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Morphometric Analysis in the Assessment of the Response of the Liver to Drugs*

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I. Introduction

The responses of cells to drugs and toxins include many complex changes in cellular morphology and biochemistry. Under one set of circumstances these changes may lead to new or improved cell vitality, whereas other circumstances may lead to cell failure and eventual death. However, this is only a single component of a much larger complexity. Toxicologists and pharmacologists are being asked to test massive numbers of chemicals with a variety of techniques and to provide interpretable results. The problem, it would seem, is as much technological as it is biological. The purpose of this report is to suggest ways in which quantitative morphological information might be helpful in pursuing the biological component of the problem.

Before considering a few experimental approaches and methods that are used to study the liver, it might be useful to look briefly at the liver itself. The liver is morphologically and biochemically a very heterogeneous organ. Its parenchyma alone, for example, consists of at least four different cell types (6, 62) with the hepatocytes assuming the major role in dealing with drugs and toxins (14, 19, 23, 25, 26, 31, 33, 35, 45, 48, 52, 61). The hepatocytes, however, are also heterogeneous. They are arranged within a functional lobule (46) where they display distinct structural (15, 39, 44, 49, 50, 58) and functional (34, 41, 46, 54, 55) differences. Moreover, membranes and organelles isolated from liver homogenates continue to display these heterogeneities (10, 12, 17, 20, 28, 43, 47). A major part of the liver complexity would thus seem to result from the fact that its hepatocytic component consists of morphologically heterogeneous cells capable of performing and responding differently. How then might one deal with all this cellular complexity?

Let us begin by defining as our principal goal an analytical approach that can detect changes occurring within a heterogeneous liver with an accuracy consistent with the identification and localization of several complex and simultaneously occurring events. The strategy of the approach is to sort the overall morphological and biochemical heterogeneities into "homogeneous" subpopulations that can be characterized quantitatively by structure-function equations.

II. General Characteristics of Morphological Information

The liver consists of cells and organelles that can be identified in light and electron micrographs. These structures can be quantitated using stereology, a morphological-mathematical method based on geometric probability theory. The validity of stereological estimates depends primarily upon: 1) tissue preservation (10); 2) the use of adequate sampling (10, 24, 53); and 3) corrections for section artifacts (40, 42, 56). In effect, the method translates measurements taken from "two-dimensional" planar sections to estimates that characterize a three-

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dimensional space. With it one can describe the liver as a volume containing different frequencies and sizes of cell types, each of which can also be characterized as to the volumes, surface areas, lengths, and frequencies of the organelles within (12, 40, 57). A property shared by these morphological parameters is that the solution of stereological equations relates these data to a similar reference, namely, a unit of volume. Volume densities, surface densities, length densities, and numerical densities all represent concentrations, that is, the total amount of volume, surface, length, or number of structures per unit of reference volume. This is a particularly useful outcome in that biochemistry likewise generates concentration data that can be related to a similar reference volume; this provides a common point for linking the two types of data. Unfortunately, the unit reference volume creates difficulties of interpretation when one wishes to pinpoint sources of change (10, 11). This problem of interpretation can often be avoided by expressing changes relative to a stereological average cell (7, 39, 57). Of particular importance is the fact that virtually everything found in an electron micrograph can be placed within morphologically defined compartments and be quantitated stereologically. Furthermore, the morphology of intact cells would seem to represent the only component of the experimental system that contains enough information to sort hepatocytes into discrete subpopulations.

III. General Characteristics of Biochemical Information

Before biochemical data can be collected, the liver must first be homogenized and fractionated and thus the spatial relationships between organelles within and/or between cells is lost. Marker enzyme activities associated with specific cellular organelles are assayed and expressed as concentrations, for example, by relating units of activity to 1 g or 1 cm^3 of the original liver (21). The power of this biochemical approach resides in the accuracy with which an enzyme activity can be measured in a given fraction and in its ability to interpret this accuracy experimentally. This is accomplished within the framework of analytical fractionation (22), which allows one to compare the amount of activity in the fractions to that of the original homogenate by calculating recoveries. This type of analysis is used to evaluate both the data and the procedures used in their collection.

Certain limitations exist, however, in the interpretation of the marker enzyme data used to characterize liver membranes. The initial homogenization of the tissue mixes membranes from a variety of cell types, including different hepatocytes. Although biochemical methods can be used to identify the presence of membrane marker enzyme heterogeneities (see; for example, references 5 and 20), the cellular origin of the heterogeneities remains obscured. It is characteristic of a marker enzyme heterogeneity that more activity can be associated with a given membrane surface area in one fraction than in another (5, 12, 37, 59). As a consequence, membrane surface area predictions based on marker enzyme activities, or vice versa, may be problematic (10).

IV. Liver Model for Assessing the Responses of the Liver to Drugs

The purpose of the discussion thus far has been to suggest that the interpretation of data collected from the liver can be subject to limitations inherent in both morphological and biochemical methods. The goal, however, is to characterize as accurately as possible the effects of drugs on hepatocytes. But, more specifically, what does this accuracy include? If it is assumed, for example, that a drug affects similar cells (hepatocytes) and similar membranes [endoplasmic reticulum (ER)] differently, then the desired accuracy includes an ability to detect these different responses morphologically and biochemically. In attempting to sort out several potential differences, a model can be constructed based on the combined characteristics of the *liver*, the *methods*, and the *data*. The model contains

two postulates, both of which the model itself is designed to test. It is assumed 1) that the liver consists of several populations of cells, which in turn consist of various subpopulations, and 2) that specific organelles (the ER, for example) are "homogeneous" within a given subpopulation. These postulates are currently being tested by addressing a series of questions that attempt to define the relationships between the methods, the data, and the liver.

