1965

Christian de Duve, 1963

The Rockefeller University

THE SEPARATION AND CHARACTERIZATION OF SUBCELLULAR PARTICLES*

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I. Introduction

In his Harvey Lecture, delivered a little over fifteen years ago, Albert Claude (1950) recalled the construction of improved microscope lenses by Giovanni Battista Amici in 1827 and pointed out that this technical development had led directly to our concept of the cell as the basic unit of living matter. "In the history of cytology," Claude wrote, "it is repeatedly found that further advance had to await the accident of technical progress."

Today, with only fifteen years to look back on, we may already safely say that few such accidents, since the days of Amici, have had such far-reaching influences on the history of cytology as those which Claude then goes on to describe in the main part of his lecture. And I refer specifically to the application of electron microscopy to the study of cells, which dates back to the first attempts of Porter, Claude, and Fullam (reported in 1945), and to the fractionation of mammalian cells by differential centrifugation, a method worked out over a number of years and first described in its entirety by Claude in 1946.

Until these "accidents" occurred, workers engaged in the exploration of living organisms had been forced to stop at the edge of a mysterious no-man's-land, bounded at the upper level of the dimension scale by the resolving power of the light microscope, and at the lower level by the applicability of chemical techniques. They knew, in a frustrating sort of way, that the area between these two boundaries contained some of the essential clues without which life would remain forever ununder-

* Lecture delivered November 21, 1963.

standable. With the technical advances mentioned, this region suddenly became accessible, both to visual examination right down to the level of macromolecules, and to chemical separation and analysis right up to the level of microscopic entities.

In his lecture at the centennial of the National Academy of Sciences, George Palade remarked on the fortunate coincidence that caused these two definitive steps to be taken almost simultaneously. It may also be counted fortunate that they were taken at a time when biology had just completed its preparations, in such fields as genetics and biochemistry, and marshaled important new forces, particularly with the development of chromatography and radioisotope techniques. The results of this formidable combination are known to every one of us today.

It has been the good fortune of the small group of investigators working at that time at the Laboratory of Physiological Chemistry at the University of Louvain to become associated at a very early stage with this invasion of the submicroscopic domain. Indeed, we can claim some sort of spiritual kinship with one of its main originators and received our first instruction in the art of fractionating cells from Dr. Claude himself. It would please me greatly to be able to say that we adopted differential centrifugation at that time because of a lucid and compelling realization of its fundamental importance as a biological tool. Unfortunately, this would not be quite truthful, and ours was very much a case of getting wise after the fact. We were first led to use the technique, in what we thought would be only a short interlude in our work on carbohydrate metabolism, by some chance observations made in the course of an attempt to purify the enzyme glucose 6-phosphatase. Other, even more accidental observations made during this early work on the unspecific acid phosphatase of liver, an enzyme which held no interest for us except in its negative property of not being glucose 6-phosphatase, caused us to linger in the centrifuge field longer than we had expected, and finally, one thing leading to another, to make centrifugal fractionation one of our major concerns.

It would also be pleasant to say that the conceptual and technical intricacies of the method became immediately clear to us and that our experiments proceeded with beautiful cartesian

order and design toward their ultimate goal. However, this again would be embellishing the facts. Actually, during those critical years when the method matured to its present status, we remained very much in the rear and owed much of our progress to the pioneering work performed in the United States by Hogeboom, Schneider, Palade, Novikoff, Thomson, Anderson, Kuff, and many other investigators. I would like particularly to recall here the name of the late George Hogeboom whose untimely death has deprived this field of one of its most creative leaders.

I have felt it necessary to record these facts because I have chosen in the rest of this lecture to sacrifice historical accuracy to the interests of clarity and brevity. Rather than describe the hesitant and rambling course of our explorations, I will immediately try to explain very briefly our present concept of centrifugal fractionation and will then review some of the results which I believe justify this concept. I have already recalled our debt to others, and it remains for me to emphasize the fact that the work which I will survey has been very much a cooperative effort in which many have participated. Especially in its technical and theoretical developments, the close collaboration of Drs. Jacques Berthet and Henri Beaufay has been invaluable.

II. General Principles

Tissues are composed of cells held together within a fibrous framework. Cells consist of a ground substance of unknown degree of organization, of membrane systems, and of structural entities which may be designated as organelles. The ground substance, membrane systems and organelles, as well as the extracellular fibers, are made of a variety of macromolecules and micromolecules. The macromolecules are themselves assembled from micromolecules, and the latter are made of atoms. At each level, these parts may be grouped in a number of distinct populations.

The populations dealt with generally by the chemist are highly homogeneous, though not perfectly so, since even the elements contain members of different weight. As the complexity and size of the molecular aggregates found in living organisms increase, so does their intrinsic variability. Individual molecules of

a given type of protein have more ways of differing from each other than, let us say, individual molecules of glucose. Similarly, we may expect to find more degrees of freedom, leading to greater dispersion within the population, in ribosomes than in proteins, more again in mitochondria than in ribosomes. Fortunately, this variability, which could easily have made our task wholly impossible, is in fact restricted to a considerable extent by the molecular prerequisites of structure and function.

