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SUMMARY

Using methods currently available, estimates for cellular changes often fail to deliver satisfactory levels of precision, accuracy, and reproducibility. This shortcoming can occur, for example, whenever cellular data are not referenced to an average cell or a fixed number of cells. The report attempts to mitigate this limitation by first figuring out how to detect local changes in the relationships of structure to function at the level of membranes. Using enzyme densities, which relate enzyme activity to membrane surface area, we can extend the data of several publications by generating diagnostic and predictive equations. These equations, which often display $R^2s = 1$, suggest that change in biology is a function of well-defined rules applied specifically to molecules populating cytoplasmic membranes. In mitochondria and the endoplasmic reticulum, for example, changes in the molecular profile of a membrane was found to be a highly controlled process. When membranes changed their surface area, they also changed the packing density (concentration) of their constituent marker enzymes and their rate constants. Enzyme densities allowed us to follow this complex process of change because they became the variables in the rate constant equations. Data drawn from four publications were used to show how hepatocytes design specific membranes when exposed to drugs (phenobarbital), surgical procedures (bile duct ligation), and multiple exposures (phenobarbital + bile duct ligation). In turn, equations were used to transform the concentration data of both morphology and biochemistry into data comparable to that of average cells – without having to count cells. These calculations allowed molecular events to be interpreted at the level of average cells. Taken together, these results suggest that new and more effective approaches to experimental biology require little more than replicating biology's rules and recipes for change. The most interesting finding of all, however, was one of connectivity. Connections between parent DNA (nuclear) and its cytoplasmic progeny (RNAs and proteins) exist as relationships of structure to function that can be read mathematically from the cell's organelles.

INTRODUCTION

A key requirement of most research papers includes an ability to demonstrate a biological change. Herein lies a problem. Change in biology occurs as a continuum of complex events occurring across many parts of an organism. Consequently, detecting a change becomes a function of knowing where, when, and how to look.

Ideally, we would like to connect molecular events occurring in the genome to the downstream expression of these events in the phenome. Such as a task, however, is easier said than done. Many would argue that our published data are simply not up to the task. Reports suggest that the findings of only 20% to 30% of research papers are likely to be correct (Ioannidis, 2005; Begley and Ioannidis, 2015) and that a crisis of confidence surrounds the accuracy, precision, and reproducibility of our published results (Baker, 2016; Collins and Tabak, 2014; Engber, 2016; Freedman et al., 2015; Roth and Cox, 2015). According to such accounts, we must be doing something wrong. What might we do to improve our prospects?

Why is biology able to solve incredibly difficult problems routinely, but we are not very good at detecting changes with convincing levels of precision and accuracy? One way of avoiding such criticism might be to copy biology's rules and solutions by fitting our published data to these rules with $R^2 = 1$ equations. Since this approach worked earlier for controls (Bolender, 2017), it might also work when cells are changing.

Accordingly, the report reworks the published data of several studies with the goal of answering questions with $R^2 = 1$ equations. It also explains how to use equations to develop a strategy for obtaining rate constants within the framework of what appears to mirror the *in vivo* setting. Recall that rate constants allow us to determine rates of change and to predict concentrations. In short, we will approach the problem of biological change as a mathematical exercise, wherein equations drive the solutions. To follow the details of this approach, calculation worksheets are bundled with the report and posted online (playingcomplexitygames.com).

METHODS AND RESULTS

GAME PLAN

STRATEGY: Biology, chemistry, and physics consists of parts and connections that change according to rules (first principles). Recall that a change exists as a package containing constants and variables subject to rules and measures of precision, accuracy, and reproducibility.

Although events in physics and chemistry can be derived routinely from first principles, biology has remained curiously resistant to such a robust approach. Why? Biology encapsulates both physics and chemistry on top of which it adds living machines capable of obeying the rules and creating new ones as the need or opportunity arises. Nevertheless, few would disagree with the argument that understanding change in biology requires access to the entire package of rules if we expect to help and to be helped by biology.

TACTICS: Fortunately for us, physics, chemistry, and biology all hide their best kept secrets the same way. Rules and first principles exist as complex relationships of constants to variables. We become privy to these secrets by figuring out the recipes being used by expressing them as equations.

Let's start with a simple example taken from physics. If we drop a ball from a given height, it takes a specific amount of time to hit the ground. This gives us two known variables (distance: d, time: t) and one unknown constant (k). The first principle (k) defines the relationship of distance to time. By expressing the recipe as an equation and solving it for k, we can explain why the distance and time variables behave as they do.

Let's do the calculation. If the ball moves 144 feet in 3 seconds, what is the principle in play?

$k = 2d/t^2 = 288\, feet/9sec^2 = 32\, feet/sec^2$

The answer is the force of gravity (k), which defines the relationship of distance to time for the falling ball. To look for precision, accuracy, and reproducibility, simply rerun the experiment with new sets of variables. The report merely applies such an approach to biological changes.

DEFINITIONS

ENZYME DENSITY: An enzyme density (ED) relates units of marker enzyme activity (U) to a unit (1 m^2) of membrane surface area (S):

$$ED = U/S. \quad (1)$$

Since an ED is expressed as a concentration (U/S), it can be used in equations to calculate activity (U) and surface area (S) thusly:

$$U = ED\ x\ S \quad (2)$$

$$S = U/ED\ . \quad (3)$$

RATE CONSTANTS: Recall that a rate equation expresses the rate at which a concentration changes over time. As concentrations, enzyme densities allow us to evaluate rate equations, which are classified according to their order.

A zeroth order equation is given as:

$$[A_i] = [A_0] \pm kt\ , \quad (4)$$

where $[A_i]$ is the concentration at time *i*, $[A_0]$ the concentration at time *0*, k the rate constant, and *t* time. When concentrations are plotted against time, a straight-line indicates a zeroth order reaction.

OVERVIEW: The report briefly summarizes the results of a given publication in the usual way and then expands its interpretation by calculating enzyme densities and rate constants. Such an exercise produces a collection of empirical equations with $R^2 = 1$ (or $R^2 \approx 1$), designed to access the rules being used by biology. The publications were selected because they described both structural and functional changes in hepatocytic membranes.

PAPER 1

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.

EXPERIMENT: Adult male rats were treated with phenobarbital (100 mg/day) for five days after which samples were taken for morphology (stereology) and biochemistry. The results displayed in Figure 1 corrected the original data for section artifacts (Weibel and Paumgartner, 1978) and referenced them to one gram of liver.

ORIGINAL DATA: Figures 1 and 2 summarize the changes in ER membranes and ER marker enzymes induced by phenobarbital.

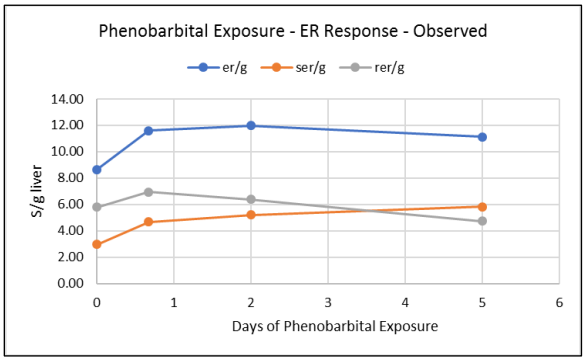


Figure 1 Phenobarbital induced changes in the surface areas of hepatocytic membranes – per gram of liver. The original data were corrected for section artifacts and related to one gram of liver. [er = endoplasmic reticulum; ser = smooth-surfaced er; rer = rough-surfaced er]

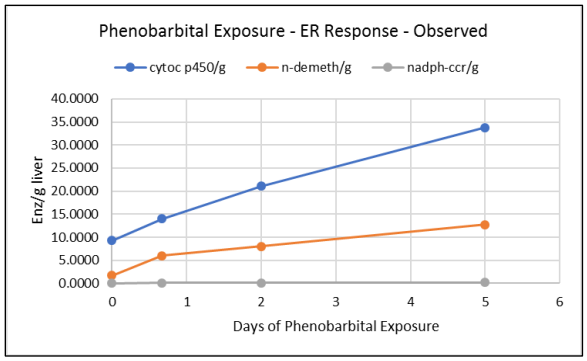
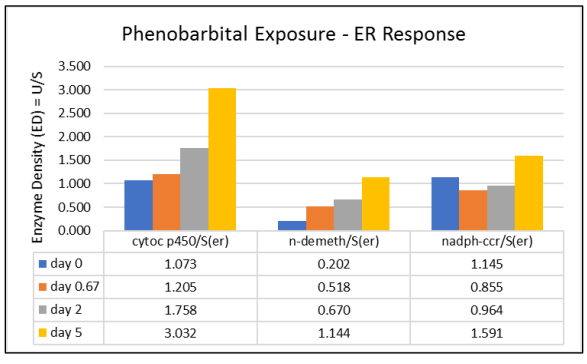


Figure 2 Phenobarbital induced changes in marker enzymes (Enz) of the endoplasmic reticulum (ER) expressed per gram of liver.