A. Can Morphological and Biochemical Data Be Related to One Another Quantitatively?

This seemed the likely question to begin with because the integration of structural and functional data ultimately rests upon an assumption of compatibility. Consider the data available to us. Stereological methods characterize morphological components such as organelles and membranes in the intact liver as well as in the homogenate and fractions. Biochemical methods characterize chemical constituents (for example, marker enzymes, protein, lipids) in the liver homogenate and fractions. Since the structural and functional data overlap in the homogenate and in the fractions, they would seem to be the most promising of the liver configurations to use for integration of the results. Although membranes from homogenized cells can be assayed biochemically with a relatively high degree of precision, much of the morphological detail seen in the intact tissue is no longer present, and thus the morphological precision is reduced to the mere estimation of total membrane surface areas and total organelle volumes (13).

Given a specific interest in hepatocytes and individual membrane compartments therein, the extent of the contaminations derived from other cell types needs assessment, as in the study of Blouin et al. (6). Figure 1 illustrates the morphological compartments of the liver parenchyma that were quantitated, and Figure 2 summarizes the partitioning of organelles between hepatocytic and nonhepatocytic sources. Of

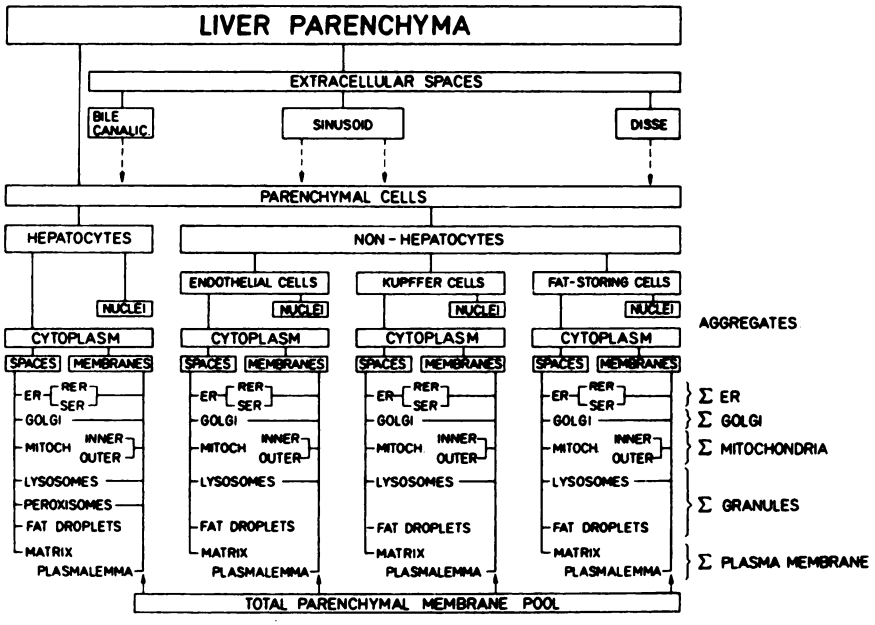

FIG. 1. Quantitative model for the liver. The liver parenchyma consists of extracellular spaces and parenchymal cells subdivided into hepatocytic and nonhepatocytic compartments. Cytoplasmic organelles are described as spaces (volumes) and membranes (surface areas) that can be characterized for individual types of cells or for the total parenchyma. [From Blouin et al. (6).]

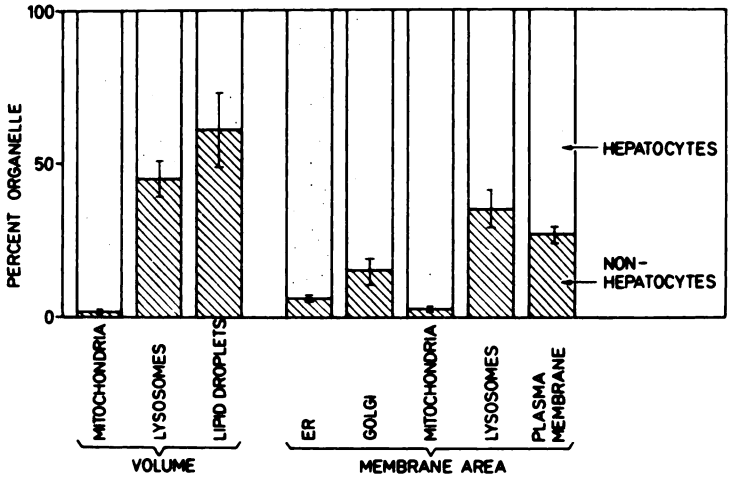

FIG. 2. Relative distributions of organelle volumes and surface areas are shown as they occur in hepatocytic and nonhepatocytic cells of the liver parenchyma. [From Blouin et al. (6).]

the total liver membranes, somewhat more than 95% was derived from hepatocytes, which included most of the liver endoplasmic reticulum (94%) and mitochondria (98%), but notably smaller proportions of Golgi (85%) and plasma membranes (74%). The substantial concentration of lysosomes (38%) and lipid droplets (55%) in cells other than hepatocytes was a most unexpected finding. Corrections for nonhepatocytic compartments could now be applied to the liver parenchyma, but the information of more immediate importance was the almost entirely hepatocytic origin of the endoplasmic reticulum of the liver. The ER, therefore, became the most likely candidate for pursuit of an answer to the first question.