Biological structures are not haphazard aggregates of macromolecular components; their assembly is governed by a combination of genetic determinism and simple chemical stoichiometry, which is reflected at the morphological level by the existence of repeating units and crystalline patterns, and at the chemical level by a remarkable uniformity in composition. Conversely, the morphological differences whereby we recognize members of distinct populations are obviously rooted in corresponding chemical differences. These undoubtedly extend to the functional level, and it is not unreasonable to expect that subcellular entities which are morphologically and chemically different also perform different functions, in other words contain different enzymes. Of course, we now know this to be true in a number of cases, but what I want to point out is that this could already have been predicted fifteen years ago. The order we see in the internal arrangement of living cells, the harmony we witness in their behavior, could not possibly arise out of biochemical chaos.

These considerations form the basis of what I have called the *postulate of biochemical homogeneity,* a working hypothesis which has guided our whole approach in this field. In its most uncompromising form, this postulate presupposes that all members of a given subcellular population have the same enzymatic composition. Now this, like the limit laws of physics, refers to an ideal situation; when applied to reality it has to be taken with a grain of salt. How big a grain of salt we rely on the experiments themselves to tell us. For the great advantage of the postulate of biochemical homogeneity is that it does not have to be true in all cases to be useful, since the experiments which it suggests are such as eventually to prove it false should it be invalid.

A second postulate which has also been very useful, even though

it is less essential than the former, is that of *single location.* It assumes that each enzyme is entirely restricted to a single site within the cell. Here again, we must be ready for exceptions, but the advantage of taking single location as our working hypothesis is that we will accept multiple location only when forced by the experimental evidence. The converse would not be true.

Granting these two postulates, especially that of biochemical homogeneity, we can now use the enzymes as markers for their host particles and conduct tissue fractionation experiments very much like any other type of chemical fractionation. We may, at least in the initial analytical phase of the work, forget all about morphological features and treat suspensions of ground cells or tissues as mixtures of different populations of physical entities to be identified, characterized, resolved, and purified, with as sole guides the enzymes. If we can reach the final preparative phase and achieve sufficient purification, then the test of our working hypotheses will come, for morphological examination will show whether our deductions were in fact valid or not. When available, cytochemical staining techniques also provide a valuable way of testing the biochemical conclusions.

III. Methods

The approach which I have just sketched is really a conceptual or philosophical one; it provides a logical framework for any type of tissue fractionation. It becomes particularly powerful in connection with the new techniques of density gradient centrifugation, which are characterized by a much greater resolving power, flexibility, and ease of application than the classical fractionation methods, and can be applied in an entirely arbitrary fashion, without any preconceived idea as to the composition of the fractions.

The experiments can be run in two ways (Fig. 1). In one type, the starting material is layered on top of a stabilizing gradient and centrifuged under conditions insufficient to cause the complete sedimentation of even the heaviest component. Thus, after the centrifuge is stopped and if the resulting pattern has not been blurred by convective artifacts, the various components are all found somewhere on their way down to the bottom of the

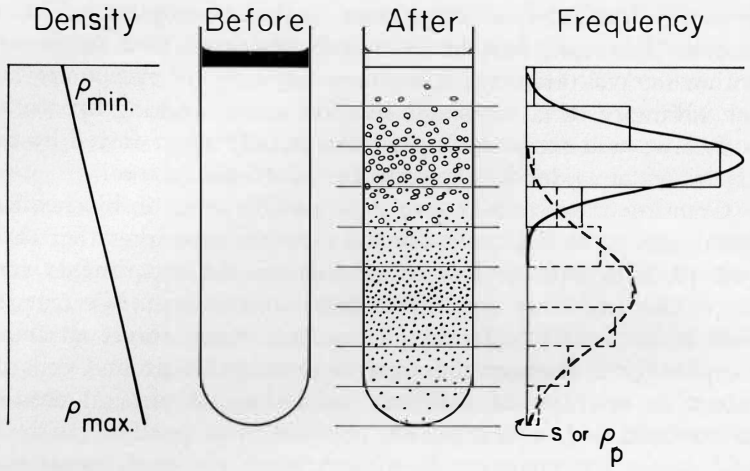


FIG. 1. Density gradient centrifugation, schematic representation. Note that equal cuts through the tube do not necessarily correspond to equal divisions on the abscissa of the frequency distribution curve. This depends on the shape of the gradient.

tube, the position at which they have become arrested depending on the rate at which they sedimented—in other words, on their sedimentation coefficient. The value of this coefficient corresponding to any position in the tube can easily be calculated from the conditions of the experiment. If we remove the tube contents layer by layer without disturbing their arrangement and assay each layer separately for a given chemical component or enzyme,

we are now in a position to plot the frequency distribution curve of this component as a function of the sedimentation coefficient, our chosen parameter in this particular case. In experiments of this type, the density gradient serves only a stabilizing purpose; its slope is usually small and its upper limit is lower than the density of any of the centrifuged components, so that, if centrifugation were pursued, they would all eventually reach the bottom of the tube. Note that the fractions which are prepared by the usual method of sedimentation and washing may in first approximation be considered as cuts through sedimentation coefficient frequency distribution curves. But the method is very laborious, and the small number of fractions which can be prepared in this way limits considerably the amount of information that can be obtained.

