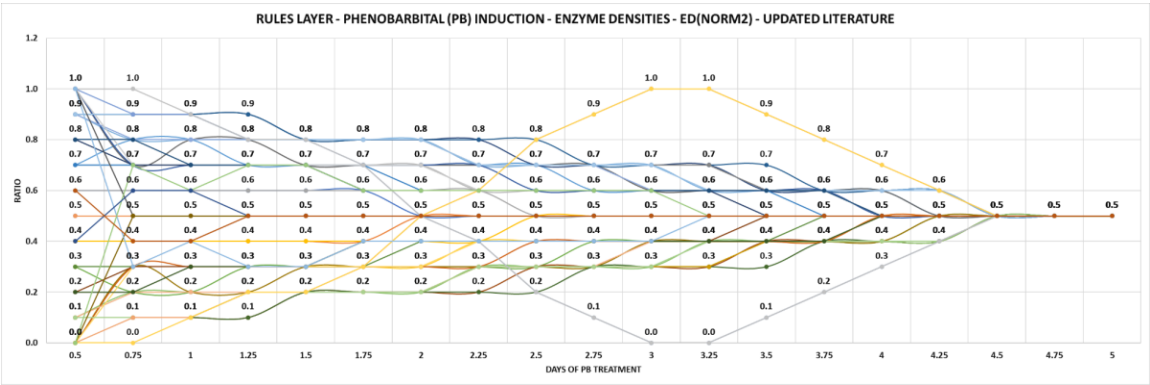
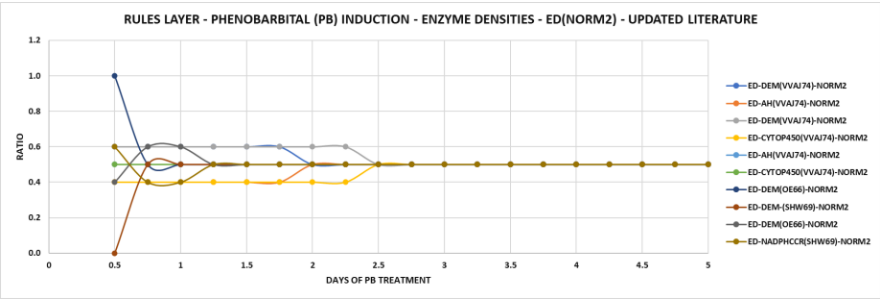


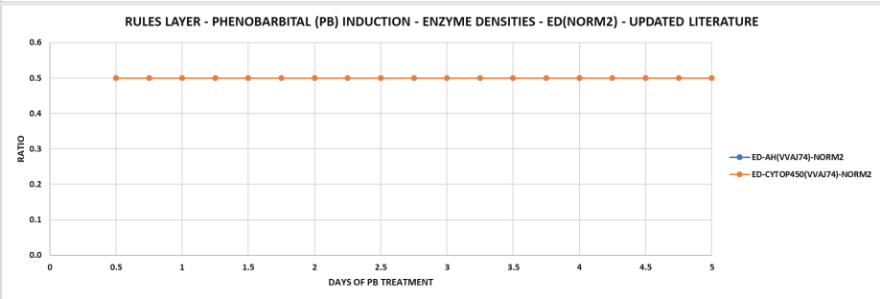
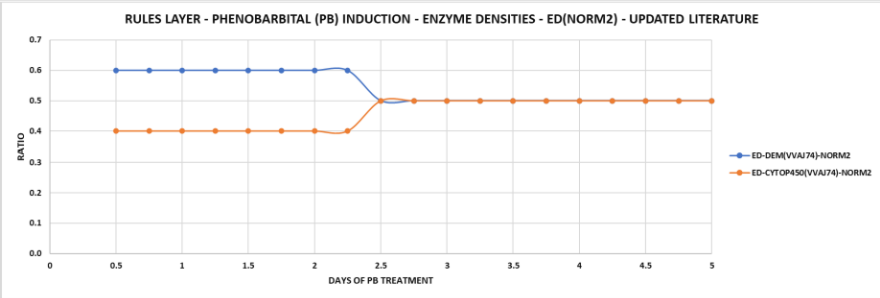
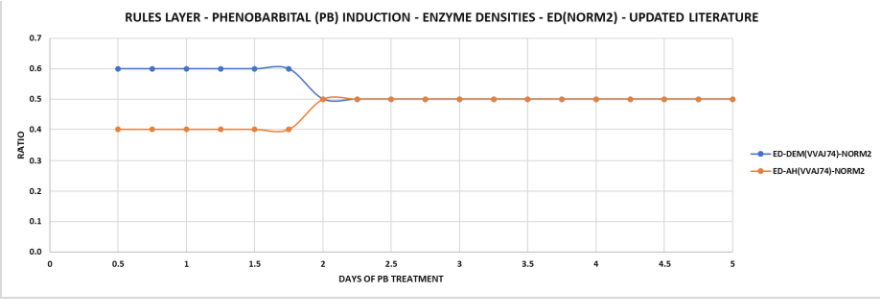
Figure 10.18 Reverse engineering the phenotype shown in Fig10.16 delivered the first subgroup (0.5:0.5). To view the details of the problem-solving process for the first subgroup, one can reverse engineer the subgroup down to the changes that occurred in a single data pair ratio.

Beginning with the small sample of the data pair ratios shown in figure 10.18, one can reverse engineer the changes down to single data pair ratios. The point? To show empirically that the biology literature can (1) identify candidate enzymes for coordinated gene expression and (2) demonstrate that the biology literature represents a primary source of reproducible results (Figure 10.19).