Confidence in biochemical data can be developed by calculating recovery (4, 18, 21), which relates the amount of marker enzyme activity in the original material [in this case the liver homogenate (H)] to the aggregate activities of the derived fractions (F). When biochemical (F/H) recoveries approximate 100%, a conclusion frequently drawn is that the enzyme activity of the homogenate has been *conserved* during the subsequent fractionation steps.

But what happens to the morphology of the same membranes to which these marker enzymes are attached? Are they likewise conserved during the fractionation? To answer these questions, membrane surface areas in the intact tissue and then in the homogenate and derived fractions were estimated as outlined in Figure 3. The 96% F/H recovery for the total liver membranes shown in Figure 4 indicates that membrane surface area is also *conserved.* What had happened, however, was that only 63% of the membranes that could be identified as belonging to a specific compartment in the intact tissue was still recognizable as such in the fractions. This represented a considerable loss in the ability to identify specific membranes morphologically. In order to integrate the morphological and biochemical data characterizing the ER, this lost resolution had to be regained and, to this end, cytochemical (8) and freeze-fracture (38) methods were incorporated into the model.

The cyotchemical procedure of Leskes et al. (36), which identifies glucose-6-phosphatase-positive membranes in fractions, was used in combination with stereology to estimate the surface area of the endoplasmic reticulum in each of the fractions (12) described in Figure 3. Fraction/homogenate recoveries calculated for these cytochemically identified membranes averaged 94% ($n = 3$), providing some assurance that the new method was at least giving results predicted from the biochemical recoveries for

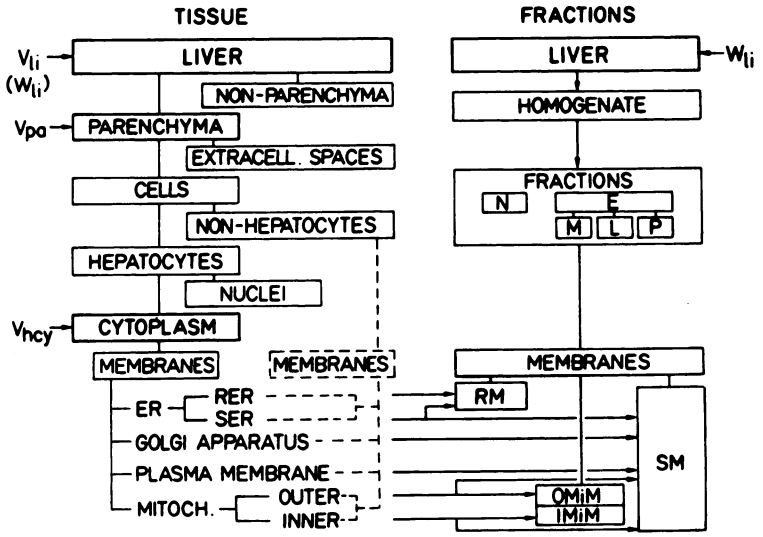

FIG. 3. The liver model identifies cellular and subcellular components associated with the intact tissue and fractions. Organelles recognizable as specific membrane compartments in the intact liver are identified in the homogenate and fractions as rough membranes (RM) when they have attached ribosomes, as smooth membranes (SM) when they are fragments without attached ribosomes, and as mitochondrial membranes when the inner and outer membranes are recognizable as such. The intact tissue reference volumes used to relate membrane surface areas (S_i) estimated at sampling stages III and IV to 1 g of liver at stage I include the hepatocyte cytoplasm (V_{hcy}) and the parenchyma (V_{pa}). The pellicle volume ($V_{pel(f)}$) represented the volume of a disc obtained by filtering a known fraction aliquot ($V_{(f)}$); the volume was calculated from the diameter and thickness of the disc. The fractions are identified according to deDuve (21); E, extract; N, nuclear; M, heavy mitochondrial; L, light mitochondrial; and P, microsomal. [From Bolender et al.(13).]

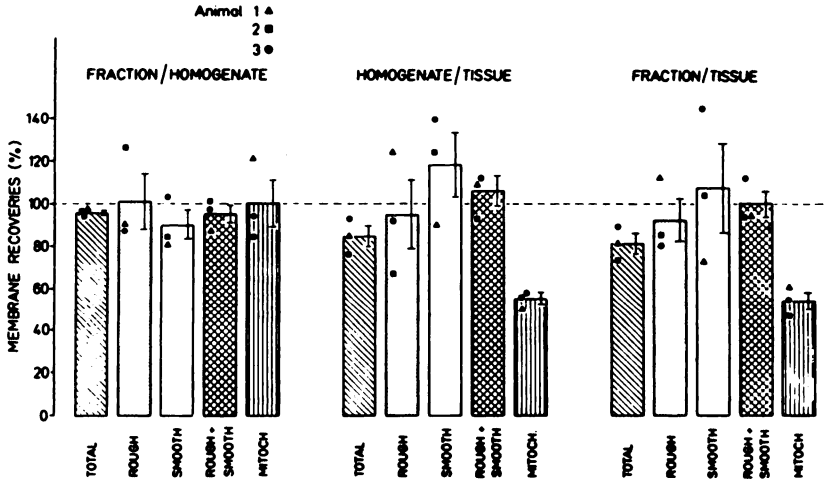

FIG. 4. Morphological membrane recoveries compare the surface areas of the same membranes estimated first in the intact tissue and then in the homogenate and fractions. The contents of the individual compartments are given in Figure 3. The 96% fraction/homogenate recovery indicates that almost all of the membrane surface area is conserved during the fractionation. The tissue recoveries are somewhat lower, but this is the result, at least in part, of an uncorrected overestimate for the intact tissue membranes. Means and standard errors are included. [From Bolender et al. (13).]

glucose-6-phosphatase activity obtained from the same animals, which averaged 96%. Subsequently, additional cytochemical methods for other hepatocyte membranes have been tried but thus far without success (unpublished observations). The fact that intramembranous particles, as seen in freeze-fracture replicas of membranes, exhibit characteristics distinct for specific membranes offered an alternative way to identify these membranes, at least in the microsomal fraction (38). The results of the freeze-fracture were particularly encouraging in that the independent estimate for ER membranes in the P fraction (63%) compared favorably with the corresponding cytochemical estimate (62%).