In the other type of experiment, one uses a much steeper gradient with an upper limit extending above the density of the components. Prolonged high-speed centrifugation of a mixture of particles in a gradient of this kind causes the particles to sediment only so far as their own density permits. They necessarily come to rest when they reach a position in the gradient corresponding to their own density. If the tube contents are now sampled and analyzed as in the previous type of experiment, the results obtained can again serve to construct frequency distribution curves, but as a function of another parameter, which is the equilibrium density of the component in the particular medium used.

There is theoretically no limit to the number of components or enzymes that can be measured simultaneously in experiments of this sort, and these therefore make it possible to compare numerous frequency distribution curves. This is where the postulate of biochemical homogeneity comes in. Armed with this postulate, we can now proceed in our interpretation of the results very much like any biochemist dealing with a chromatogram or with an electrophoresis diagram. If two frequency distribution curves differ significantly from each other, we may conclude tentatively that the two components belong to different particles. If they coincide, we take this as an indication that the components assayed may be associated with the same type of particle, but not

as proof of this, for the coincidence could be a fortuitous one. Such fortuitous coincidences are well known to the chemist, who tries to recognize them by repeating his fractionation in a number of different systems, for instance by using buffers of different pH or ionic strength in electrophoresis experiments, or different mixtures of solvents in chromatography experiments. We have tried to apply the same principle in density gradient centrifugation. Fortunately, the physical characteristics that determine the sedimentation coefficient or the equilibrium density of subcellular particles vary with the composition of the suspension medium, and this relationship is not the same for each type of particle. This makes it possible to verify to some extent whether an observed association is truly structural or accidental.

Eventually, when enough analytical resolution has been attained, purification followed by morphological examination can be attempted. However, as I hope to show later, a considerable amount of detailed information can already be gathered long before this final goal has been reached, and this by a variety of methods, some very simple ones. Conceptually, they have in common that they all rely on the enzymes themselves to serve as guides in the design and interpretation of the experiments.

I do not wish to go further into the logical aspects of this approach, which have been discussed extensively elsewhere (de Duve, 1964), nor into the manifold practical as well as interpretative difficulties that are common to all types of tissue fractionation and have been the subject of numerous reviews. But I would like to point out that the experimental design as I have just sketched it has rarely been applied to the study of the larger intracellular entities, even though it is a standard procedure in macromolecular chemistry and is now used extensively in investigations on ribosomal particles. However, when it comes to entities of the size of mitochondria or nuclei, it has been customary to aim first at purification on the basis of morphological criteria, and then to proceed with biochemical characterization. There is no doubt that we owe much of our present knowledge of cellular organization to the latter type of approach and it has obviously many virtues. It has, however, one defect, in that it leaves no room for the unexpected.

IV. Identification and Separation of Particles

The first observations which served to orient the course of our work were made in a number of laboratories during the early years which followed the description of the technique of centrifugal fractionation. Ten years ago, Berthet and I had an opportunity to review those earlier results (de Duve and Berthet, 1954). In this paper and, more explicitly, in a subsequent one (de Duve, 1957), attention was called to the fact that some enzymes show what we called simple distributions, whereas others have complex distributions. Leaving out the problem of soluble activities, which is to some extent irrelevant to the present discussion, what we meant by a simple distribution was that of an enzyme which was recovered to a large extent in a single fraction, usually either the mitochondrial fraction or the microsomal fraction; so much so that the amounts found in the other fractions could be ascribed to contamination and that the enzyme could be considered as occupying a single site within the cell. Early examples of simple distributions were provided by cytochrome oxidase, an enzyme found by Hogeboom, Schneider, and Striebich (1952) to be associated exclusively with the mitochondria, and glucose 6-phosphatase, an enzyme belonging entirely to microsomal elements (Hers, Berthet, Berthet, and de Duve, 1951). These findings played an important role in causing us to formulate the postulates of single location and of biochemical homogeneity.

One of the first examples of a complex distribution was given by acid phosphatase, which was studied in several laboratories. Work conducted independently by Novikoff, Podber, Ryan, and Noe (1953) and by the Louvain group (de Duve, Gianetto, Appelmans, and Wattiaux, 1953; Appelmans, Wattiaux, and de Duve, 1955) led to the finding that a particularly large proportion of the total acid phosphatase activity of liver could be isolated in a small intermediate fraction made up essentially of the lighter mitochondria and heavier microsomes. While Novikoff and his co-workers attempted to interpret this observation in terms of what they could see in the fractions by means of phase contrast microscopy, we ourselves were influenced in our reasoning by our previous observations on glucose 6-phosphatase and

proposed a tentative interpretation based on single location. Subsequent experiments guided by this hypothesis provided additional support for it, and we arrived at the conclusion that acid phosphatase is probably associated with a special type of particle entirely distinct both from mitochondria and from microsomes. We then turned our attention to other enzymes showing similar complex distributions, using the new centrifugation scheme developed for the study of acid phosphatase (de Duve, Pressman, Gianetto, Wattiaux, and Appelmans, 1955). Some enzymes, for instance the cytochrome c reductases, turned out to have truly bimodal distributions; these, however, did not contradict the postulate of single location since evidence was obtained that different systems were responsible for the activities found in the mitochondria and in the microsomes. On the other hand, several enzymes were found to resemble acid phosphatase and to be particularly concentrated in the small intermediate L fraction. More recent work has shown this property to be common to at least fifteen different enzymes. These are listed in Fig. 2. They include a number of acid hydrolases as well as the nonhydrolytic enzymes urate oxidase, catalase, and D-amino acid oxidase.