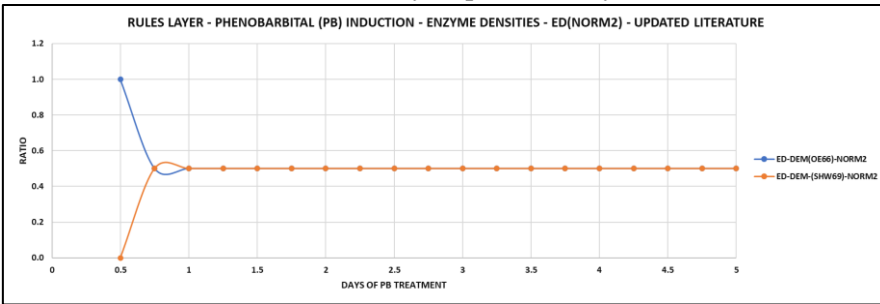
Subset of Data Pair Ratios



Individual Data Pair Ratios (Identify Coordinated Expression – One Study)



Individual Data Pair Ratios (Identify Reproducibility – Two Studies)



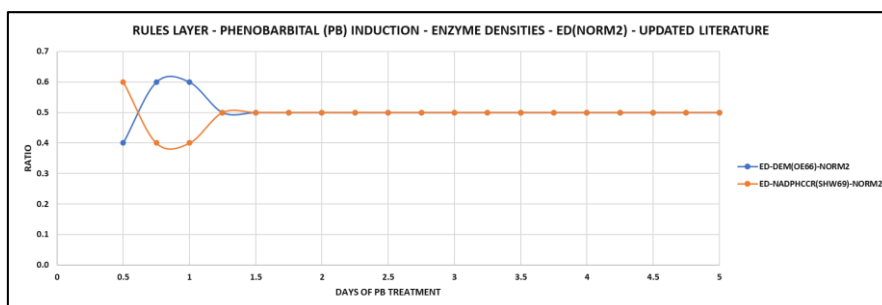


Figure 10.19 Starting with a subset of data pair ratios with a 0.5:0.5 solution, reverse engineering the subgroup to single data pair ratios, created opportunities (1) to predict coordinated gene expression and (2) to demonstrate reproducibility based on data taken from the biology literature. Notice that SHW69 reproduced 95% of the values reported by OE66 and was 89% compatible with another enzyme involved with drug metabolism. In effect, one can use the reproducibility of rules to demonstrate a biological change. See Figure 10.20 for additional examples.

- c. **Publications Duplicating the ED Solutions During the Last Three Data Points of Phenobarbital Treatment:** Although the solution to the phenobarbital problem for this subgroup was the 0.5:0.5 ratio, different data pairs found the solution at different times. The histogram shown in Figure 10.20 identifies the data pairs with persistent solutions for three consecutive time points (days 4.75 to 5.0). The same data pair ratio coming from two different publications becomes a measure of reproducibility.

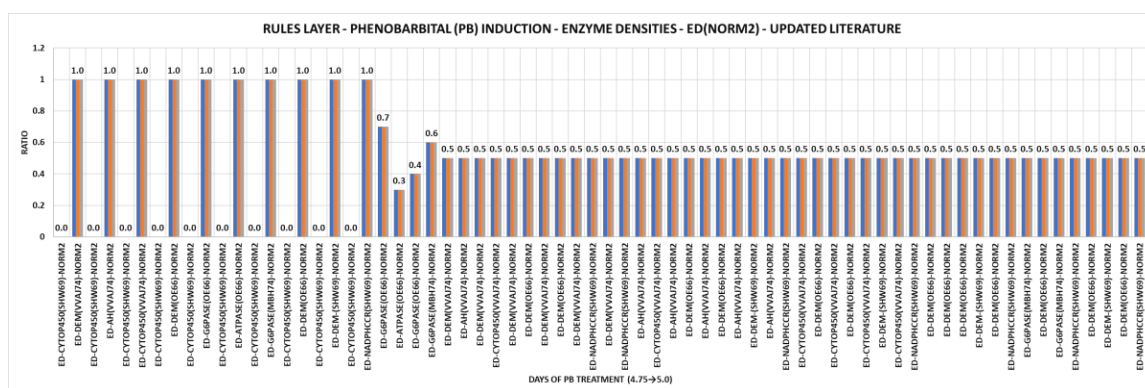


Figure 10.20 Data pair ratios calculated from enzyme densities demonstrated the widespread presence of reproducibility across publications as duplicated data pairs. Since different author codes demonstrated the same solution (0.5:0.5), the histogram offers empirical evidence that would seem to suggest that hepatocytes have solved their reproducibility problem globally (across individuals and populations). By copying the hepatocyte's solutions, we can also show that our published results are likewise reproducible. Note that OE66 included two publications.

10.9 Standardizing Slopes

Chapter 2 used slopes to compare ER data across eleven different studies. Two of the publications, however, included mitochondrial data but not ER. Using data from Blouin et al. (1977) as a standard, membrane organelles expressed as a ratio and percentage served as a lookup table (Table 10.11). Entering an ER surface area (as a seed value) resulted in a string of standardized values. For the mitochondrial membranes, entering the mitochondrial surface area (IMIM) displayed a standardized value for the missing ER.