Notice in Figures 1 and 2 that the ER membranes and their constitutive marker enzymes changed differently at different rates, suggesting the absence of a relationship between structure and function. When, for example, ER surface area was plotted against the activity of cytochrome P450, the resulting regression line showed a weak correlation (R^2 = 0.05).

NEW DATA: If, however, we use the data in Figures 1 and 2 to calculate enzyme densities (U/S), distinct patterns of connectivity begin to appear (Figures 3 and 4). Over time, biology responds to phenobarbital by increasing the concentrations of its membrane-bound marker enzymes in the ER.



	cytoc p450/S(er)	n-demeth/S(er)	nadph-ccr/S(er)
day 0	1.073	0.202	1.145
day 0.67	1.205	0.518	0.855
day 2	1.758	0.670	0.964
day 5	3.032	1.144	1.591

Figure 3 In response to the phenobarbital treatment, the enzyme densities increased over time. To display the changes, the nadph-ccr values were multiplied by 100.

When plotted in Figure 4, the experimental data points listed in Figure 3 produce linear curves with R^2s equal to or approaching 1. This tells us that biology is controlling – very precisely - the concentration of enzymes in its ER membranes. In effect, the linear equations captured several parts of the recipe biology used to solve its phenobarbital problem.

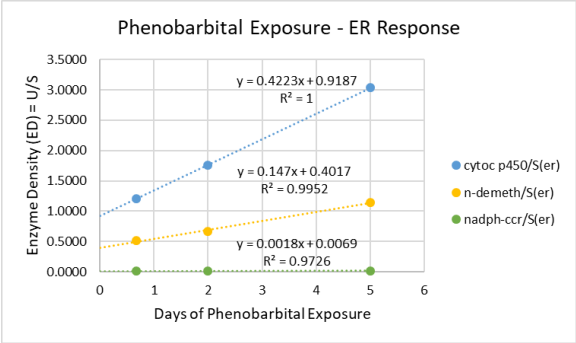


Figure 4 When changes in membrane surface areas and enzyme activities are expressed as enzyme densities, distinct relationships of structure to function become apparent. Notice that the linear equations serve as the constants for the variable data points.

Figure 4 identifies missing values for days 3 and 4. Two options exist. We can either take these missing values directly from Figure 4 or calculate them from their rate constants (k), which represent the slopes of the curves. Working through the calculations of the latter option will serve to illustrate the simplicity of the approach.

PREDICTIONS: Calculate enzyme densities for days 3 and 4 of phenobarbital exposure.

Note that Day 3 of the original paper becomes Day 2 here because Day 1 = Day 0 in the rate equations.

Note that the slope is positive ($+k$) because the enzyme densities (concentrations) are increasing.

DAY 3

Cytochrome P450 (5)

Cytochrome P450 (5)

$$[ED_3] = [ED_0] + kt$$

$$[ED_3] = [1.341] + 0.4223/d * 2\ d$$

$$[ED_3] = 2.1856$$

n-demethylase (6)

$$[ED_3] = [ED_0] + kt$$

$$[ED_3] = [0.5487] + 0.147/d * 2\ d$$

$$[ED_3] = 0.8427$$

NADPH Cytochrome c reductase (7)

$$[ED_3] = [ED_0] + kt$$

$$[ED_3] = [0.0087] + 0.0018/d * 2\ d$$

$$[ED_3] = 0.0123$$

Note that Day 4 of the original paper becomes Day here because Day 1 = Day 0.

DAY 4

Cytochrome P450 (8)

$$[ED_4] = [ED_0] + kt$$

$$[ED_4] = [1.341] + 0.4223/d * 3\ d$$

$$[ED_4] = 2.6079$$

n-demethylase (9)

$$[ED_4] = [ED_0] + kt$$

$$[ED_4] = [0.5487] + 0.147/d * 3\ d$$

$$[ED_4] = 0.9897$$

NADPH Cytochrome c reductase (10)

$$[ED_4] = [ED_0] + kt$$

$$[ED_4] = [0.0087] + 0.0018/d * 3\ d$$

$$[ED_4] = 0.0141$$

This completes the data set (see Figure 5).

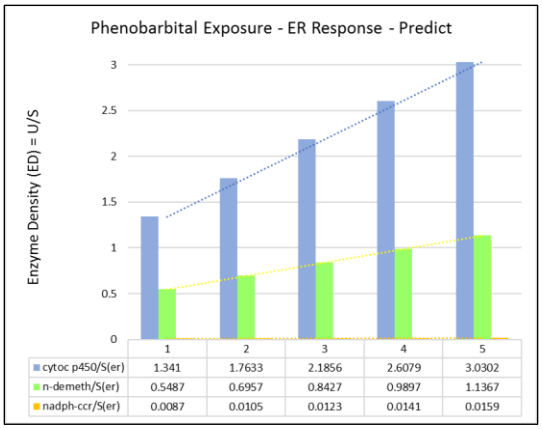


Figure 5 Using enzyme densities and rate constant equations, the missing data points for days 3 and 4 were predicted.

Next, we can use the enzyme densities of Figure 3 to look at another level of complexity, one defined by the relationship of one enzyme density to another. This will tell us something about how different marker enzymes are being packed in the same ER membrane. Figure 6 plots pairs of enzyme densities to show quantitatively the relationship of one enzyme activity to another. The slope of the regression line identifies the ratio of one enzyme activity to another. For example, the ratio of n-demethylase to cytochrome oxidase is 1:2.5. As noted earlier (Bolender, 2017), enzyme ratios can be used to connect and predict biochemical data sets routinely.

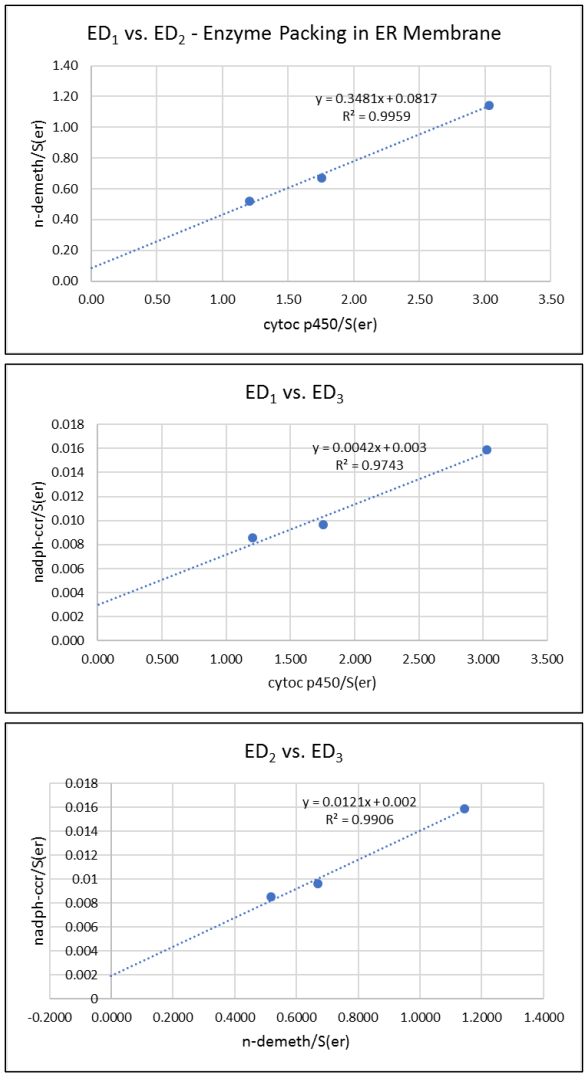


Figure 6 The R^2 values of the linear equations suggest that the packing of enzymes in the ER membrane follows distinct patterns (rules) defined by ratios.

When the ratios of the enzyme pairs in Figure 6 are plotted together in Figure 7, we can see how the ER enzymes and membranes responded to the phenobarbital. Notice how quickly the membranes designed specifically to detoxify the drug became operational.

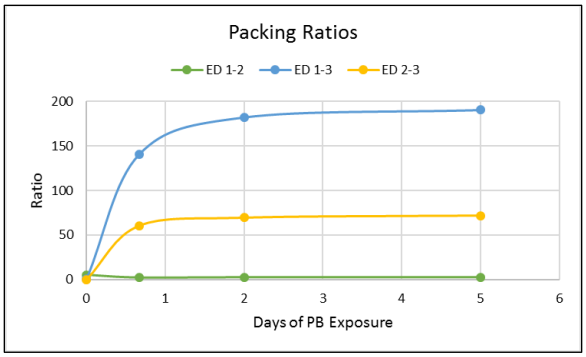


Figure 7 The relative amounts (proportions) of cytochrome P450, n-demethylase, and NADPH cytochrome c reductase change in response to phenobarbital. As reported earlier (Bolender, 2011-2017) such ratios are both dignostic and predictive.