These findings were put into more quantitative terms by means of density gradient centrifugation experiments, which clearly showed that these enzymes all had unimodal and relatively similar frequency distribution curves of sedimentation coefficients; these curves were displaced significantly toward the lower end of the abscissa scale with respect to those of truly mitochondrial enzymes (Fig. 3). There were small differences between the individual distributions, suggesting that the postulate of homogeneity might not be perfectly applicable to the new particles, but there was no compelling indication of the existence of more than a single population.

However, when the fractions were subjected to density equilibration in a number of different density gradients, the group was found to dissociate into two subgroups, one containing all the acid hydrolases, and the other including urate oxidase, catalase, and D-amino acid oxidase. This phenomenon is illustrated in Fig. 4, which also brings to light the remarkable influence exerted by the composition of the medium, particularly its sucrose concentration, on the equilibrium density of subcellular particles.

With the usual aqueous sucrose gradients, in which the particles are exposed to very high sucrose concentrations, the mitochondria equilibrate around a density value of 1.19, the acid hydrolases show a flat distribution with a peak in the neighborhood of 1.22,

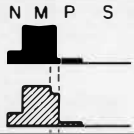
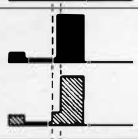
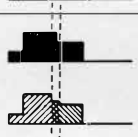
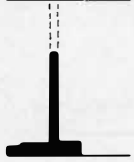
Scheme	Pattern	Distribution	Enzymes
Old New		Mitochondrial	Cytochrome oxidose, rhodonese, succinate-cytochrome c reductase, dehydrogenases (glutamate, malate, β-hydroxybutyrate), alkaline DNase
Old New		Microsomal	Glucose 6-phosphatase Esterase (aryl-sulfotose C)
Old New		Complex 1. Mit. – Micr.	NADH – cytochrome c reductase NADPH – cytochrome c reductase Monoamine oxidase
New		2. Peak in L-fraction	Acid phosphatase, cathepsin, acid RNose, acid DNase, β-glucuronidase, β-N-acetyldeoxyglucosidase, β-galoctosidose, α-monnosidose, α-glucosidase, (arylsulfatases A and B, phosphoprotein phosphatase, phosphatidate phosphatase) Urote oxidose, catolase, D-amino ocid oxidose

FIG. 2. Distribution patterns of enzymes in rat liver. The diagrams shown are constructed according to de Duve *et al.* (1955), but with the soluble activities left out. They illustrate in a schematic manner the type of distribution shown by the enzymes in each group. The enzymes mentioned were all studied in the author's laboratory, with the exception of those listed in parentheses. References: de Duve *et al.* (1955), Underhay *et al.* (1956), Beaufay *et al.* (1959a), Sellinger *et al.* (1960), Lejeune *et al.* (1963), Baudhuin *et al.* (1964). For other enzymes see: Roy (1958), Paigen and Griffiths (1959), Wilgram and Kennedy (1963).

and urate oxidase, catalase, and D-amino acid oxidase form sharper bands with peaks between 1.23 and 1.25. In an isoosmotic gradient of glycogen with 0.5 *M* sucrose as solvent, the latter three enzymes are now concentrated in the least dense region, with median densities ranging between 1.11 and 1.12,

at a considerable distance from the acid hydrolases, which show median equilibrium densities of 1.13 to 1.14; the mitochondria lie in between the two groups, around a value of 1.125.

From these and other results which have been published in detail by Beaufay *et al.* (1959b, 1964), it was concluded that rat liver contains two distinct populations of special particles: one, which we have named lysosomes, characterized by the acid

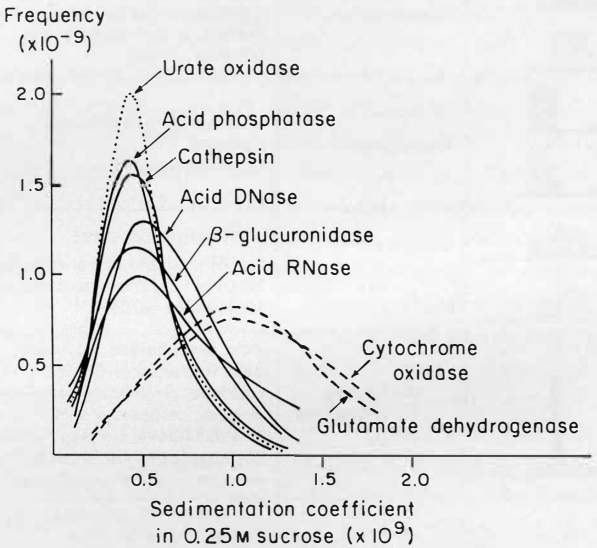


FIG. 3. Frequency distribution of sedimentation coefficients. Mitochondrial fraction from rat liver centrifuged in a linear gradient of 0.25 to 0.5 *M* sucrose. Results of Beaufay *et al.* (1959b).

hydrolases; the other containing urate oxidase, catalase, and D-amino acid oxidase. By removing head or tail fractions from appropriate gradients, it was possible to prepare relatively pure samples of each subgroup and to examine them in the electron microscope. In confirmation of early investigations made in collaboration with Novikoff (Novikoff, Beaufay, and de Duve, 1956) and of a number of subsequent cytochemical observations, the lysosomes could be identified with the pericanalicular dense bodies (Fig. 5). The particles described by Gänsler and Rouiller

(1956) under the name "microbodies" (Fig. 6) were the most conspicuous components of the sample enriched in urate oxidase.