Table 10.11 Using a standard data set, a value for the IMIM (3.4300) generated the missing ER surface area (5.3198) generated

STANDARD DATA SET (HEPATOCYTE) EXPRESSED AS A MEMBRANE SURFACE AREA PER GRAM OF LIVER											
HEPATOCYTE		UNITS	PM	ER	RER	SER	MIM	OMIM	IMIM	GOLGI	SUM
		m ² /g	0.4983	3.5799	2.2383	1.3430	3.3042	0.9971	2.3082	0.1518	14.4209
		%	0.0346	0.2482	0.1552	0.0931	0.2291	0.0691	0.1601	0.0105	1.0000
	ENTER A SEED VALUE										
	↓										
PM	0.4983	m ² /g	0.4983	3.5799	2.2383	1.3430	3.3042	0.9971	2.3082	0.1518	14.4209
ER	3.5799	m ² /g	0.4983	3.5799	2.2383	1.3430	3.3042	0.9971	2.3082	0.1518	14.4209
RER	2.2383	m ² /g	0.4983	3.5799	2.2383	1.3430	3.3042	0.9971	2.3082	0.1518	14.4209
SER	1.3430	m ² /g	0.4983	3.5799	2.2383	1.3430	3.3042	0.9971	2.3082	0.1518	14.4209
MIM	3.3042	m ² /g	0.4983	3.5799	2.2383	1.3430	3.3042	0.9971	2.3082	0.1518	14.4209
OMIM	0.9971	m ² /g	0.4983	3.5799	2.2383	1.3430	3.3042	0.9971	2.3082	0.1518	14.4209
IMIM	3.4300	m ² /g	0.7405	5.3198	3.3261	1.9956	4.9100	1.4816	3.4300	0.2256	21.4293
GOLGI	0.1518	m ² /g	0.4983	3.5799	2.2383	1.3430	3.3042	0.9971	2.3082	0.1518	14.4209

Fitting the highlighted values to a linear regression produced a linear equation with a slope (1.486) (Figure 10.21).

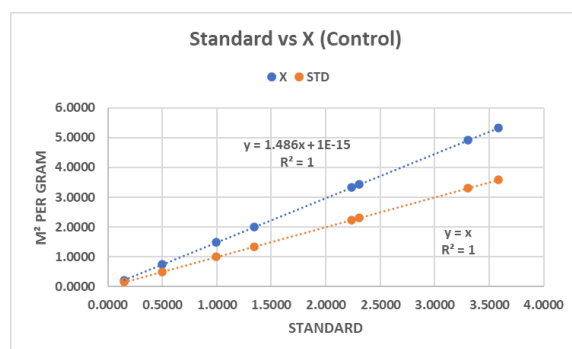


Figure 10.21 A single mitochondrial surface area predicted a value for the missing ER (5.3198) and generated a slope (1.486).

10.10 Making the Transition from Simple to Complex

Traditionally, we took cells apart to study their structures and functions and to detect significant differences between control and experimental data points. Because of the overwhelming complexity of biology and the absence of advanced technologies, focusing just on the parts was by itself a remarkable undertaking. But our technologies and methods have caught up to the challenges of biology's complexity to the point where we can begin to copy and reverse engineer our way to a first principles approach. Although meta-analyses report that the literature may be wobbly statistically, it nonetheless harbors a wealth of information that we can update to serve our current needs and interests.

We took biology apart and created a monumental collection of isolated parts – the biology literature. The time had come to put the parts back together, to return the connections and the complexity. The primer started with two disciplines (biochemistry and morphology), a road map (the postulates of biochemical homogeneity), and a set of goals (detect biological changes, reproduce results, and derive biology from first principles empirically).

Figure 10.22 summarizes the move from simplicity to complexity. The rules layer model reconnected the parts, supports the view that living systems operate by rules based on ratios, and reveals that cells change to solve problems by growing into the solutions. A biological change has many moving parts with the goal of redefining complex relationships of structure to function. Detecting, reproducing, and explaining the complexities of a biological change is not a simple task. It's far removed from demonstrating a significant difference between two data points.

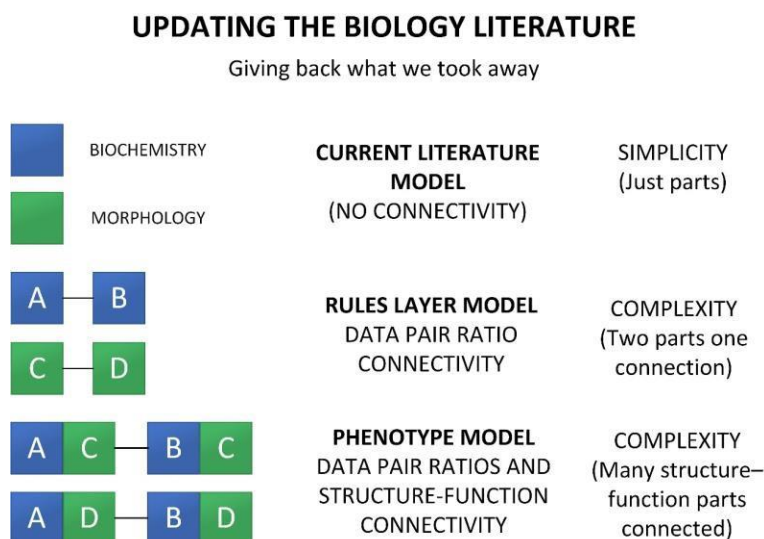


Figure 10.22 Parts, connectivity, rules, and relationships define biological changes. Models provide new strategies for detecting and interpreting changes.

10.11 Reference Table for Enzyme Locations

Table 10.12 Abbreviations and putative locations of marker enzymes as suggested by authors and others.