SUMMARY: The ER membranes of hepatocytes undergo four distinct changes in response to phenobarbital. They:

1. Increase their membrane surface area (Figure 1).
2. Increase the activity of their membrane bound marker enzymes (Figure 2).
3. Increase the packing densities (enzyme densities) of their marker enzymes in the membrane (Figures 3, 4).
4. Change the proportions of their membrane bound enzymes in the membrane (Figures 6, 7).

Thus far, the analysis of Paper 1 was done entirely with concentration data (expressed per gram of liver). Although we have quantified the downstream effects of gene expression, the results relate just to cytoplasmic membranes. However, this is only the first part of the story (see Discussion).

PAPER 2

Denk, H. Eckerstorfer, R., Rohr, H. P. (1977) The endoplasmic reticulum of the rat liver cell in experimental mechanical cholestasis. Correlated biochemical and ultrastructural-morphometric studies on structure and enzyme composition. Exp Mol Pathol:193-203.

EXPERIMENT: In adult male rats, double ligation and transection of the common bile duct was used to induce cholestasis. The goals of the study included determining the influence of cholestasis on the structure and function of the ER and on the activity of its electron transfer systems.

ORIGINAL DATA: Figures 8 and 9 summarize the changes in membrane and enzymes in response to the experimental cholestasis. When related to a gram of liver, bile duct ligation increased the ER surface area, but appeared to decrease the enzyme activities. Once again, we start with seemingly incompatible results.

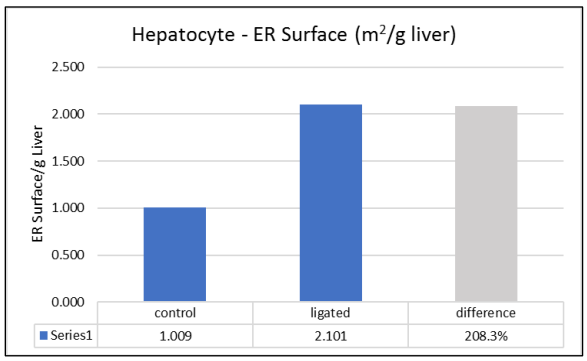


Figure 8 The surface area of the ER (From Table I; Denk et al., 1977) were corrected for section artifacts according to Weibel and Paumgartner (1978) and related to one gram of liver.

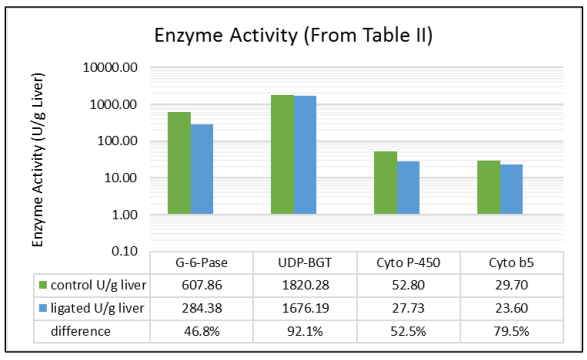


Figure 9 Enzyme activity data from Table II (Denk et al., 1977) were related to a gram of liver.

NEW DATA: If, however, we use the data in Figures 8 and 9 to calculate enzyme densities, a uniform pattern appears. The concentration of the enzymes in the ER membranes – as indicated by the enzyme densities – showed a persistent decrease in response to the ligation (Figure 10).

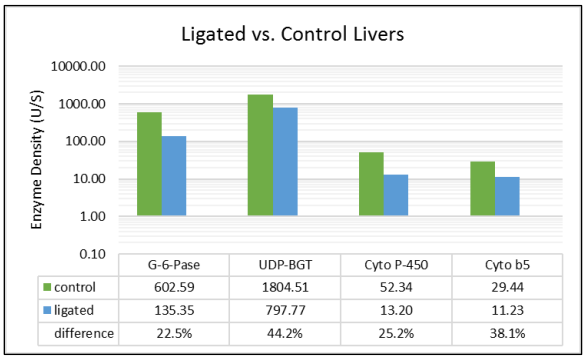


Figure 10 The enzyme densities were calculated using the data given in Figures 8 and 9. Notice that the packing density of the ER marker enzymes decreased substantially.

When the ED data of Figure 10 were plotted (Figure 11), however, the R^2 value was only 0.9715. If we treat glucose-6-phosphatase as an outlier (not a drug-metabolizing enzyme), then the R^2 value approached 1 (0.9999).

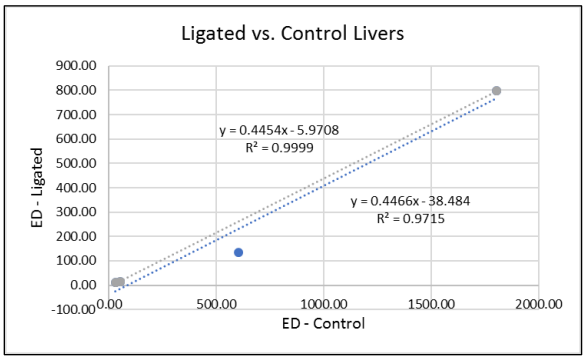
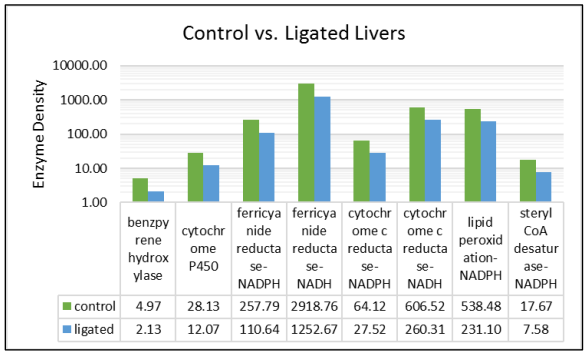


Figure 11 The data of Figure 10 are plotted as regressions.

Paper 2 included a second study that focused on the enzymes of the electron transfer pathways (see Table IV in the original paper). Similarly, these enzymes were related to the ER surface areas of Figure 8 (see Table I in the original paper) to generate enzyme densities (Figure 12). When the eight enzyme densities were plotted as a regression (ligated liver vs. control), the R^2 was equal to one (Figure 13). Notice that both experiments (Figures 11 and

13) produced linear equations with similar slopes (0.4454 vs. 0.4292).



	benzpyrene hydroxylase	cytochrome P450	ferricyanide reductase-NADPH	ferricyanide reductase-NADH	cytochrome c reductase-NADPH	cytochrome c reductase-NADH	lipid peroxidation-NADPH	steryl CoA desaturase-NADPH
control	4.97	28.13	257.79	2918.76	64.12	606.52	538.48	17.67
ligated	2.13	12.07	110.64	1252.67	27.52	260.31	231.10	7.58

Figure 12 The enzyme data (from Table IV) were expressed as enzyme densities using the corrected ER surface areas shown in Figure 8.

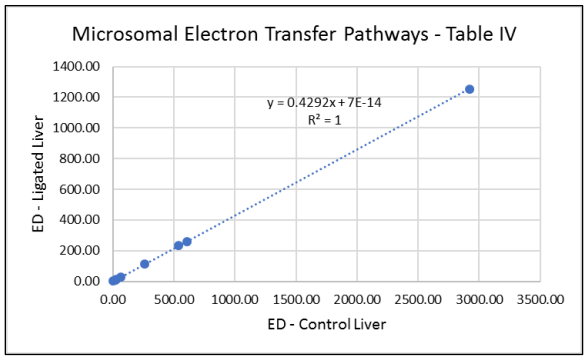


Figure 13 When plotted as a regression, the enzyme densities (ED) of Figure 12 displayed a $R^2 = 1$ equation, which illustrates the precision and reproducibility of the data. Notice that the relationship of ligated to control livers defined the change mathematically as a single ratio (1:2.33).