These experiments illustrate up to its final conclusion the approach outlined above, including the important necessity of varying the fractionation conditions before attaching a structural significance to an observed similarity in behavior. They have also served to underline some of the weaknesses of the approach, in

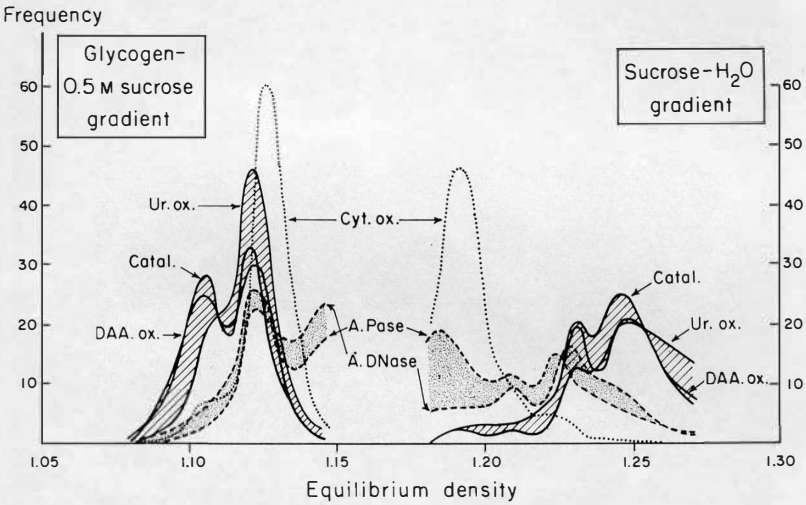


FIG. 4. Frequency distribution of equilibrium densities. Mitochondrial fractions from rat liver equilibrated in a gradient of glycogen (initially linear from 0 to 30.6 g. per 100 g. water) in 0.5 *M* sucrose, and in a linear gradient of sucrose (from 59.7 to 117.0 g. per 100 g. water). Results of Beaufay *et al.* (1964).

particular the limitations of the postulate of biochemical homogeneity. So far, this postulate appears to apply in a rather faithful manner to rat liver mitochondria, which in none of our experiments showed evidence of significant heterogeneity. It also seems to apply to catalase and D-amino acid oxidase, which almost invariably show very similar distributions. On the other hand, the distribution of urate oxidase does not coincide with that of the other two enzymes, and the results obtained in density equilibration experiments suggest that this enzyme might be preferentially