NADPHCCR	ER	NADPH CYTOCHROME C REDUCTASE
CYT0-P450	ER	CYTOCHROME P-450
N-DEM	ER	N-DEMETHYLASE
G6PASE	ER	GLUCOSE 6 PHOSPHATASE
5'NUC	PM	5' NUCLEOTIDASE
MAO	OMIM	MONOAMINE OXIDASE
CYOX	IMIM	CYTOCHROME C OXIDASE
ATPASE	IMIM	ATPASE
CYOX	IMIM	CYTOCHROME C OXIDASE
MAO	OMIM	MONOAMINE OXIDASE
ATPASE	MI MATRIX	ATPASE
G6PASE	ER	GLUCOSE 6 PHOSPHATASE
ESTERASE	ER	ESTERASE
NADPHCCR	ER	NADPHCCR
ATPASE	IMIM	ATPASE
CYOX	IMIM	CYTOCHROME C OXIDASE
MAO	OMIM	MONOAMINE OXIDASE
GLUTDH	MI MATRIX	GLUTAMATE DEHYDROGENASE
G6PASE	ER	GLUCOSE-6-PHOSPHATASE
UDP-BGT	ER	UDP-BILIRUBIN GLUCURONYL TRANSFERASE
CYT0-P450	ER	CYTOCHROME P-450
CYT0-B5	ER	CYTOCHROME B-5
AMD	ER	AMINOPYRINE-N-DEMETHYLASE
BPH	ER	BENZOPYRENE HYDROXYLASE
FR(NADPH)	ER	FERRICYANIDE REDUCTASE (NADPH)
FR(NADH)	ER	FERRICYANIDE REDUCTASE (NADH)
CYT0-C-R(NADPH)	ER	CYTOCHROME C REDUCTASE (NADPH)
CYT0-C-R(NADH)	ER	CYTOCHROME C REDUCTASE (NADH)
LP(NADPH)	ER	LIPID PEROXIDATION (NADPH)
SCOD(NADPH)	ER	STEARYL CO A DESATURASE (NADPH)

EPILOG

Changes in living systems occur chiefly in cells. This means that understanding how cells change becomes basic to understanding the rules and principles of living systems. Currently, we believe that we can detect changes in cells by demonstrating a significant difference between the same part measured under control and experimental conditions. Meta-analyses (Ioannidis 2005, Ioannidis et al., 2015), however, have openly challenged this belief by showing that most or all the results published in the biology literature are incorrect - at least statistically. Moreover, we continue to struggle with the task of reproducing experimental results. Since both problems map back to a biological change, could our inability to detect a biological change correctly account for both shortfalls? What if a significant difference is not a change and the absence of reproducibility is the absence of change? This would mean that solving the biology literature should begin by updating the way we detect biological changes. Since this required a return to the basics, a primer became a logical place to put such questions to the test.

We have at least three options. We can (1) fix our statistical problem by increasing our sample sizes, (2) figure out how to reproduce experimental results, or (3) avoid options one and two by deriving biological changes from first principles empirically. The primer chose the easiest option (first principles) because it only required figuring out how to copy the way cells change. This meant going directly to the source (to biology via an updated version of the literature). Besides, Harold Morowitz and many in the Biomatrix group were convinced that organizing published data was the key to a first principles approach and Harold M. Schoolman (NLM) pointed out that one can organize published data simply by generating tables of data ratios (personal communication). Since Weibel and Paumgartner (1978) with Roger Miles solved the section thickness problem of biological stereology, an empirical approach to first principles was eminently doable. Ironically, the first two options would have worked only if the original cell changes were correct to begin with. They were not.

By updating published data and copying the basics of a biological change from cells, the primer became a road (albeit bumpy) leading to a first principles approach based empirically on forward and reverse engineering cell phenotypes. Although lacking the argument of an effect size calculation, the updated publications still managed to identify changes and complex patterns in cells and phenotypes reproducibly. Since a statistical approach was out of reach (no raw data), reproducibility became the surrogate referee for validating changes. The case studies routinely used reproducibility, recoveries, and patterns to test the proposed models and methodological workarounds. By example, they show how to update the literature.

By taking a rule-based approach to designing and interpreting experiments, we can minimize the negative effects of our methods and begin to approach the cell as a complex mathematical entity, one that runs communication systems and advanced algorithms with computational awareness. A cell change is not about the difference between two data points, it's about complex problem solving the likes of which extend well beyond our current understanding. The challenge now becomes one of figuring out how cells have corralled quantum mechanics to perform remarkable feats in our familiar world of classical mechanics. Knowing where, when, and how to look for "impossible" outcomes will supply the clues needed to copy our way to a postulated bridging theory sitting between classical and quantum mechanics. Cells have already demonstrated their skills by accomplishing photosynthesis, visual systems, brains, optimization, and complex problem solving - no doubt by applying mathematically elegant solutions.

Everything we could ever know or understand either comes from or through our biology.

In cells, the essential features – rules, principles, changes, reproducibility, relationships of structure to function, and complexity – all come from ratios.

Complexity derives from parts, connections, and rules. Without connections cells have no rules, without rules cells have no change. Give the connections back to the parts and the cell's rule-based changes return. Updating published results solves the biology literature by giving back to the cells back what we took away because at the time it was our only option. Since other options now exist, our future becomes a question of choice.