SUMMARY: Bile duct ligation (cholestasis) and hepatocytic ER (based on concentration data):

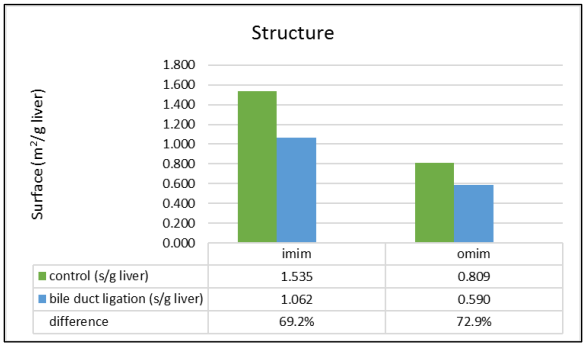
1. Ligation increased the surface area of the ER (Figure 8), but decreased the enzyme activities (Figure 9).
2. Enzyme densities suggested a decrease in enzyme activity per m^2 of ER (Figure 10).
3. When plotted (Figures 11, 13), the enzyme densities (control vs. ligated) displayed R^2 = 1 or ≈ 1 (0.9999).
4. Since cholestasis decreased the packing density of the electron transfer enzymes in the ER by roughly half, the hepatocytes compensated for the loss by doubling their ER surface area (Figure 8).

PAPER 3

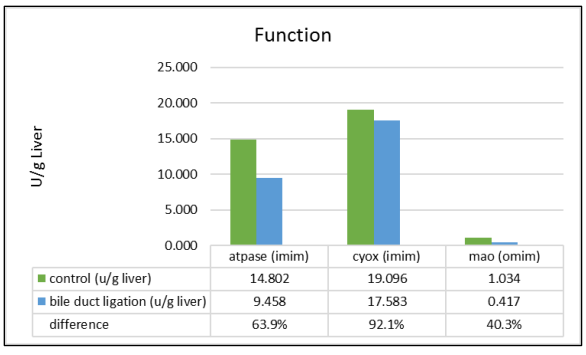
Krähenbühl, S., Krähenbühl-Glauser, S., Stucki, J., Gehr, P., Reichen, J. (1992) Stereological and functional analysis of liver mitochondria from rats with secondary biliary cirrhosis: Impaired mitochondrial metabolism and increased mitochondrial content per hepatocyte. Hepatology 15: 1167-1172.

EXPERIMENT: The mitochondrial functions of adult male rats with secondary biliary cirrhosis – induced by bile duct ligation – were studied *in vivo* and *in vitro*. The goal of the study was to determine the mechanism leading to hepatic decompensation.

ORIGINAL DATA: Figure 14 summarizes the changes in mitochondrial membranes (inner = imim; outer = omim) and their marker enzyme activities five weeks after bile duct ligation. Everything was related to one gram of liver.



	imim	omim
control (s/g liver)	1.535	0.809
bile duct ligation (s/g liver)	1.062	0.590
difference	69.2%	72.9%

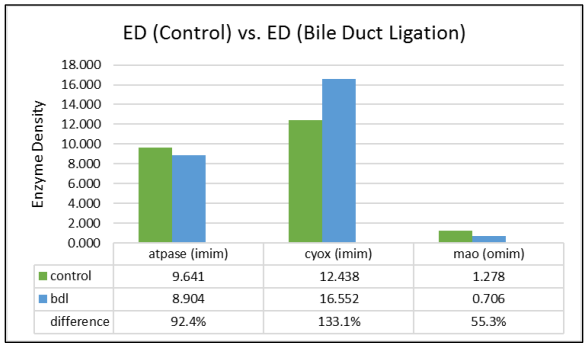


	atpase (imim)	cyox (imim)	mao (omim)
control (u/g liver)	14.802	19.096	1.034
bile duct ligation (u/g liver)	9.458	17.583	0.417
difference	63.9%	92.1%	40.3%

Figure 14 Top: Animals with secondary biliary cirrhosis suggest a decrease in mitochondrial membrane surface

area. Bottom: Similarly, mitochondrial marker enzymes suggest a decrease in enzyme activity.

NEW DATA: Starting with the data in Figure 14, we can calculate enzyme densities to see how the relationship of structure to function changed in response to bile duct ligation. Figure 15 suggests that the concentration of the enzymes in the mitochondrial membranes underwent a mixed response to the bile duct ligation. Two enzyme densities decreased in concentration (atpase and monoamine oxidase), whereas one increased (cytochrome c oxidase).



	atpase (imim)	cyox (imim)	mao (omim)
control	9.641	12.438	1.278
bdl	8.904	16.552	0.706
difference	92.4%	133.1%	55.3%

Figure 15 The enzyme densities show how bile duct ligation affects the concentration of marker enzyme activities in mitochondrial membranes.

When we plot the data of Figure 15 as a regression (Figure 16), we find the best fit identifies an exponential equation instead of a linear one. This, however, was to be expected.

By including data from both the inner and outer mitochondrial membranes in the same plot, we are mixing the results of two different control mechanisms - one associated with nuclear DNA and the other with mitochondrial DNA. The result is a complex curve of our own making (Figure 16). By treating the membrane compartments separately, however, the underlying equations are likely to be linear. As noted earlier (Bolender, 2017), analyzing mitochondrial membranes can be problematic.

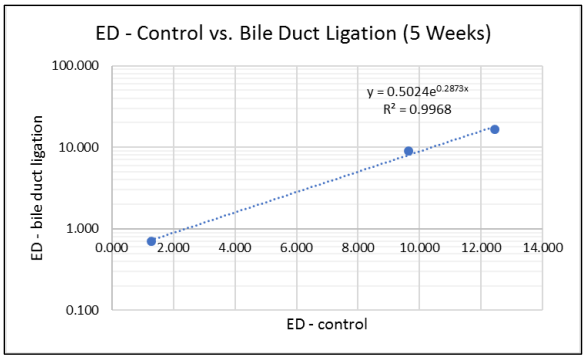


Figure 16 When the data of inner and outer mitochondrial membranes are combined, the relationship of structure to function fitted an exponential curve.

Summary: Cirrhosis induced by bile duct ligation affects mitochondria.

1. It decreased the surface area and the marker enzymes of the inner and outer mitochondrial membranes (Figure 14).
2. The enzyme densities, however, showed a mixed response. They decreased for atpase (imim) and mao (omim), but increased for cyox (imim); as shown in Figure 15.
3. The enzyme density plot (control vs. ligated liver) fit an exponential curve with and R^2 = 0.9968. Such a result suggests that several separable complexities may be in play (Figure 16).

Paper 4

Krähenbühl, S., Reichen J., Zimmernamm, A., Gehr, P., Stucki, J., (1990) Mitochondrial structure and function in CCl_4 induced cirrhosis in the rat. Hepatology 12: 526-532.

EXPERIMENT: The goal of the study was to determine whether the impairment of mitochondrial function in cirrhosis resulted from a decrease in liver cell mass (hepatocytes) or from an alteration in mitochondrial membranes (inner and outer). Cirrhosis was induced by long-term exposure to CCl_4 vapors and phenobarbital; some experiments used liver perfusion. The study included both *in vivo* and *in vitro* experiments.

ORIGINAL DATA: In Figure 17, we begin with the *in vivo* data related to the liver, which serves as an indicator of liver cell mass. The phenobarbital and CCl_4 exposure produced a decrease in the surface area of mitochondrial membranes and in their constitutive marker enzymes. The same pattern seen earlier (Paper 3).

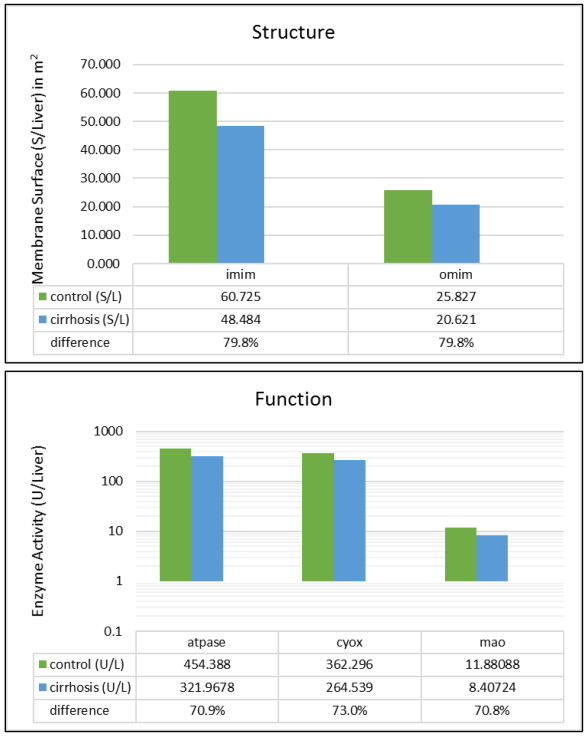


Figure 17 Both structural and functional data show a loss of mitochondrial membranes and their associated marker enzyme activities – about 20 - 30%.

NEW DATA: When the data of Figure 17 are related to a gram of liver, we can calculate the mitochondrial enzyme densities (Figure 18). The response of hepatocytes to cirrhosis was similar for the imim (atpase, cyox), but not for the omim (mao).