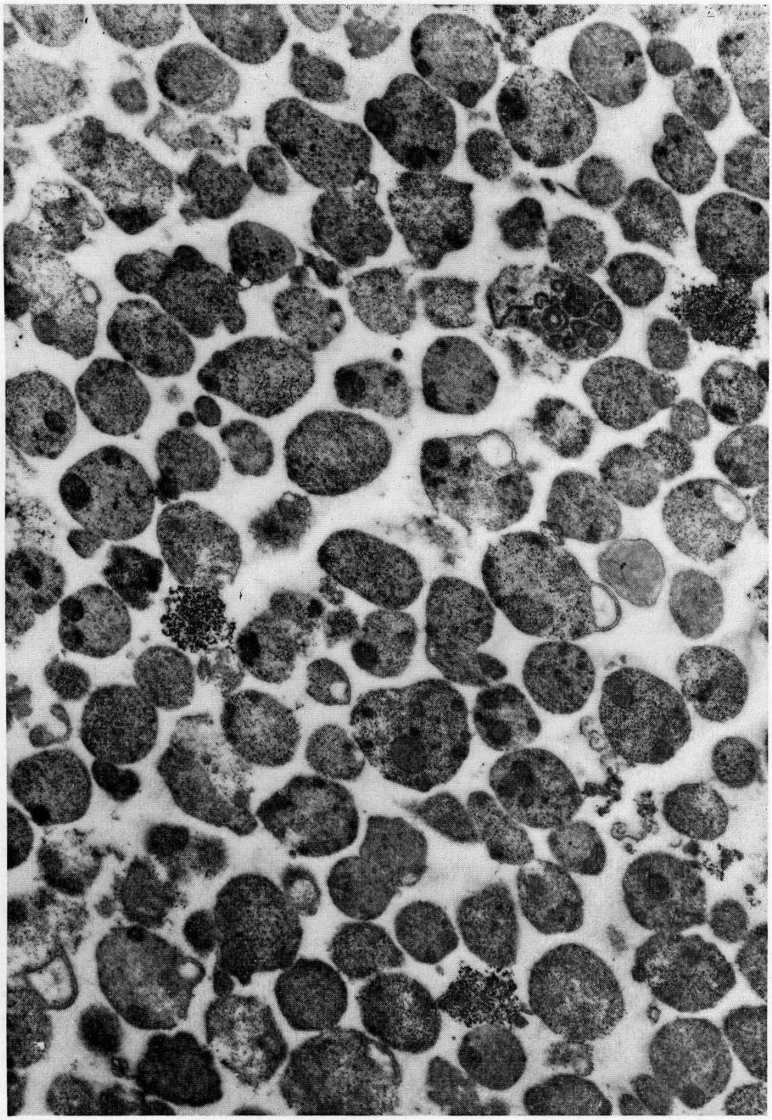


FIG. 5. Electron micrograph of a thin section of a lysosome-rich sediment isolated from a particulate fraction from rat liver by density equilibration in a glycogen-0.5 *M* sucrose gradient (Baudhuin and Beaufay, 1963). Most particles in the field are typical pericanalicular dense bodies. Note the presence of numerous spherules inside the particles. Electron-dense dots are ferritin Lead stained. Magnification: × 20,700.

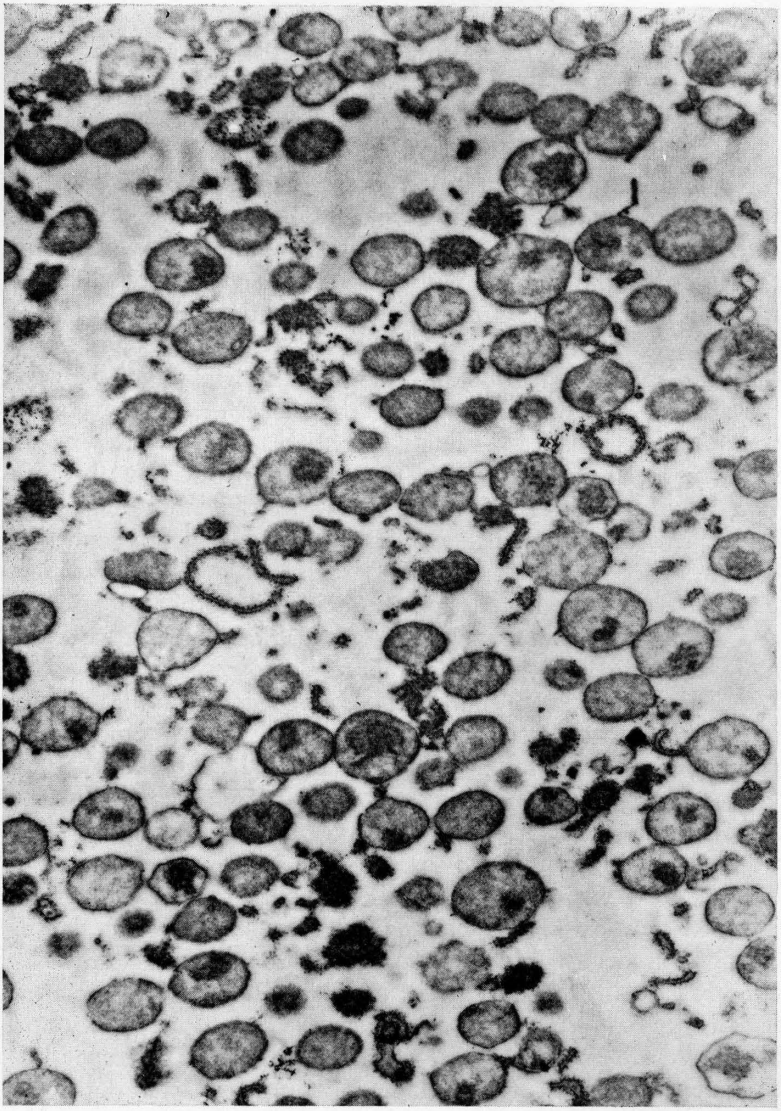


FIG. 6. Electron micrograph of a thin section of a sediment enriched in urate oxidase isolated from a particulate fraction from rat liver by density equilibration in an aqueous sucrose gradient (Baudhuin and Beaufay, 1963). Most particles in the field are "microbodies" (Gänsler and Rouiller, 1956). Note the presence of a dense core in the particles. At higher magnification, this core appears as a system of parallel tubules or rods arranged in an hexagonal lattice. Lead stained. Magnification: × 12,100.