Freeze-fracture confirmation of the cytochemical method plus the earlier findings that morphological and biochemical F/H recoveries were at the 95% level suggested that at least a partial answer to the first question was possible, i.e., in a given fraction, the activity of an ER marker enzyme could be linked quantitatively to the surface area of the membrane to which it was attached. As shown in Figure 5, activity of glucose-6-phosphatase is related to the surface area of the ER in each of the membrane-containing fractions. The amount of enzyme activity per unit of membrane surface area, that is, the enzyme density (12), is different for all four fractions, varying as much as 70% from one fraction to another. In view of earlier studies (5, 20, 26, 37, 59), this finding of a heterogeneous distribution of glucose-6-phosphatase across the membranes of the ER came as no surprise; rather the intriguing fact is that just a differential centrifugation was enough to uncover such a broad range of heterogeneities. Since the individual fractions contain in effect an "ER homogenate," the actual range must be far greater. The implication of this finding is that a unit of glucose-6-phosphatase activity predicts a different amount of ER membrane surface area for each of the four fractions.

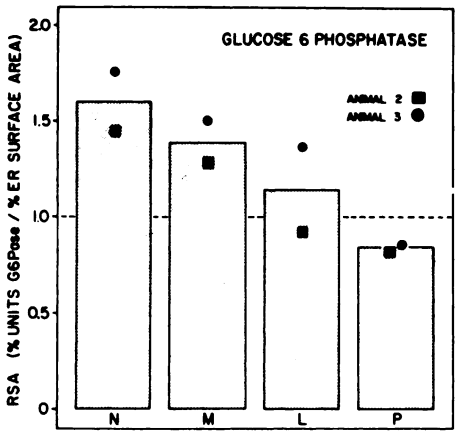

FIG. 5. Relative specific activity (RSA) of glucose-6-phosphatase using endoplasmic reticulum (ER) surface area as its reference. The figure illustrates that glucose-6-phosphatase activity is not uniformly distributed across the membranes of the ER in the various fractions. See text for details. [Adapted from Bolender et al. (12).]

The strategy of the model thus far has been to interpret morphological data within the same framework of analysis used for biochemistry by calculating recoveries. In following this approach, morphological methods have proven to be useful: 1) for identifying and quantitating a specific membrane compartment in fractions; 2) as a reference system for biochemical data; and 3) as a means to define quantitatively biochemical heterogeneities in fractions. The immediate practical use of enzyme densities, which link the morphological and biochemical data, is that only one measurement is needed to predict the other. Such heterogeneity information is particularly useful 1) for determining the amount of ER contamination in a given fraction and 2) for extrapolating data from the fractions to the original liver. It should be emphasized, however, that the enzyme densities of Figure 5 reflect the fractionation method (13).

The demonstration of a marker enzyme membrane heterogeneity suggests that ER membranes can play different functional roles, at least with respect to glucose-6-phosphatase. But how can these membrane heterogeneities be related to hepatocytes as they occur in the liver lobule? The next question considers a framework of analysis in which such relationships might be considered.

B. What Changes Can Be Detected Morphologically and Biochemically?

The somewhat surprising answer to this question is that morphological and biochemical results depend almost as much on how one chooses to interpret the data as on the data itself. In retrospect, this point now seems obvious, although the extent of the effect was not anticipated at the outset of the experiments.

As mentioned earlier, the solution of stereological equations can provide morphological estimates related to a cubic unit of reference volume (CURV). This reference volume can be chosen to characterize the entire liver, the parenchyma, hepatocytes, or just the hepatocytic cytoplasm (6, 13, 40, 57). Furthermore, stereological estimates for volume, surface, and length are represented as aggregate values, that is, they can detect only the total compartmental volume, surface, or length of a particular component found within the CURV. While the method supplies quantitative morphological information, the interpretation of this information, *particularly when used to describe changes*, can be severely limited by ambiguities associated with the CURV (10, 11). A closer look at these ambiguities is even more strongly indicated in view of the fact that a unit reference weight (1 g of liver) has just served as the mechanism for linking the structure-function data (13) (see Fig. 3).