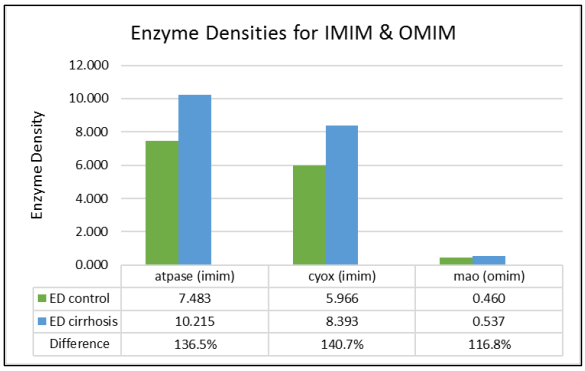


Figure 18 Cirrhosis had a similar effect on the enzyme densities of the inner mitochondrial membrane, but not on the outer.

When the enzyme densities of Figure 18 were plotted as a regression, they fit a power curve with an $R^2 \approx 1$ (Figure 19).

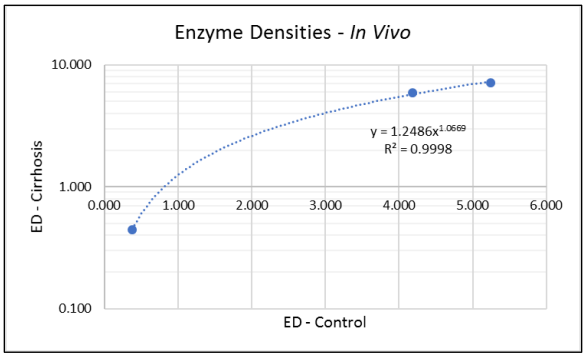


Figure 19 The best fit to a power curve describes the relationship between the inner and outer mitochondrial membranes and enzymes.

Figure 20 summarizes the results of the *in vivo* experiment. It shows that cirrhosis produced a 39% loss of liver cell mass, which was compensated for by increasing the packing density of the enzymes (i.e., EDs) in the inner mitochondrial membrane by 38.6%.

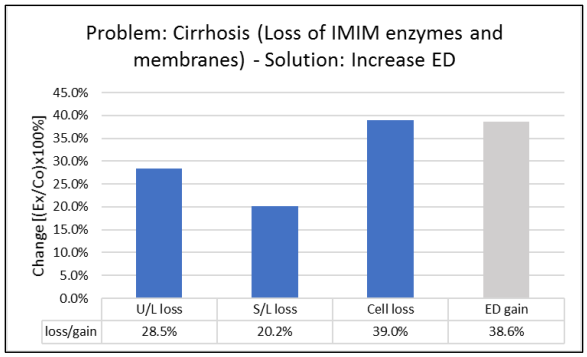


Figure 20 In response to the structural and functional deficits caused by cirrhosis, the remaining hepatocytes increased the enzyme densities of their inner mitochondrial membranes.

The *in vitro* experiment produced patterns comparable to those of the *in vivo* study, as seen in Figure 21. Enzyme densities showed similar differences and both experiments produced similar power curves.

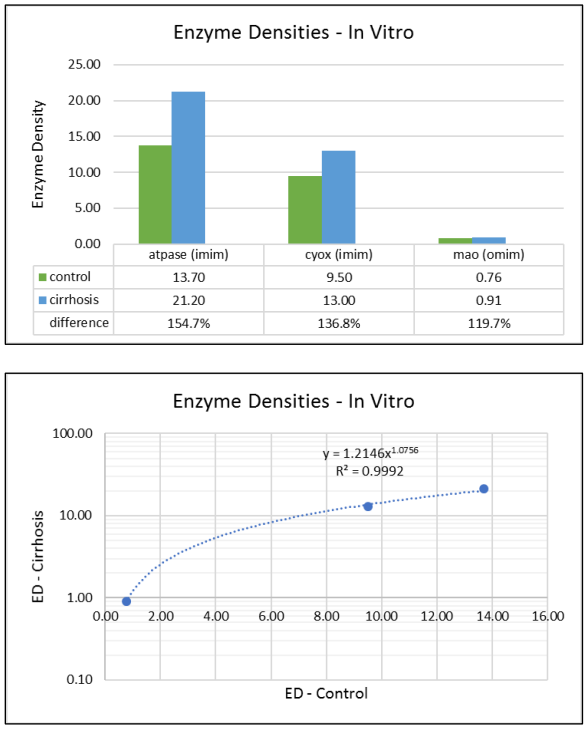


Figure 21 The in vitro experiment showed patterns similar to the in vivo one (Figures 18 and 19).

Summary: Cirrhosis induced by exposure to CCl_4 and phenobarbital created three problems for the liver hepatocytes to solve simultaneously, the loss of liver function (cirrhosis) and the removal of two xenobiotics (CCl_4 and phenobarbital).

1. The lengthy period of exposure to phenobarbital (PB) and carbon tetrachloride (CCl_4) produced a loss of mitochondrial function (Figures 16, 17), which was compensated for by increasing the enzyme densities of the inner and outer mitochondrial membranes (Figure 18).
2. The similarity of the *in vivo* and *in vitro* results speaks to the compatibility of the experimental designs.
3. Taken together, Papers 3 and 4 identify the packing densities of the membrane-bound marker enzymes as a key player in the compensatory machinery of hepatocytes.
4. The response of hepatocytes to cholestasis depends importantly on the experimental methods used to create the disorder (Paper 3 vs. Paper 4).

DISCUSSION

THE BIG PICTURE

Biology is a quick study. As shown in the report, it swiftly detected, analyzed, and mounted responses to experimental exposures by manufacturing new arrays of membranes and enzymes capable of dealing with each new challenge. It did so by exercising several options. It modulated the amounts of membranes locally (per hepatocyte) and globally (per liver), the packing densities of enzymes in ER and mitochondrial membranes (enzyme densities), and determined the rate at which each part changed (rate constants).

Data taken from the four papers recruited for the report helped to show that a biological change represents a highly choreographed and interrelated series of events. Presumably, the

scripts responsible for triggering a change reside in the nuclear DNA, but the process of creating and executing the specialized recipes capable of producing such complex changes in the cytoplasm remains largely a mystery.

CHANGE REVISITED

Currently, our definition of a biological change all too often refers to whatever can be reduced to a numerical value and, in turn, shown to be significantly different from something else. The validity of such a result is often "confirmed" by quoting an independent study with similar results. Although such practices may be convenient, they are not necessarily convincing.

If instead, we argue that a change is what biology does in response to a challenge, then we are compelled to consider how our methods relate to the precision, accuracy, and reproducibility of our results. Specifically, what do we have to know about biology to detect its changes and to offer reasonable explanations thereof? Since even a simple biological change represents the aggregate of many interconnected events, our understanding of change requires a much greater attention to detail. Table 1 suggests what some of these details might include.

Table 1 A biological change involves:

1	Relationships of structures to functions (EDs)
2	Data related to cells (average, fixed number)
3	Connectivity of parts (ratios)
4	Data synergies (local and global patterns)
5	Parts expressed as concentrations (X/Y)
6	Parts expressed as absolute values (X,Y)
7	Corrections for experimental biases
8	Precision, accuracy, and reproducibility

In short, understanding a biological change becomes a process of defining new relationships of constants to variables – expressed locally and globally. In effect, Table 1 puts us in the business of deriving biology from first principles.

CHANGING THE FOCUS

In keeping with our ongoing strategy, we begin with what we want to do rather than what we can do. Specifically, we wanted to find a rule-based pathway for detecting biological changes, one that extended from livers to hepatocytes to organelles to membranes to enzymes to RNA to DNA. Moreover, we wanted to do this without the usual limitations imposed by data sets subject to low levels of precision, accuracy, and reproducibility. To this end, we elected to copy biology's approach to change, which consists of working out solutions with rules derived from first principles.

In the methods and results section, we used concentration data to detect changes in membranes by identifying a rule at each time point (an ED) and then fitted the local rules to an equation that identified a first principle (e.g., a rate constant). This approach worked because we replaced the varying contents of a gram of liver over time (unstable cell numbers) with an unvarying membrane surface area (1 m^2). In effect, we nailed down the data reference.

This, however, was only part 1 of the story. We left the larger problem of figuring out how to detect biological changes by comparing concentrations unresolved. Now let's see what we can do when we want to solve this problem.

CHANGE – PART 2

A biological change generates a swirling cloud of variables by putting many parts and connections in play. Therefore, progress

toward understanding the complexity of change is likely to accelerate rapidly as we begin to shift our experimental strategy from following a few variables to following as many as possible – across multiple disciplines.