associated with the denser members of the population. This, however, could be an artifact. We have found that catalase and D-amino acid oxidase are easily released in soluble form from their host particles, whereas urate oxidase is firmly attached to their insoluble framework. Our latest results suggest that the intact microbodies could be homogeneous with respect to the three enzymes and that the displacement of urate oxidase reflects the fact that the injured particles which have lost catalase and D-amino acid oxidase are slightly denser than the intact ones (Beaufay *et al.*, 1964). It is unlikely that artifacts alone explain the differences in distribution that have been observed between the various acid hydrolases. Also, these enzymes show very flat and often extremely irregular distribution curves in most of the systems we have investigated. The indications are that the lysosomes are characterized by a fairly considerable degree of heterogeneity, both with respect to their size and other physical characteristics and with respect to their enzymatic composition. This heterogeneity is reflected in their polymorphic appearance and has become more easily understandable now that we know more about their biological significance. However, it is a somewhat disquietening phenomenon to the biochemically minded investigator accustomed to dealing with relatively homogeneous populations. To those who might be tempted to share these misgivings some results obtained recently by Wattiaux, Wibo, and Baudhuin (1963a,b) will, I hope, bring reassurance. These workers have found that a single intravenous injection of 170 mg. of Triton WR-1339 at 2 to 4 days before the animal is killed causes a remarkable and selective change in the density distribution of the hepatic lysosomal hydrolases, as studied by equilibration in aqueous sucrose gradients. Instead of their usual flat distribution around a median density value of 1.22, they now all congregate closely together around a sharp peak corresponding to a density of 1.11 (Fig. 7). In these preparations, the distributions of the mitochondria and of the enzymes believed to be associated with the microbodies were entirely unchanged. Electron micrographs of intact liver sections and of isolated fractions, as well as biochemical studies on animals injected with labeled Triton have shown conclusively that the decrease in the density of

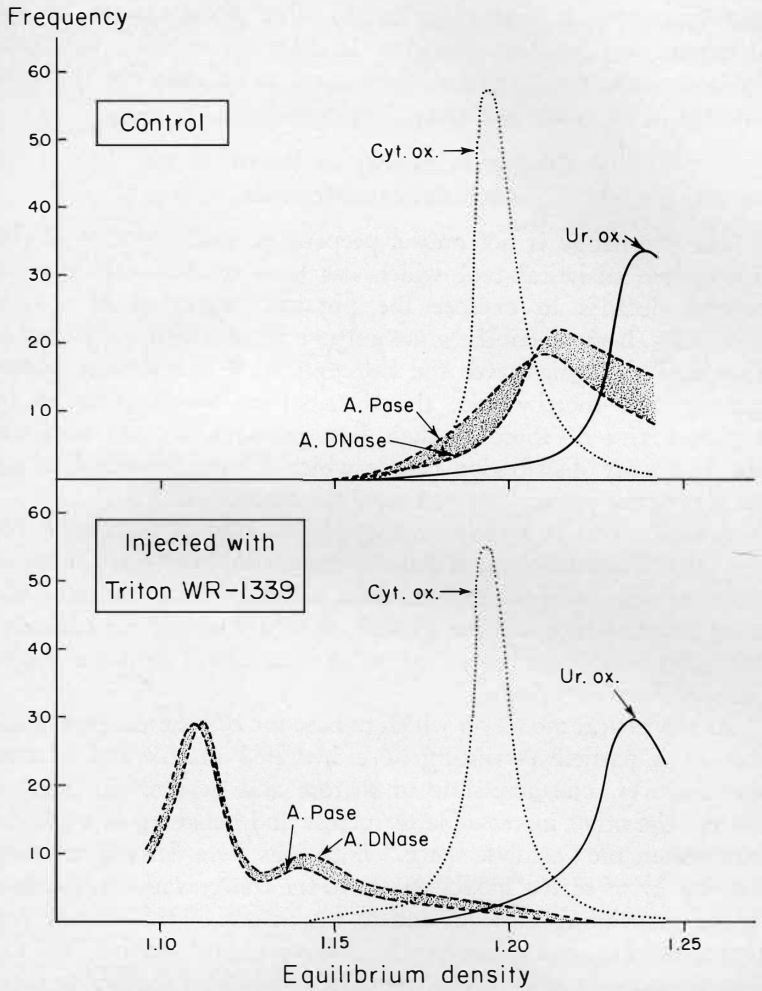


FIG. 7. Effect of a previous injection of Triton WR-1339 on the equilibrium density of particulate enzymes. Density equilibration of mitochondrial fractions from rat liver in an aqueous sucrose gradient. Upper graph: control; lower graph: animal injected intravenously 4 days previously with 170 mg. of Triton WR-1339. Note the selective shift of the lysosomal hydrolases. Constructed from results of Wattiaux *et al.* (1963a,b).

the lysosomes is due to a considerable accumulation of the detergent within these particles. In addition to their biological interest, these results undoubtedly serve to validate the lysosome concept in its purely biochemical definition.

V. Characterization of Particles by Centrifugal Methods

The centrifuge is not only a preparative instrument, it is also a sensitive analytical tool which has been used successfully for several decades to explore the physical properties of macromolecules. It can serve the same purpose with subcellular particles. Provided we again make the assumption of biochemical homogeneity and thereby take the distributions of enzymes to be representative of those of their host particles, we can now use the frequency distribution curves which I have described to get an idea of the physical properties of these particles.

Actually, this is a somewhat optimistic way of putting it, for the calculations involved require so many simplifying assumptions that one may well wonder at their validity or significance. We have nevertheless made the attempt, mostly to satisfy our curiosity, and the results have turned out more meaningful than one might perhaps have expected.

As theoretical model on which to base the calculations, we chose that of a particle consisting of a hydrated matrix and of two water spaces, one accessible to sucrose and designated "sucrose space," the other inaccessible to sucrose and behaving as a perfect osmometer, the "osmotic space." Equations were derived, relating the density of such a model system to the concentration of sucrose in the suspending medium and to the nature of the solvent (de Duve, Berthet, and Beaufay, 1959; Beaufay and Berthet, 1963). These equations involve four particle parameters: the dry density, the hydration ratio of the matrix, the size of the sucrose space, and the content of the osmotic space in osmotically active solutes. The particle size represents a fifth parameter, which can be derived from the sedimentation coefficient and density by means of the classical Svedberg equation as applied to a spherical particle.