KEY FINDINGS

1. **Same data, two interpretations.** (1) Adaptability Layer: single point data gives three possible outcomes ($\uparrow, \downarrow, \leftrightarrow$), and (2) Rules Layer: two data points give five possible data pair ratios (0.1: 0.9, 0.2: 0.8, 0.3: 0.7, 0.4: 0.6, 0.5:0.5) where two parts and one connection (the ratio) define a complexity. The single value of the adaptability layer accommodates a wide range of values while remaining subject to the data pair ratios of the rules layer. The rules layer enables a first principles approach wherein ratios become the basic rules, which are reproducible.
2. **Change as a strategy for problem solving.** The cell changes from one phenotype to another to solve a problem. In short, the cell becomes the solution by changing its structure-function recipes. Such recipes generate or contribute to the emergent properties of the cell (still undefined and unpredicted).
3. **Postulates of biochemical homogeneity.** Tested twice using recoveries; passed both times.
4. **Relationships of structure to function.** When cells change, they instigate wide-scale changes to their parts by redefining relationships of structures to functions. By allowing more than one solution to the same problem, complexity adds redundancy to increases survivability.
5. **Forward and reverse engineering cells requires a first principles approach.** A cell change involves sets of specialized changes occurring by rule within multiple subgroups. The cell continues to change its parts and connections until it finds a persistent rule-based solution.
6. **Complexity Theory.** Based on first principles copied from cells, the theory now posits that the connectivity of data pair ratios adheres to the notion of classical entanglement wherein by knowing the value of one part, one automatically knows the value of the other. Entanglement would explain (1) the near perfect timing of the production and distribution of RER and SER membranes, the interacting polynomial wave functions 180° out of phase, and the just in time solutions displaying identical values with opposite signs (+ or -) and (2) the changes in the data pair ratios that occur as phenotypes subcontract the larger change to smaller subgroups, which redefine new relationships of structure to function. The reach of quantum mechanics into classical complexity theory seems to have surfaced in hepatocytes using a combination of entanglement (RER:SER) and the cellular equivalent of our mean value theorem of calculus to solve a production and distribution problem. If cells use "calculus" to change, then the combination of entanglement and optimizing routines might begin to explain the mechanism behind the problem-solving event.
7. **A mashup and normalized data.** By sharing data (mashup) and removing disruptive data units (normalization), published data became suitable for forward engineer cell phenotypes. While the initial assumptions of data compatibility seemed unlikely, extensive testing proved the opposite. Although unconventional workarounds became necessary but certainly not recommended, the literature - as the sole source of data - drove the solutions. A first principles approach to change eliminates such cumbersome fixes.

7. **Reproducibility.** As a major product of the rules layer, reproducibility becomes fundamental to practically everything. Reproducibility, which is simply detecting the same change a second time, occurs abundantly throughout the updated biology literature. In the adaptability layer, reproducibility has the best chance of working by relating enzyme or membrane data to the volume (or weight) of the parent organ or gland. Concentration data (per mg protein or per gram) can be expected to detect a biochemical or morphological change correctly only about 50% of the time.
8. **Published data interpret cell changes as just biological.** However, published results typically include two sets of equally important changes (1) changes produced by cell parts and (2) changes produced by experimental methods. Updating the biology literature mitigates the damage (changes) coming from the methods.
9. **A biological change alters relationships of biochemistry to morphology.** Estimated separately, a biochemical or morphological change detects only part of the larger and more complex event.
10. **Detecting a change as a significant difference.** While investigators can detect a biological change by demonstrating a significant difference between two data points, cells cannot because they must execute a change as a complex event involving many parts and connections.
11. **Perception of a Change.** Our training teaches us to see change statistically as a binary event (yes or no), whereas cells see the same event as many changes contributing to a larger solution. Since cells know how to change correctly by rule, the primer swapped the convenience of experimental assumptions for the guidance coming from cells.
12. **The Reality:** In cells, the essential features – rules, principles, changes, reproducibility, relationships of structure to function, and complexity – all come from ratios. The ratios are the rules.

RECOMMENDATIONS

1. **Publish raw data.** Data characterizing individuals contain critical information that disappears into mean values. Individuals contain unique patterns related to health and disease and belong to distinctive populations. Following changes in rules becomes a diagnostic and predictive tool.
2. **Organize published data using named phenotypes.** A cell phenotype combines published data related to the same topic (development, disease, treatments, aging, etcetera). By allowing data to combine across publications, the larger datasets become more effective when detecting changes and identifying the algorithms in play. This leads to the development of large-scale simulations.
3. **Derive biology from first principles empirically.** By recovering the original complexity of cells, the rules, principles, and algorithms used by living systems become available empirically. By interacting with the cells mathematically, we can copy, detect changes, and get help from cells.
4. **Shift the focus of research from simple to complex.** Since biology exists as a complexity, copying and studying cells as complexities leads to more realistic outcomes.
5. **Extend the reach of a research paper beyond the demonstration of a significant difference.** Currently, identifying a significant difference terminates the experiment because the data trail abruptly ends when the p values appear. Without access to the details of a complex change, authors cannot explain how or if the cells solved the problem being studied by the experiment.
6. **Design and execute experiments around a central core of reproducibility.** Reproducibility is at the heart of experimental biology – from sampling data to detecting changers to verifying outcomes.
7. **Approach biology as a complex information processing entity.** Cells operate within the framework of a big data system, one that's based on biological complexity and subject to rules and principles of classical and quantum mechanics. Know how to detect a change and you know how to simulate it.
8. **Identify and put currently permitted assumptions to the test.** The biology literature is awash with untested assumptions protected largely by reductionism. By restoring their complexity, cells quickly reveal the mischief created by reducing biology to a simplicity (a collection of isolated parts).
9. **Organize the biology literature as a renewable resource.** Update it regularly.

GLOSSARY

ABSOLUTE DATA – Data expressed as volume, surface, length, or number of parts.

ADAPTABILITY LAYER – Original data with corrections applied for section thickness and compression. It's interpreted and reproduced with ratios in the rules layer.

ALGORITHM – A step-by-step sequence of operations used to perform a specific task.

ALPHANUMERIC – A set (or string) of characters containing letters and numbers (e.g., a mathematical marker).

ANALOGUE – One thing comparable to another.

BIAS – Identifies something that introduces systematic variation in research data, a regular not an irregular distortion (variable error).