This shift in perspective, however, becomes disruptive. Investigators unfamiliar with the mathematical properties of biological parts – as they exist in control and experimental settings - are more likely than not to suffer the consequences of being blind-sided. For example, when looking for a significant difference between control and experimental time points, no one would never dream of comparing cell data taken from time points derived from representative tissue samples containing largely different numbers of cells. And yet, this is exactly what is being done roughly 50% of the time when we use concentrations to detect biological changes (Bolender 2001A; Bolender 2016: Figure 1.7). Although the ambiguity surrounding concentration data is widely understood by many investigators, even the best journals routinely publish papers reliant largely on concentration data.

FIRST PRINCIPLES: We can do better by implementing a best practices approach. Let's begin by setting as our goal a solution to the problem of detecting changes in the liver from the liver's first principles – starting with the allegedly unstable concentrations of morphology and biochemistry.

We can begin by assuming *a priori* that first principles define equations that solve problems:

$1^{st}\ Principles \rightarrow Equations \rightarrow Solutions.$

For our purposes here, two principles will be in play:

1. Biology defines the relationship of structure to function for its membranes by rule, and that…
2. The number of hepatocytes in the liver remains essentially constant – in both control and experimental settings. This second principle can be expected to apply to adult animals except when an exposure specifically induces important changes in cell number.

Along with the principles, we need to make two assumptions:

1. Estimating the number of hepatocytes in experimental settings using current stereological approaches becomes problematic because (1) reference volumes are unstable and (2) changes can occur in the number of nuclei per hepatocyte. Recall that stereological methods count nuclei, not cells.
2. Estimating biochemical activities by relating them to a mg of protein reference works equally well in both control and experimental settings. To wit, if biases exist, they remain constant.

To demonstrate the effectiveness of a first principles approach, we will use it to figure out how to stabilize the concentration data of Paper 1 (Stäubli et al., 1969).

THE DATA SET: Figure 22 plots the structural and functional data sets of Paper 1 related to a gram of liver. Notice that all the enzymes continued to increase (with two of them in parallel), but that the ER surface area increased and then decreased (statistical arguments aside).

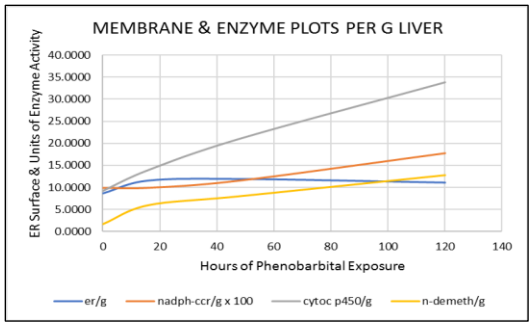


Figure 22 Hepatocytes exposed to phenobarbital change the surface area of their ER membranes and the activity of their membrane-bound marker enzymes.

Why do the results given in Figure 22 differ substantially from those in the original paper? [Paper 1 reported that the amounts of membranes and enzymes increased far more than Figure 22 suggests.]

Paper 1 used a different reference system for the data. By relating the original concentration data to 100 grams of body weight, the authors showed that the hepatocytes produced larger amounts of new ER membranes and enzymes to metabolize and eliminate the drug to which they were being exposed (phenobarbital). Their Figures 6, 8, 13, and 16 showed roughly a 50 to 60% increase in ER surface area with increases considerably greater for the enzyme activities. The purpose in drawing attention to such differences is that it focuses squarely on the problem we're trying to solve. Apparently, everyone gets to decide how to detect and interpret a change. Biology does it its way, we do it our way.

Before continuing, it will be helpful to introduce some background information on the liver. Recall that hepatocytes are largely responsible for mounting responses to foreign substances (drugs, toxins, and mutagens). These cells are unusual in that they represent a collection of mono and binucleated cells, often displaying polyploid nuclei (>2N). To increase the efficiency of its synthetic output, for example, a polyploid nucleus can undergo fission and become two nuclei. In effect, one cell effectively becomes "two" without resorting to cell division, thereby avoiding the risk of DNA damage that might otherwise occur during mitosis in the presence of toxic substances. Once a problem is solved, the two nuclei can fuse to become one.

UNSTABLE DATA REFERENCES: Back to Figure 22. If the hepatocytes get bigger, but the total number of hepatocytes in the liver remains the same, what happens to the liver? It gets bigger and weighs more. This is what happened in Paper 1 (see its Table I).

Now for the telling question. In the phenobarbital study of Paper 1, what happened to the number of hepatocytes needed to fill a cm^3 or gram of liver? As the hepatocytes enlarged by adding new membranes, fewer and fewer cells were needed to fill the reference space (a cm^3 or g of liver). How did this affect the experiment? By diluting the number of cells in the reference space (the cm^3 or gram of liver), the amounts of membranes and enzyme activities were likewise diluted. By relating the data to a gram of liver instead of to 100 grams of body weight, the results given in Figure 22 underestimate the extent of the changes by a wide margin.

STABLE DATA REFERENCES: To detect cellular changes reliably, the experimental rules in play require that we relate our data to an average cell or to a fixed number of cells. Herein lies a problem. Since counting hepatocytic nuclei is not the same as counting cells, estimating average cell data becomes problematic. In effect, counting cells with stereology is not a tempting option.

What is the solution? If our inability to count cells in intact tissue puts average cell data out of play, we are left with the option of keeping the number of cells associated with a cm^3 or gram of liver constant throughout the course of

an experiment. Recall that data related to a constant number of cells or to an average cell both detect cellular changes equally well. In effect, what we need is an equation that solves the problem for us.

CORRECTED CONCENTRATION (CC) EQUATION: Think a moment. What do we really want? Ideally, we would like to have a single, straightforward solution that applies equally well to the concentration data of both stereology and biochemistry. Such a solution would require collecting concentration data from the same number of hepatocytes, which, in turn, were extrapolated to the whole liver.

Since we know that the packing of hepatocytes in a cm^3 of liver changes in response to changes in hepatocytic volume, how might we account for these volume changes? If we assume that the observed increases in liver volume (or weight) are due entirely to increases in hepatocytic volumes, then all we would have to do is multiply the concentration by the change in liver volume – at each time point (Equation 11). Recall that a concentration can be related to a weight (gram) or volume (ml or cm^3) and that volumes and weights become interchangeable using the density (ρ) equation ($\rho = Weight/Volume$). A liver density of 1.0651 g/cm^3 was used for our purposes here.

The corrected concentration (CC) equation using liver weights is given as:

$$CC_{(t_i)} = C(t_i)\, x\, \frac{W_L(t_i)}{W_L(t_0)}\ , \qquad (11)$$

where $CC_{(t_i)}$ is the corrected concentration at experimental time (t_i), C concentration, and W_L the weight of the liver at t_0 and t_i. Equation 11 corrects for the concentration bias that occurs when the hepatocytes filling the reference space change their volumes in experimental settings. The equation assumes that the changes in liver volume apply entirely to changes in hepatocytic volumes. In effect, the corrected concentrations of Equation 11 detect changes comparable to those related to the total liver weight or volume. This is precisely the outcome we wanted because changes related to the liver are equivalent to changes related to average cells. In effect, this gives us access to average cell information without having to count cells.

The next set of figures illustrate changes detected with equation 11. Notice in Figure 23 that data related to a gram of liver detected the smallest change in the hepatocytic volumes, whereas the same data related to the liver with equation 11 detected the largest; data related to 100 grams of body weight gave intermediate values. In effect, Figure 23 shows how the results of the same experiment can tell three very different stories.

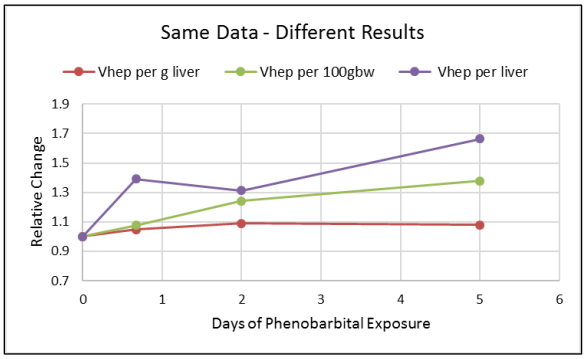
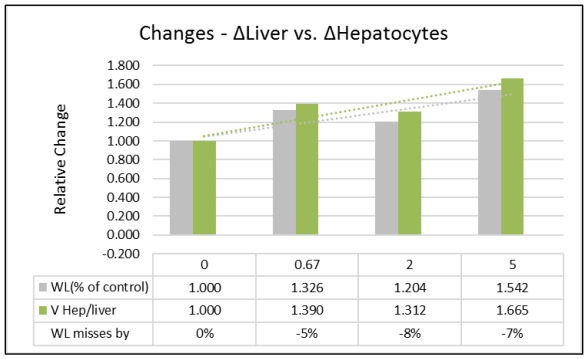


Figure 23 The same data set from Paper 1 produced different results depending on the choice of the data reference.