Figure 6 illustrates changes occurring within hypothetical hepatocytes each containing a single spherical nucleus. The standard unit of reference volume is represented by six large cubes, all of which have identical volumes. In the *top row*, the nuclei within the unit references increase in volume moving from left to right. The nuclei in the *bottom row* maintain a constant volume, but the number of cells (and nuclei) packed into the cubic unit of reference volume increases from one, to two, to four. When the volume densities of the nuclear compartments are calculated for the top and bottom rows, the left, middle, and right

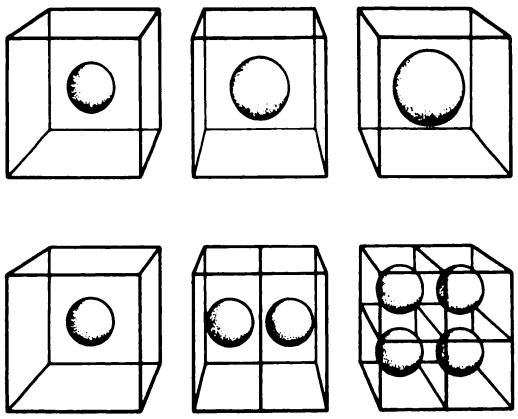

FIG. 6. "Stereological volume densities" using a unit of reference volume (large cubes) can detect only changes in the total volume of a compartment (spheres). In the *top row*, the volume of the sphere in the cube increases, whereas in the *bottom row* the volume of the individual spheres remains constant while the number of spheres per reference volume (large cube) increases from one to two to four. Although the "morphological events" responsible for the changes in the compartmental volume of the sphere(s) are quite different, the volume densities for both rows are identical. [From Bolender (10).]

vertical sets of cubes are "stereologically" indistinguishable. Although the "cellular events" producing the changes in the top row were quite different from those in the bottom, the changes in volume densities for both rows are identical. From this example, one can develop an understanding of how a change in a stereological volume density, or, for that matter, surface and length densities as well, may be influenced by an actual change in a cellular organelle or by a change in the number of cells filling the reference volume. More often than not both events occur simultaneously. The point of particular importance here is that stereological densities *can* detect what is happening within a cubic centimeter (or gram) of liver, but they *cannot* pinpoint the source of the change(s).

The seemingly obvious solution to this problem of interpretation is to determine the numbers of hepatocyte nuclei per unit of reference volume, and then use this estimate in combination with the volume, surface, and length densities to calculate average cell values (10, 40, 57). Describing

changes per average cell would seem to give the most interpretable results in that this derived reference presumably detects *only* changes in cellular organelles. Using this approach to estimate an average hepatocyte volume, however, is not straightforward. The assumption that each "average cell" contains a single nucleus is not fulfilled by hepatocytes: roughly 20% are binucleate in adult rats (16). Consequently, when their aggregate cell volume (per cm^3) is divided by their nuclear numerical density, which is, of course greater than the cellular numerical density, an underestimate for the average cell volume results. Conditions under which the relative frequencies of binucleated hepatocytes change are therefore expected to reduce the effectiveness of the average cell volume reference for detecting hepatocytic changes (10). There is a second and perhaps an even more important problem. Numerical density estimates require 1) likening the nuclei to geometrically defined models and 2) knowing the size frequency distributions of the nuclei (24, 53). These requirements are not only difficult to satisfy, but the errors produced by biological structures often not well characterized by such models are even more difficult to detect. A brief example will serve to illustrate just how sensitive a numerical density method is to only one of several measuring errors.

An average cell volume can be derived from an estimate for average nuclear diameter (7). The method consists of measuring the diameters of nuclear profiles and then converting the distribution of profiles to one of nuclear diameters stereologically (2, 24, 53, 60). A relatively small error in measuring the diameters of the nuclear profiles, however, can lead to misleading estimates for average nuclear volume, and the average derived cell volume. These measurements are influenced, for example, by 1) section thickness (27, 30), 2) section compression (30), and 3) choice of a "representative diameter" (10). Rat liver nuclei are usually spherical, but when sectioned for electron microscopy ellipsoidal profiles are produced that have, on the average, a compression (major/minor profile diameter) of about 20% (6, 13, 15, 40). Assuming, as done earlier by Loud et al. (40), that only one diameter of the nuclear profile is distorted during the sectioning, namely the minor diameter, then to use a diameter less than the major one would be expected to lead to an underestimate for the average nuclear diameter. For example, a spherically reduced profile, represented by a circle having an area equal to that of an elliptical one having 20% compression, would underestimate the diameter of the noncompressed nuclear profile by more than 10%. A 10% measurement error here, however, results in a 30% underestimate when the average cell volume is calculated. The 30% error is transferred to cytoplasmic organelles when this underestimated average cell is used as their reference.

Numerical density estimates seem to be most reliable when two conditions are satisfied: 1) the nuclei are spheroidal (1); and 2) each cell contains a single nucleus (10). Hepatocytes fulfill the former requirement but not the latter. In reporting average hepatocytic volumes, the effect of the binucleated cells on these estimates has been recognized (29, 40, 50, 57), but a correction has not been forthcoming that would account for the error associated with treating a single binucleated hepatocyte as two separate cells. Carriere (16) and Wheatley (58) have published estimates that put the relative proportion of binucleated hepatocytes in the adult liver at about 20%. Using this value to correct the average hepatocytic volume for binucleated cells, one finds that the noncorrected estimates contain a 17% error. Combining just the errors associated with compression (30%) and binucleated hepatocytes (17%), the average volume for a "hepatocyte" can easily be underestimated by almost 50%. While such an estimate for the potential error associated with an average cell volume may be instructive, it is not particularly useful except to illustrate that numerical densities can compromise interpretations of average cell data. A

new approach was thus needed for estimating average cell values, one that did not depend on prior determination of the number of nuclei and cells.

The surface area ratio method (9–11, 51) seems to offer some promise in this respect in that it can detect relative changes in average cells and requires only a special interpretation of surface densities. The method depends on being able to identify a membrane compartment that maintains a constant surface area to which changing membrane compartments are then compared.