ENZYME DENSITY (ED) – A complex data type relating a biochemical constituent to a morphological component. An enzyme density ($ED = U/S$) allows changes in morphological surface area (S) and in units of biochemical activity (U) to occur relative to a constant reference (one square meter of membrane surface area) – at a time when everything else can be changing.

BIOLOGICAL CHANGE – It defines a complex rule-based event extending across hierarchical levels of complexity within a phenotype, which includes, for example, subatomic particles, molecules, organelles, cells, and organs.

BIOLOGICAL VARIATION – normal differences between individuals due to differences in genomes, health, and environmental factors.

BLACK BOX – A construct involving a collection of components hidden from the observer.

BLUEPRINT – A detailed outline or plan of action; a design.

BUTTERFLY – In chaos theory, the butterfly effect exemplifies the dependence of events on initial conditions wherein minor changes can cause large effects. For example, the disorder created by the flapping of a butterfly's wings can trigger a distant storm.

GRAPH TO NUMBER EQUATION – used to extract numerical data from graphs: $y \text{ value} = (((y_{top} \times y_{length}) / (y_{units})) - y_{from_top}) \times (y_{top} / ((y_{top} \times y_{length}) / (y_{units})))) -$ (Bolender, 2019)

CASE STUDY - Updates published data to (1) detect biological changes quantitatively, (2) combine published data across studies, and (3) generate new forms of information from old.

HEPATOCYTES (CENTRAL) – Occur in the middle portion of the liver lobule.

HEPATOCYTES (PORTAL) – Occur in the portal portion of the liver lobule.

CASE STUDY – Updates a published study by applying rules and principles copied from biology.

CHANGE MODELS – A reductionist model defines change as a significant difference between two biological parts, whereas a complexity model copies biology’s problem-solving approach to change.

CHAOS THEORY – A branch of mathematics that deals with complex systems. Such systems display an underlying order, wherein small events can trigger large and complex outcomes. The theory identifies the “edge of chaos” as the condition wherein unexpected things can happen. The “data rounded” plots of the rules layer approximate the condition.

CLASSICAL COMPLEXITY THEORY – Includes the rules and principles biology uses to design, produce, maintain, and change relationships of structure to function.

CLASSICAL COMPLEXITY THEORY (INFLUENCED BY QUANTUM THEORY) – Identifies or posits the properties of quantum mechanics detected within the boundaries of classical mechanics.

CLASSICAL ENTANGLEMENT (BIOLOGICAL) – Posits that properties attributed to quantum mechanics appear in biological settings consistent with classical mechanics.

COEFFICIENT OF DETERMINATION – A measure of fit between dependent and independent variables in regression analysis; abbreviated R^2 .

COMPLEX DATA TYPES – Identifies a biological data type that can function as (1) an equation that defines a change (enzyme density), (2) a set of variables that define a solution as a recipe, and (3) pairs of connected parts (simple or complex) that transform one cell phenotype into another.

COMPLEX DATA REFERENCE – Defines a virtual space as a constant reference, which becomes the result of dividing an enzyme activity by a membrane surface area before or after normalization.

COMPLEX SYSTEMS – Composed of connected parts. They exhibit properties that emerge from the interaction of parts, which one usually cannot predict from the properties of individual parts.

COMPLEXITY MODEL – A model used to detect biological changes by copying the way cells change.

COMPLEXITY THEORY – Complex outcomes emerge from simple rules that produce large networks of interacting parts and connections.

CONCENTRATION – The amount of a constituent (or component) divided by the total volume of the reference or containing space; expressed per unit volume. Reference spaces can also include surface, length, and number. The amount of a part contained within a defined space.

CELL PACKING PROBLEM (CONCENTRATION TRAP) – Identifies a methodological error that can occur when attempting to detect changes in cells with concentration data. While the volume of the reference compartment (the denominator) remains constant, the number of cells needed to fill the reference volume changes. In effect, both the numerator and denominator behave as variables. The trap – widely unknown – can reduce the reliability of experimental results by 50% or more (Bolender, 2019). Investigators routinely assume that the number of cells contained within a unit of reference volume remains constant.

CONFOUNDING VARIABLES – Represent contaminating variables. When ignored, they become assumptions built into the experimental design. Since experimental biology adheres to a reductionist model, the negative effects of such variables are difficult to detect and correct.

CONNECTION – Something that connects two or more things. In biology, connections can represent ratios derived from two or more parts.

CORRECTION FACTORS – Used to correct biased measurements and methodological errors.

DATA LAYERS – Published data occur in the (1) adaptability layer as single values and in the (2) rules layer as data pair ratios.

DATA PAIR (DATA PAIR RATIO) – A ratio of two numerical values.

DATA PAIR RULE – A quantitative relationship exists between two parts (A, B) when expressed as a ratio (A:B).

DATA REFERENCE – When expressed as a concentration (A/B), a numerator (variable) becomes divided by a denominator (constant). In experimental biology, however, the denominator often becomes a variable. See **CELL PACKING PROBLEM**.

DATA ROUNDED – Found in the rules layer, it expresses ratios with a single decimal place (e.g., 0.5). This speeds the task of finding, comparing, and analyzing complex patterns. It treats change as ratios of whole small numbers. For example, $0.5:0.5 = 1:1$ and $0.4:0.6 = 2:3$.

DATABASE – A structured dataset usually accessed, managed, and updated with computers.

DATA-DRIVEN – Progress propelled by data, rather than by methods.

DENSITY – A term used in stereology to describe a concentration.

DESCRIPTIVE BIOLOGY – A qualitative approach to biology.

DESIGN-BASED SAMPLING – Sampling independent of size, shape, orientation, and distribution of a given set of parts. Designed to minimize sampling bias, the method samples every part of a structure with the same probability.