CORRECTED CONCENTRATION CORRECTED (CCC) EQUATION: Equation 11, however, uncovered another problem. Figure 24 shows that the

relative changes were greater for hepatocytic volumes than for liver weights.



	0	0.67	2	5
WL(% of control)	1.000	1.326	1.204	1.542
V Hep/liver	1.000	1.390	1.312	1.665
WL misses by	0%	-5%	-8%	-7%

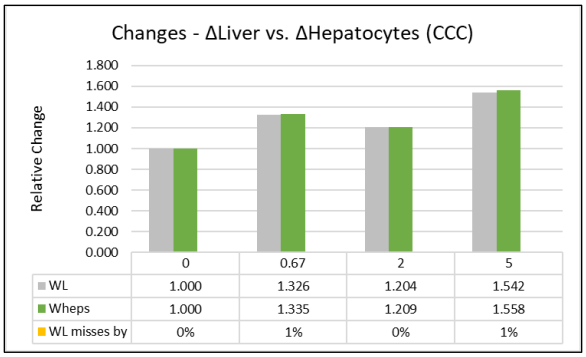
Figure 24 By assigning all the changes in liver weight to the hepatocytes, these cells showed the greatest increases. Relating the concentration (Vhep/g) to the liver (Vhep = (Vhep/g) x W_L underestimated the change by as much as 7%. [Hepatocytic volumes or weights (W = ρ x V; ρ = 1.0651 g/ml) produced similar overestimates.]

Note that the overestimates for hepatocytic volumes were the result of applying all the liver changes to just hepatocytes and to the absence of the liver volume data coming from the step 1 sampling (see Paper 1), which was included in the first paper of the series (Weibel et al., 1969). This explains why Equation 11 needed a second correction.

Equation 12 removes the weight of the extrahepatocytic space (everything except hepatocytes) from the calculation as follows:

$$CCC_{(t_i)} = C(t_i)\, x \frac{W_L(t_i) - W[EHS(t_0)]}{W_L(t_0) - W[EHS(t_0)]}\ , \qquad (12)$$

Where $CCC_{(t_i)}$ is the corrected concentration corrected at time i, $C(t_i)$ the concentration at time i, and $W[EHS(t_0)]$ the weight of the extrahepatocytic space (EHS) at time 0 (t_0). The equation assumes that this space remains constant throughout the experiment. When the data of Figure 24 were recalculated with Equation 12, the expected agreement was found (Figure 25). The value used for the $W[EHS(t_0)]$ correction came from (Weibel et al., 1969).



	0	0.67	2	5
WL	1.000	1.326	1.204	1.542
Wheps	1.000	1.335	1.209	1.558
WL misses by	0%	1%	0%	1%

Figure 25 By removing the effect of the extrahepatocytic compartment from Figure 24, all the changes in liver weight were applied just to hepatocytes.

The next three figures use Equation 11 to illustrate the price we pay for using uncorrected concentration data (X/g) to detect biological changes. Imprecise estimates - missing the mark by 30% to 50% - diminish both the reliability of a result and no doubt dulls the effectiveness of statistical tests when looking for significant differences.

Figure 26 reports that the concentration of hepatocytes in a gram of liver (V_{hep}/g) remained largely unchanged during the 5 days of the experiment, but that the liver became bigger and weighed more. In responding to the phenobarbital challenge, hepatocytes enlarged to accommodate the growing populations of organelles. Consequently, fewer of them could fit into a gram (or cm^3) of liver. This hepatocytic change, however, was detected in Figure 26 as a 30% increase in liver weight – not as an increase in cell volume (or weight).

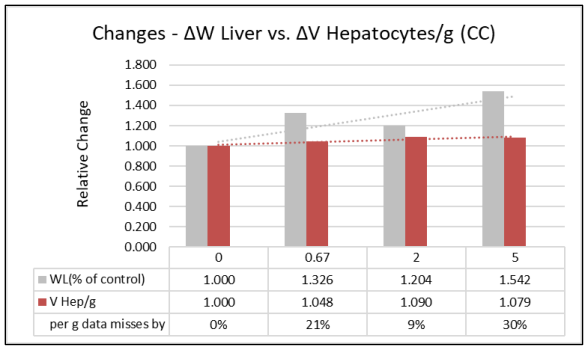


Figure 26 The differences in the changes shown can be explained by enlarging hepatocytes exiting the gram of liver, in numbers approaching 30%.

We can readily see the mischief being created by the uncorrected concentrations in Figure 27. Cytochrome P450 showed a 3.6-fold increase at day 5 when related to a gram of liver, but jumped up to 5.6-fold when related to the liver with Equation 11 – a considerable difference (54%).

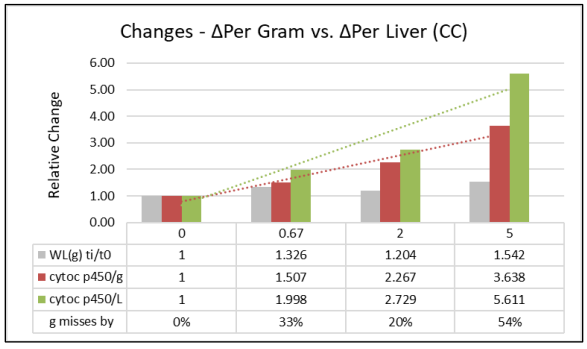


Figure 27 Relating biochemical data to a gram of liver, which is actively losing large numbers of hepatocytes, results in underestimating the enzyme activities by more than 50%.

Figure 28 compares estimates for the surface area of the ER data related to a gram of liver and to a gram of liver corrected with Equation 11. Once again, Equation 11 delivered the better results.

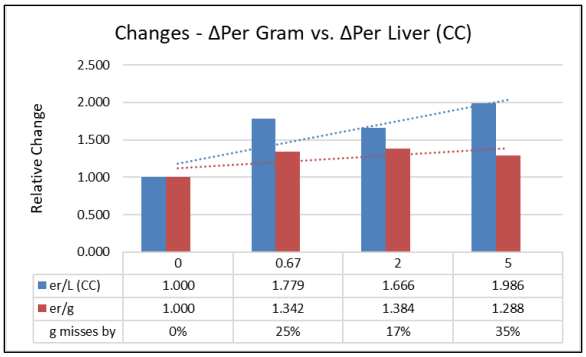


Figure 28 Changes in cytoplasmic organelles (ER) related to a gram of liver progressively lost hepatocytes as the size of the cells increased. The corrected data (CC) recovered these lost cells by assigning the changes in liver weight (or volume) to the changes in cell weight (or volume).

TESTING THE CCC EQUATION: If the changes in liver volume were allocated to the hepatocytes correctly with Equation 12, then the enzyme densities based on a gram of liver or on the total liver weight should be the same. Figure 29 shows this to be the case. It duplicates the results shown in Figure 4.

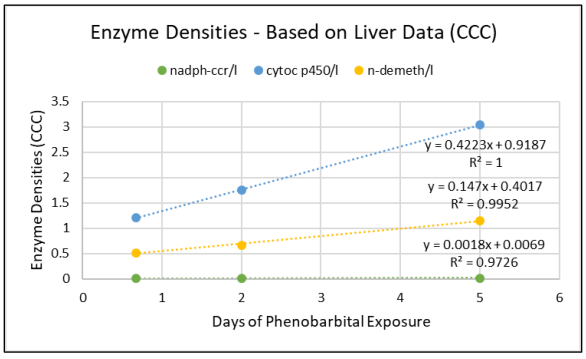


Figure 29 The results in Figures 4 and 29 are the same.

PRECISION AND ACCURACY: A solution to the concentration problem (Equation 12) also helps to alleviate the precision and reproducibility problems that often arise when detecting biological changes. By increasing the separation between control and experimental time points, equation 12 can be expected to improve outcomes when looking for significant differences. It would not be surprising to find many published P values shifting from >0.05 to ≤0.05 or better.

Consider, for example, the accuracy in reporting changes in cytochrome P450 activity. Figure 30 plots the way changes in this enzyme are usually reported (per gram of liver) and compares it to the same data scaled to the increases in liver weight (Equation 12). Notice that the amount of change differed by a unsettling 100%, whereas the rates of change (the slopes) differed by about 70%. Might such an example shed some light on our current problems with precision, accuracy, and reproducibility? Most likely, yes.