The method has been tested with the guinea pig pancreas (10). This tissue was chosen because the exocrine cells contain *single* nuclei that closely approximate a *spherical* shape, the ideal conditions for estimating average cell volumes from numerical densities. With the in vitro tissue slice system of Jamieson and Palade (32), exocrine cells in the process of releasing zymogen granules were analyzed using both numerical density and ratio methods (9, 11). Relative changes in four membrane compartments after 20 minutes exposure to a secretagogue were compared to controls, as illustrated in Figure 7. The changes detected by the two average cell methods, one based on the average cell surface (ratio method), the other on average cell volume,

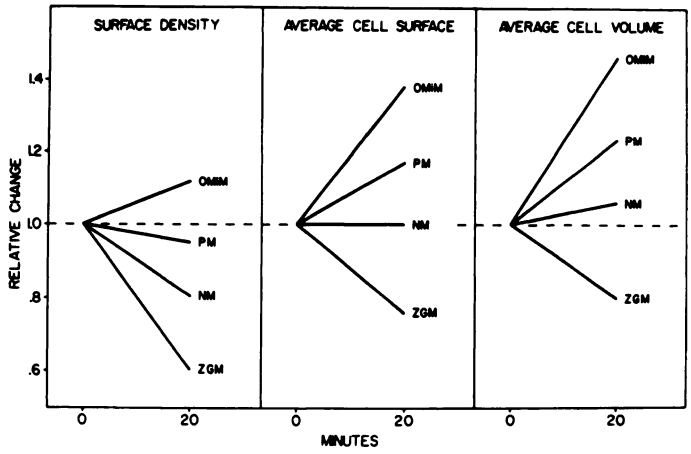

FIG. 7. Relative changes in the surface areas of four membrane compartments of pancreatic exocrine cells exposed to a secretagogue for 20 minutes: outer mitochondrial membrane (OMiM), plasma membrane (PM), nuclear membrane (NM), zymogen granule membrane (ZGM). Results are similar for the average cells using either nuclear surface area or nuclear volume references, but markedly different for the surface densities related to 1 cm^3 of reference volume. [From Bolender (11).]

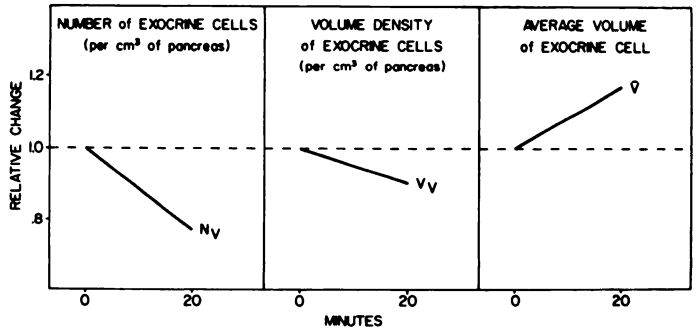

FIG. 8. The discrepancies between the surface density and average cell changes seen in Figure 7 are explained by three concurrent events: 1) the number of exocrine cells per cm^3 (N_V) decreases; 2) the relative volume of exocrine cells (V_V) decreases; and 3) the average exocrine cell volume increases. [From Bolender (11).]

were essentially identical. The changes based on the usual interpretation of surface densities, however, gave very different results (Fig. 7). Such a discrepancy was, in fact, predicted in the earlier discussion of stereological density ambiguities. Using the data from the pancreas model, the sources of these ambiguities are easily pinpointed. Figure 8 indicates that during the 20 minutes of incubation, the number of cells filling the unit of reference volume, i.e., their numerical density (N_V), decreased by more than 20%. Consequently, at 20 minutes, fewer than 80% of the cells measured in the controls were still contributing to the aggregate membrane surface areas that characterize the surface densities (S_V) of the four membrane compartments. The 20% decrease in the number of cells was produced by two events: 1) a decrease in the relative volume of the exocrine cells (V_V); and 2) an increase in the average exocrine cell volume ($\bar{V}$); n.b., the 20% increase in average cell volume meant that fewer cells could be packed into the cubic centimeter reference.

Figure 7 illustrates that one's choice of reference system in describing these membrane changes during secretion, in fact, determines the interpretations that follow. For example, the average cell data suggest that the zymogen granule (ZG) compartment is being depleted, while at the same time the surface area of the plasma membrane (PM) increases. Such an observation would be consistent with membrane transfer (exocytosis) known to occur during exocrine secretion. The surface density data, however, describe a far greater reduction in the ZG compartment and miss the increase in PM surface area altogether.

But how is the surface ratio method as described for the pancreas (9, 11) applicable for detecting average hepatocytic changes in the liver? Under experimental conditions where the validity of the reference membrane can be confirmed (11), the interpretation of the surface ratio data is expected to be equally acceptable. It is already known, for example, that the nuclei of phenobarbital-induced hepatocytes are similar to those of the controls (14, 52) and they appear to be quite suitable as a reference membrane under such experimental conditions (unpublished observations).

Thus far, it would seem that the most readily interpretable reference for detecting changes stereologically is the one based on an average cell volume or surface. The potential ambiguities associated with the stereological densities can be avoided almost entirely by using such reference systems, particularly when one's purpose is to detect cell changes. But how can these morphological findings be useful for interpreting biochemical data? For example, when relating marker enzyme activity to 1 g of liver [1.07 g = 1 cm^3 (14)] the biochemical reference system is, in fact, the same as the one characterizing the stereological densities. Does this mean that this biochemical reference is likewise potentially uninterpretable when used for detecting changes in cells? The unfortunate answer to this question is yes. For example, the identical situation exists biochemically as shown for the morphological changes in Figures 6 and 7. Replace the changes in surface densities illustrated in Figure 7 with units of marker enzyme activity (per g) that characterize these specific compartments, and, assuming marker enzyme homogeneity, a similar discrepancy between the density and average cell data would be expected. The conclusion that a change in the number of hepatocytes packed into 1 cm^3 or g of liver can have the same undesirable effect on interpreting both stereological and biochemical data seems unavoidable. The morphological and biochemical data using the gram of liver as a common linking reference (13) are therefore not expected to be readily interpretable references when hepatocytes, for example, are induced by drugs. The more useful reference for detecting structural and functional responses would still seem to be the average cell. Given the characteristics of the two experimental methods, only the morphological average cell can be directly obtained although it may be possible to

infer the biochemical average cells from the morphological ones.