DEVELOPMENTAL PHENOTYPE – Documents developmental changes with data pair ratios extending across multiple levels of complexity.

DIAGNOSE – Identify a normal or abnormal state by examining symptoms or quantitative patterns.

DIAGNOSTIC DATABASES: Based on data pair ratios, these databases use unique or duplicate markers to identify outcomes.

DISEASE – A disorder of structure or function (or both), often producing specific signs, symptoms, and quantitative patterns.

DISORDER – A malady or dysfunction.

DISRUPTION – To break apart or alter, thereby altering the normal state.

DISTORTED – Not representing reality.

DUPLICATE – One of two or more identical parts.

DUPLICATED PATTERNS – When one pattern superimposes another in part or totally.

ELECTRON TRANSPORT ENZYMES – Involved in the production of ATP.

EMERGENT PROPERTY – Connected parts display new properties equal to more than those of the individual parts: (1) the whole becomes greater than the sum of the parts and (2) properties do not reduce to those of the individual parts.

EMPIRICAL – Identifies outcomes based on testing or experience instead of being determined theoretically.

ENZYME DENSITY (ED) – A ratio (U/S) defining the relationship of an enzyme activity (U) to a morphological reference (usually a surface area (S)). For example, it provides the amount of activity associated with one square meter of membrane surface area or its equivalent.

ENZYME DENSITY MODEL – Relies on a structure-function equation ($ED = U/S$) to detect and interpret biological changes originating in cells. By encapsulating the postulates of biochemical homogeneity, it turns theory (the quantitative relationship of morphology to biochemistry: $ED = U/S$) into practice (detecting and interpreting biological changes).

ENZYME DENSITY RULE: Defines a quantitative relationship that exists between units of marker enzyme activity (U) related to a standard unit of membrane surface area (S): $ED = U/S$.

EQUATION – An expression stating that two things are equal.

ERROR – A deviation from accuracy or correctness.

EXPANDED DATA – When fitted to equations with regression analysis, few data points can generate many data points.

FIRST PRINCIPLE: A first principle can be a law (1) upon which others rely or (2) used to derive laws.

FIRST PRINCIPLES APPROACH – Biology operates by rule according to its first principles. Copying the way biology changes, for example, becomes a first principles approach.

FORWARD ENGINEERING – For the literature update, it describes the aggregation of biological parts into a phenotype by applying rules based on biology's first principles.

GENERALIZATION – A general statement, law, principle, or proposition.

GENOTYPE – Genetic constitution of an individual.

GLOBAL – Involving all of something.

GLOBAL PHENOTYPE MODEL – Includes rule-based procedures for integrating data across publications when reconstructing (forward engineering) and deconstructing (reverse engineering) phenotypes.

GOLD STANDARD – A standard used to judge or evaluate others.

HIERARCHY – A series of ordered groupings.

HOMOGENATE – Biological tissue ground up and mixed (mechanically disrupted).

INDUCTION PHENOTYPE – Designed to analyze and compare the responses of cells to drugs and toxins within and across publications.

INFORMATION PROCESSING UNIT (THE CELL) – Since cells continually process information by rule, they contain facilities consistent with such activities. The primer predicts the presence of algorithms, advanced mathematics, and computational devices in cells.

INTACT TISSUE – Complete, undamaged, and not homogenized.

INTERNET BRAIN VOLUME DATABASE (IBVD)– MRI data of patients expressed as volumes (Kennedy et al., 2012).

LITERATURE UPDATE – Reinterprets published data by shifting from a reductionist approach to one based on complexity as defined by biology. In effect, it treats published data as a renewable resource, one that continues to provide new forms of information well-suited to the task of solving current problems of complexity.

LIVER CAPACITY – The sum of the capacities of individual cells and representing an amount adequate to the task at hand. Used as an indicator and test of a successful outcome (change).

LIVER LOBULE – A subdivision of the liver consisting of subpopulations of hepatocytes surrounded by vascular and glandular components.

MARKER ENZYME – An enzyme (1) associated with a specific morphological location and (2) adhering to the rules of biochemical homogeneity.

MASHUP – A method for generating additional information by combining data from one or more sources.

MASHUP MODEL – Used by the update to share data within and across publications.

MATHEMATICAL MARKER – An alphanumeric string designed to capture the basic units of complexity specific to a given location and/or condition.

MEAN VALUES – The average of a set of numbers.

MEMBRANE DENSITY (MD) – A ratio (S/U) defining the amount of membrane surface area (S) supporting one unit (U) of enzyme activity.

META-ANALYSIS – A statistical method that uses pooled data to test for significance.

METHODOLOGICAL CHANGES – Changes that run parallel to those of biology, which can display similar or different magnitudes and directions. They distort and limit access to biological changes.

METHODOLOGICAL VARIABLES – Introduce errors and biases into experimental results. Examples include: (1) data collected from a changing number of cells during an experiment, (2) failure to adhere to theoretical requirements (stereological data collected from thick and compressed sections), (3) biased sampling, and (4) failure to check results by calculating recoveries.

METHODS-DRIVEN – An activity based on improving outcomes by updating methods.

MIRROR IMAGE SYMMETRY – Reflection, about an axis.

MORPHOLOGICAL DATA REFERENCES – Data related per gram, per organ, and per meter squared (ED) can lead to sustainable results when detecting and reproducing biological changes.

NESTED COMPLEXITY – Complexity embedded in complexity. Unfolding and refolding nested complexities identifies a major undertaking of complexity theory.

NETWORK – A system of interconnected parts.

NORMALIZED DATA – Data reorganized in standard form. For example, a normalized biological change displays values ranging from 0 to 1 and no longer carries data units.