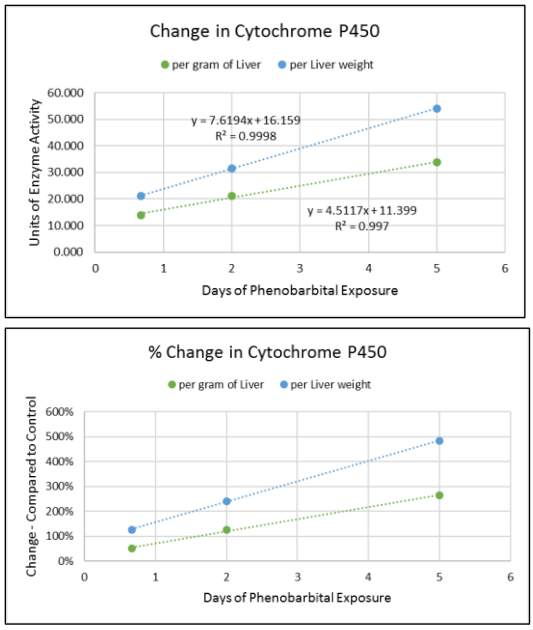


Figure 30 Collecting data from the same number of hepatocytes (per liver) detected twice as much change (compared to the usual gram of liver reference).

MOVING UPSTREAM: PROTEIN TO MRNA

Since we can use enzyme densities to generate rate constants for enzymes in membranes, will these data also allow us to generate rate constants for the messenger RNAs responsible for their production? In other words, does a quantitative relationship exist between the copy numbers of proteins (e.g., enzymes) and their progenitor mRNA molecules?

A recent study by Schwanhaüsser et al. (2011) undertook a global quantification of mammalian gene expression wherein they looked at the relationship between the synthesis rates of mRNAs and proteins for thousands of genes (> 5,000). They reported that housekeeping genes, such as those described in this report, tend to have stable mRNAs and proteins. In mouse fibroblasts, for example they found that the ratio of mRNA molecules to those of protein was 1 to 2800 with the translation rate of 1000 proteins per mRNA per hour.

If this translation efficiency for mRNA is hard-coded in the genome and shared by different cell types, then dividing one of our enzyme densities by 2800 produces a *very rough estimate* for the amount of mRNA associated with a given population of protein molecules. The result of such a calculation for cytochrome P450 is shown in Figure 31.

If the units of activity of the enzyme densities are expressed as numbers of molecules and if the protein to mRNA ratio holds, then it might be possible to estimate rate constants for mRNAs *in situ*.

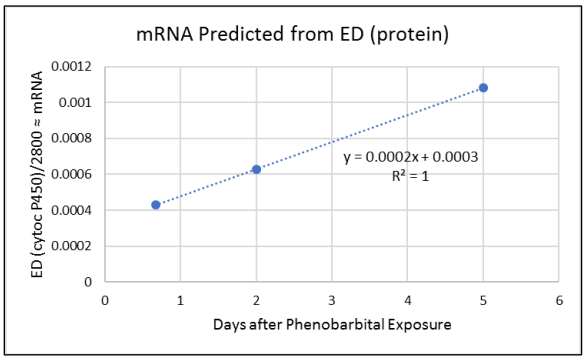


Figure 31 Note that this predicted curve for mRNA runs roughly parallel to the original cytochrome P450 curve shown in Figure 4.

Figure 31 was included to introduce a process. By extending the reach of phenome into the genome, rules detected downstream can be translated into upstream predictions.

Rethinking the traditional direction of information flow now becomes a compelling idea. Will it be faster and easier, for example, to discover biology's rules for gene expression by moving information from the phenome to the genome instead of the usual downstream approach? Will the phenotype with its ability to deliver biology's rules as empirical equations give us an edge when we set out to solve fundamental problems?

Paper 1 has yet another hidden gem. The authors reported that average hepatocytes responded to phenobarbital by increasing the surface area of the RER and the number of ribosomes attached to it (Stäubli et al., 1969: Figures 8 and 9; Table II). If these ribosomes newly attached to the ER are loaded with mRNAs specific to the hepatocyte's drug metabolizing recipe for phenobarbital, then the rate of ribosomal attachment also reflects the recipe's rate of mRNA synthesis (transcription). If we use the first and second time points (0 h, 16 h), the rate constant for the attachment of ribosomes to the ER predicts the arrival of 1,511 new ribosomes per hour per average hepatocyte. If, as Schwanhaüsser et al., (2011) reported for fibroblasts, hepatocytes also produce about 1000 proteins per ribosomal-bound mRNA, then the biological recipe for responding to phenobarbital during the first 16 hours of exposure might produce as many as 1.5 million proteins (i.e., drug metabolizing enzymes) per hour per hepatocyte. In effect, estimating rate constants for molecules in intact tissues - per cell or per organ – might provide a host of new insights into the control mechanisms of gene expression.

CHANGE – THE MANY OPTIONS

When we compare the results of several different data references commonly used to detect changes in the liver, the one that detects the greatest amount of change – starting with the same data - is the liver (Table 2). Regrettably, the least successful reference is the one most likely to be reported – changes per gram of liver.

Notice in Table 2 that biochemical data routinely display R^2 values considerably better than those of the morphology. Unfortunately, this is to be expected because of the volume distortions associated with specimen preparation for stereology. As shown in an earlier report (Bolender, 2013), data characterizing living samples tend to perform better than those from nonliving sources.

Table 2 Data references determine the amount of change detected, along with precision and accuracy. The underlined values represent the largest change or best R^2 value. Data adapted from Paper 1.

% Change and Choice of Data Reference				Linear
Days of PB	0.67	2	5	R^2
S(er)				
per 100gbw	138%	158%	165%	0.7783
per g liver	134%	138%	129%	0.5299
per liver	178%	167%	199%	0.6230
U(cyto P450)				
per 100gbw	356%	535%	941%	1.0000
per g liver	151%	227%	364%	0.9970
per liver	200%	273%	561%	0.9875
ED(cytoch P450)	112%	164%	283%	1.0000
U(demethylase)				
per 100gbw	156%	260%	469%	0.9992
per g liver	345%	460%	731%	0.9999
per liver	457%	554%	1127%	0.9712
ED(demethylase)	257%	332%	567%	0.9952
U(nadphccr)				
per 100gbw	103%	134%	228%	0.9959
per g liver	100%	117%	179%	0.9888
per liver	133%	140%	276%	0.9348
ED(nadphccr)	75%	84%	139%	0.9726

NEW INSIGHTS

By applying a first principles approach to our research programs, biology becomes progressively hardened as a science. When applied to the data of Papers 1 to 4, for example, biological rules allowed us to burrow into the mechanisms of change thereby giving us a much better idea of how to interact effectively with published data.

PRODUCTS OF RULE-BASED DATA (DERIVED FROM PAPERS 1-4)

1. **Biochemical Homogeneity** undergoes changes according to rule ($d[x]/dt = \pm k$). Data fitted to such rules display diagnostic and predictive properties (Figures 4, 5, 7).
2. **Rate Constants** define relationships of structure to function – consistent with biology operating by rule (Equations 4-10).
3. **Unique Signatures** occur when several enzyme densities combine to form distinct patterns as ratios (Figures 7, 19, 21).
4. **Control Mechanisms** expressed as nonlinear plots suggest that multiple rules are in play (Figure 19, 21).
5. **The similarity** between *in vivo* and *in vitro* equations provides reassurance (Figures 19, 21).
6. **An adaptive response** to the loss of membrane function can include an increase in the enzyme packing density of the remaining membranes (Figure 20).
7. **Morphological and biochemical concentrations** can be corrected to mimic average cell data with the same equation (Equation 11 or 12).
8. **Interpreting complex biological changes** requires mathematical collaboration between morphology and biochemistry (Figures 3-7, 10-13, 15-16, 18-20, 21, 29, 32).

CONCLUDING COMMENTS

Designing an experiment is akin to preparing a test question, which we then give to biology to answer. After running the experiment, our job is to use the resulting data to figure out how biology answered the question. A second test belongs to the investigator, who decides on what data to collect and how to interpret them.

The papers included in the report demonstrated that biology delivered first rate answers to all the questions we asked, often quite precisely ($R^2 = 1$ or $R^2 \approx 1$). Biology followed a two-step procedure. First, it created a recipe for a new membrane to answer the question and then grew the answer over time by expanding its surface area and composition. It changed by changing the packing densities of membrane-bound enzymes, the amounts of membranes and organelles, the volumes of cell compartments, and the weights of livers. Some events were local, others global.

It's fair to say that biology's answers to our questions required the creation of complex, quantitative relationships of structure to function. We captured these relationships as enzyme densities (concentrations) and used them to calculate rate constants, which, in turn, allowed us to predict morphological and biochemical data and to workout solutions to otherwise troublesome problems.

Since we know that biology plays an $R^2 = 1$ game based on clearly defined principles, our job now becomes one of capturing these principles and applying them as problem solving tools. In effect, we now know what to do and how to do it.

ACCORDING TO BIOLOGY, CHANGE CHANGES THE WAY CHANGES CHANGE...

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