Given as background the discussion above, a few examples taken from the literature might serve to amplify the importance of the reference system in interpreting changes. The effect of phenobarbital induction (5 days; 100 mg/kg) on the surface area of the ER and one of its marker enzymes, oxidative demethylase, is illustrated in Figure 9; it represents data adapted from a study by Stäubli et al. (52). The data are "integrated" by being related to the same reference and then plotted as regression lines. The slopes of the lines indicate that the three references detect the rate of change similarly, but the lengths of the lines show marked differences with respect to the amount of change. The average cell (hepatocyte) reference detects changes in membranes 18% lower than the cubic centimeter and 40% lower than the 100-g body weight reference, but 32% and 82% lower, respectively, for the chemical changes. If, on the other hand, mixed morphological and biochemical references are used, new problems of interpretation arise (Fig. 10). Data based on units of enzyme activity per mg of protein in a microsomal fraction (45) are related to morphological estimates coming from the intact liver (14). These experiments describe the recovery of hepatocytes from 5 days of phenobarbital treatment (100 mg/kg/day). By keeping the biochemical reference system constant and varying the morphological one, none of which match the biochemical milligram of protein, the resulting curves show very different amounts and rates of removal for the ER surface area and marker enzyme activity. Although all three curves suggest a parallel decrease in both the ER surface area and its constituent drug metabolizing enzyme, which one is the most correct interpretation? The question seems almost unanswerable in that the relationship between a biochemical activity related to a milligram of protein in a microsomal fraction and the surface area of the ER membranes in intact hepatocytes is quite unknown.

In answer to the original question regarding the meaning of change: 1) it appears that both morphological and biochemical

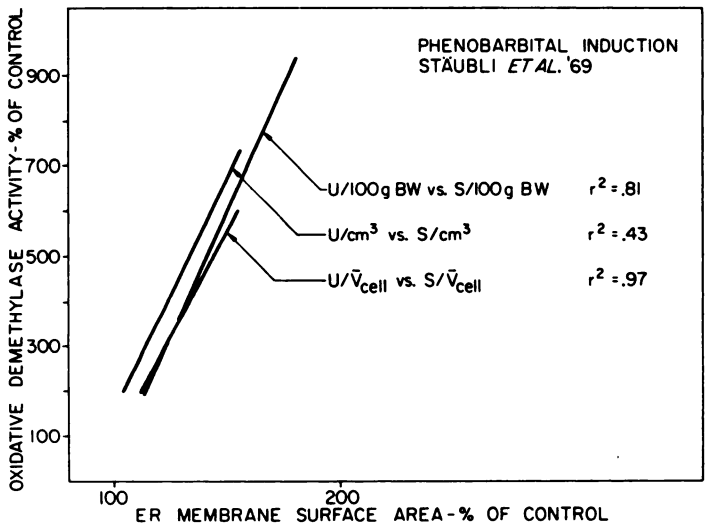

FIG. 9. Biochemical and morphological data related to similar references—100 g of body weight (BW), cubic centimeter of liver, and the volume of an average "hepatocyte" ($\bar{V}_{cell}$)—are related to one another by calculating regression lines; r^2 is the coefficient of determination. The changes in the units of enzyme activity (U) and endoplasmic reticulum (ER) membrane surface area (S) were produced by phenobarbital induction. [From Bolender (11).]

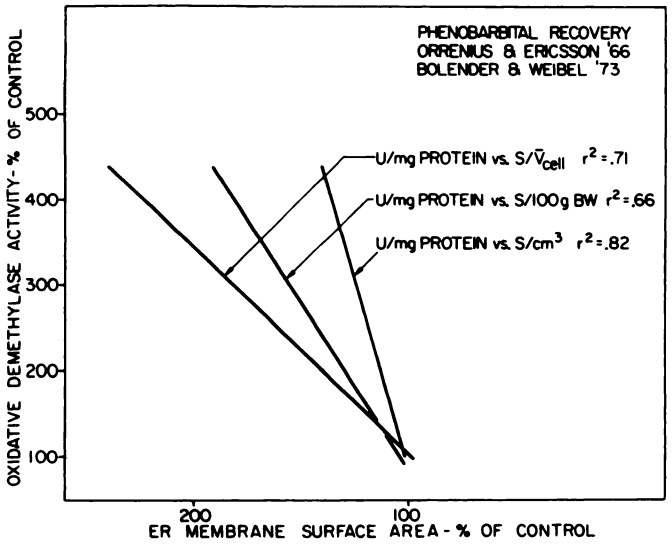

FIG. 10. Using the morphological references described in Figure 9, units of enzyme activity (U) per mg of protein, measured in rat liver microsomal fractions, are related to the surface areas of the endoplasmic reticulum membranes in the intact tissue. The changes characterize the recoveries of hepatocytes from phenobarbital treatment (14, 44); see text for details. [From Bolender (11).]

data are subject to similar ambiguities of interpretation when expressed as concentrations (per unit volume or unit weight); and that 2) any attempt to integrate these data with the purpose of combining quantitatively morphological and biochemical changes would seem to depend on the use of an average cell reference.