NORMALIZATION FORMULA – See Wikipedia.

OBJECTIVE – Not influenced by personal feelings or opinions; identified with quantitative approaches.

ORIGINAL DATA – For the update, they include data as published with morphological corrections applied (unless stated otherwise).

PARALLEL COMPLEXITY – A collection of mathematical markers serving as a proxy for biology; a construct designed with a specific goal in mind (e.g., diagnosis). It serves as a strategy for copying biological rules and changes.

PATTERN – A repeated design; an arrangement or sequence; things arranged by rule.

PERMUTATION – The way in which to order a set of numbers or items.

PHENOBARBITAL PHENOTYPE – Changes occurring within and across sets of subgroups that together become a general solution to the problem of exposing hepatocytes to phenobarbital.

PHENOTYPE – The physical appearance of an organism or some portion thereof. For the update, it represents the downstream expression of a genetic code, represented by the observable characteristics of a cell as defined by its morphology, biochemistry, physiology, and behavior.

POLYNOMIAL EQUATION – Algebraic equations containing variables, exponents, and constants.

POSTMORTEM – After death.

PRECISION – The extent to which a given set of estimates of the same sample agree with the mean value.

PREDICT – To tell in advance, using, for example, extrapolation, equations, patterns, or specific information.

PRIMER – A book containing basic information about a topic of interest to a beginner.

PROPORTION – A quantitative relationship among parts.

PROXY – A substitute for another.

PUBLICATION CODE – Used to identify authors and date of publication. It identifies the original source of the data in figures and text. ABC (2001): initials of authors last names (ABC), date of publication (2001).

QUALITATIVE – Characteristics based on qualities.

QUANTITATIVE – Characteristics based on quantities.

QUANTUM MECHANICS – A basic theory of physics describing the behavior of small particles.

RATIO – Relative amounts of two or more parts.

RECIPE: A set of outcomes defining a solution or the progress thereto.

REDUCTIONISM – Explaining complex events in terms of simple events.

REDUCTIONIST THEORY – Assumes that one can understand complex systems from the properties of individual parts.

RELATIONSHIPS OF STRUCTURE TO FUNCTION – Play a key role in defining the complexity of a cell phenotype before, during, and after a biological change. Expressed as ratios (biochemistry to morphology), such relationships define the strategies used by cells to solve problems by changing.

REPRESENTATIVE SAMPLE – Identifies a population that accurately reflects the members of the entire population.

REPRODUCIBILITY – To produce again; to duplicate.

REPRODUCIBILITY (UPDATED DEFINITION) - An ability to duplicate a biological complexity with minimal methodological interference.

REVERSE-ENGINEERING – Involves taking something apart to see how it operates. For biology, it creates opportunities for exploring the complexity of change by unfolding a newly created phenotype into multiple subpopulations of changes, which together define the change. In effect, it provides access to biology's complex set of change algorithms. Such algorithms derive from first principles.

RULE-BASED – A system based on rules for storing, manipulating, and interpreting information in a useful way.

RULES LAYER - Original data expressed as ratios (data pair ratios).

SCIENCE – Extends the knowledge of rules, fundamental principles, and practical applications. Quantitative sciences derive mathematically from first principles and operate within constructs defined by theories.

SHRINKAGE – The amount by which something decreased in volume, a reduction in value.

SIGNIFICANT DIFFERENCE – A measure of the likelihood of drawing a false conclusion in a statistical test; too closely correlated to explain by chance alone; implies a measurable difference between two groups wherein the probability of obtaining that difference by chance is small (e.g., <0.05 ; $<5\%$).

SIMULATION – Act of imitating the behavior of a situation or process; to create a representation or model.

SIMULTANEOUS EQUATIONS – Includes a method for finding solutions based on the intersection of two linear equations. Such a strategy can (1) deliver solutions for optimization problems (e.g., linear programming), (2) detect enzyme densities of membrane subcompartments, and (3) separate different cell types into subpopulations. They leverage the mathematical order of living systems.

SPOF – A single point of failure (SPOF) identifies a design flaw causing a system to collapse. Applying a SPOF analysis to the problem of detecting biological changes from published data quickly reveals the shortcomings of an experimental design.

STANDARD – A basis for comparison.

STANDARDIZE – To conform to a representative (or established) pattern.

STEREOLOGY – A collection of mathematical methods for estimating 2D and 3D structures quantitatively from lower dimensions.

STEREOLOGY LITERATURE DATABASE (SLDB) – published research data stored in a relational database (Bolender, 2019).

STOICHIOMETRY – Relationships existing as ratios of small whole numbers (integers).

STRUCTURE-FUNCTION CHANGE RULE – Identifies a biological change as a relationship of structure to function.

SUBJECTIVE – Coming more from an observer than from observations.

SWELLING – An increase in volume.

THEORETICAL – Concerned with theories, rather than practical applications.

THEORY – A working explanation of properties found in the natural world.

UNBIASED – Identifies the absence of systematic error.

UNBIASED SAMPLING – A method designed to remove bias from the sampling procedure (e.g., design-based sampling).

UNIT – A standard of measurement, appended to numerical values.

UNSTABLE – Lacking stability, subject to change, variable, unpredictable, and ambiguous.

VALUE – A numerical quantity.

VARIABLE – A quantity that can assume different values.

VIRTUAL WINDOW – Provides a stable reference for detecting biological changes when everything else is changing.

VOLUME DISTORTIONS – Introduce errors that distort biological data.

WEIBEL-PAUMGARTNER CORRECTION – Corrects stereological estimates for the biases produced by section thickness and compression. Stereological estimates assume that the sections used to collect data have no thickness.

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