C. Can Heterogeneous Populations of Cells Be Sorted Morphometrically?

In attempting to answer this question, the strategy has been to use morphology to locate specific changes in average hepatocytes, assuming that the liver consists of several subpopulations of average hepatocytes that are more or less homogeneous. Once again, however, the liver was not the ideal tissue model and preparations of lymphocytes collected from the spleen and lymph nodes of Lewis rats were used instead to test the sorting equations (1). To this end, the pellicle method of Baudhuin et al. (3) was adapted for cell suspensions and lymphocytes, polymorphonuclear leukocytes (polys), and plasma cells were collected on Millipore filters by filtration and subsequently prepared for electron microscopy (13). The total volume of the cells in the resulting disc-shaped pellicle is readily determined by morphometry, and the average cell volume by dividing this aggregate cell volume by the known number of cells filtered. This average cell volume, however, represented a mean value for the combined lymphocytes, polys, and plasma cells. It reflected two sets of unknowns: 1) the average volume for each of the three cell types; and 2) the relative proportions of each cell type. The problem was to estimate 1) and 2) without resorting to the traditional numerical density methods.

The solution was to choose a morphological ratio that can be measured in all three cell types; the nuclear-cytoplasmic volume ratio (NCVR) was selected because it appeared to be unique for each cell type. The ratio served as a quantitative morphological marker. The total number of cells in the pellicle and the NCVRs for each cell type *and* the combined cells represent the known variables, and the frequencies of each cell type represent the unknowns. The unknowns were obtained simply by solving simultaneous equations. The results of such a calculation are given in Figure 11. Once

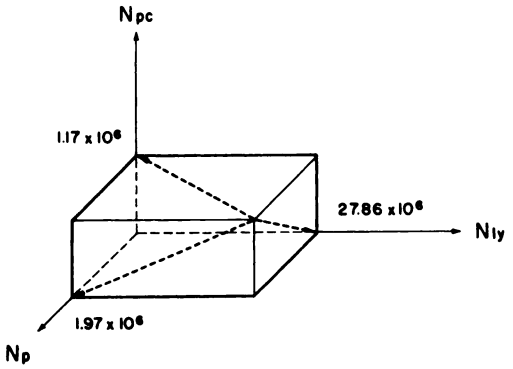

FIG. 11. Three subpopulations of cells found in a lymph node suspension are sorted stereologically. The simultaneous solution of three equations, one for each subpopulation, is shown graphically as a point that is used to locate the amounts of lymphocytes (N_{ly}), polys (N_p), and plasma cells (N_{pc}) in the pellicle. (Unpublished data.)

the frequency of each cell type is known, the average cell volumes can be calculated by dividing the total volume of the specific cell in the pellicle by its frequency.

The equations and a sample calculation for sorting two subpopulations of cells are included as a means of illustrating the method. A cell suspension consists of two subpopulations of morphologically distinct cells, x and y. The combined number of cells in a pellicle (N_{x+y}) and the individual $(V_n/V_{cy})_{x,y}$ and combined $(V_n/V_{cy})_{x+y}$ nuclear cytoplasmic volume ratios are known. Two equations can be written and their simultaneous solution provides the number of cells in each subpopulation.

$$N_x + N_y = N_{x+y} \tag{1}$$

$$N_x(V_n/V_{cy})_x + N_y(V_n/V_{cy})_y = N_{x+y}(V_n/V_{cy})_{x+y} \tag{2}$$

When $N_{x+y} = 120{,}000$ cells, $(V_n/V_{cy})_x = 0.5$, $(V_n/V_{cy})_y = 0.3$, and $(V_n/V_{cy})_{x+y} = 0.35$, the simultaneous solutions of equations 1 and 2 gives $N_x = 90{,}000$ cells and $N_y = 30{,}000$ cells ($x = 75\%$, $y = 25\%$). Having an aggregate pellicle volume of 0.01 cm^3 and 0.02 cm^3, respectively, the average cell volume for cell $x = 1.1 \times 10^{-7}$ cm^3 and for cell $y = 6.7 \times 10^{-7}$ cm^3.

Subpopulations of hepatocytes can be identified visually (26, 39, 50) and then characterized quantitatively with volume or surface area markers (unpublished data), but a reliable stereological method for estimating the number of hepatocytes per cm^3 or gram of liver does not yet appear to exist. How then can the sorting method be applied to hepatocytes? Fortunately, equations 1 and 2 can be solved by substituting any number at all for N_{x+y}; the relative proportions (frequencies) of the different hepatocytes as well as the average hepatocyte volume (or surface) ratios remain exactly the same. It would, therefore, appear that the sorting method can supply at least the relative distributions of hepatocyte subpopulations and their average cell volume ratios without having to know the number of hepatocytes in 1 cm^3. Changes in these subpopulations can be estimated with the surface ratio method (11).

V. Morphometry and the Future

Technologies of the future will undoubtedly include computer modeling systems capable of diagnosing and predicting short- and long-term effects of drugs and toxins on cells. In developing such systems, however, difficult questions arise as to what will be stored in the computer memories and what types of inputs are to be evaluated. The purpose of the foregoing discussion has been to consider ways in which morphometric methods can be helpful in defining such inputs. Particular attention has been given to stereological indicators of change and to experimental systems that favor the integration of morphological and biochemical data. It might be postulated that sets of structure-function equations will become one of the many inputs needed to diagnose and predict cellular behavior.

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