To provide a sufficient number of independent data for the computation of these parameters, we measured sedimentation rates in 0.25 to 0.5 *M* sucrose gradients (see Fig. 3) and equilibrium

densities in gradients of sucrose in H_2O, of sucrose in D_2O, and of glycogen in sucrose solutions of several different concentrations (see Fig. 4). These experiments have been described by Beaufay *et al.* (1959b, 1964). Typical values, usually the median, or,

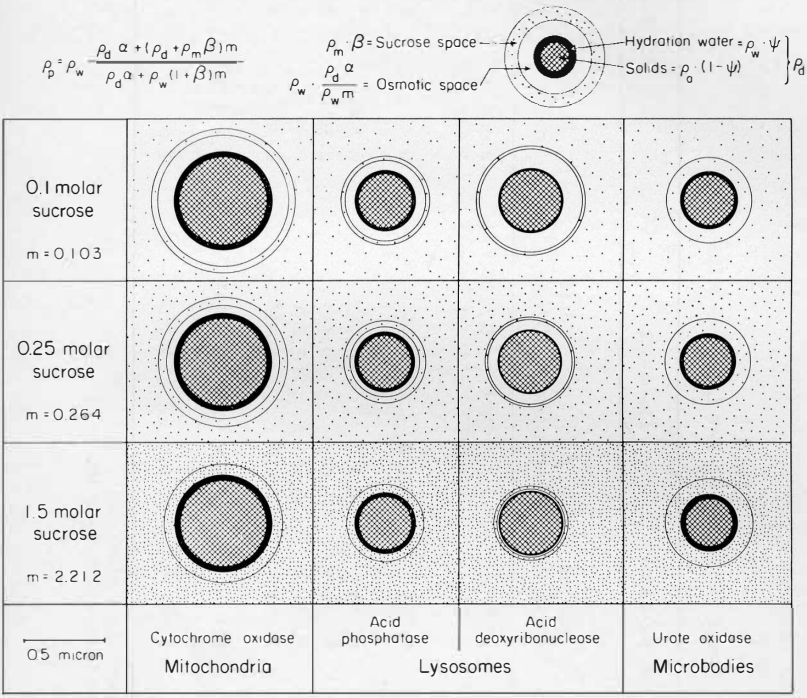


FIG. 8. Schematic representation of the physical properties of rat liver particles in sucrose solutions of different concentration, as calculated from the formula shown, with the parameters given in Table I. Particles are represented to scale by an inner sphere of solids surrounded by concentric shells picturing the three water compartments. Symbols refer to the density (ρ) multiplied by the volume of each compartment relative to that of the hydrated matrix taken as unity. For further details, see de Duve *et al.* (1959), Beaufay and Berthet (1963).

exceptionally, the principal mode, were derived from the observed distribution curves and fitted to the theoretical equation by a method of least squares (Beaufay and Berthet, 1963). The particle parameters arrived at in this manner are listed in Table I. Figure 8 shows in a schematic fashion how each group of particles

TABLE I

PHYSICAL PROPERTIES OF RAT LIVER PARTICLES[a, b]

Parameter	Mitochondria	Lysosomes		Microbodies		
Reference enzyme:	Cytochrome oxidase	Acid phosphatase	Acid DNase	Urate oxidase	Catalase	D-Amino acid oxidase
Dry weight (μg.)	10^{-7}	2.7×10^{-8}	3.6×10^{-8}	2.4×10^{-8}	—	—
Dry density	1.315	1.300	1.331	1.322	1.319	1.315
Osmotically active solutes (milliosmoles/g. dry weight)	0.157	0.128	0.334	0	0	0
Water compartments (cm.^{3}/g. dry weight)						
Hydration	0.430	0.256	0.212	0.214	0.295	0.296
Sucrose space	0.905	1.075	0.330	2.51	2.68	2.54
Osmotic space in 0.25 *M* sucrose	0.595	0.485	1.265	0	0	0
Total in 0.25 *M* sucrose	1.930	1.816	1.807	2.724	2.975	2.836
Sedimentation coefficient in 0.25 *M* sucrose (Svedberg units)	10^4	4.4×10^3	5×10^3	4.4×10^3	—	—
Diameter in 0.25 *M* sucrose (μ)	0.8	0.51	0.56	0.54	—	—
Density in 0.25 *M* sucrose	1.099	1.103	1.100	1.095	1.088	1.090

[a] Computed from results of density gradient centrifugation experiments.

[b] According to Beaufay *et al.* (1959b, 1964) and Beaufay and Berthet (1963).

is believed to respond to changes in sucrose concentration. By combining these data with what we know of the structure of the particles, we arrive at the more realistic but still quite hypothetical representation shown in Fig. 9.

I have to refer to the original publication of Beaufay and Berthet (1963) for a complete discussion of the results of these calculations and wish only to draw attention to a few salient facts.

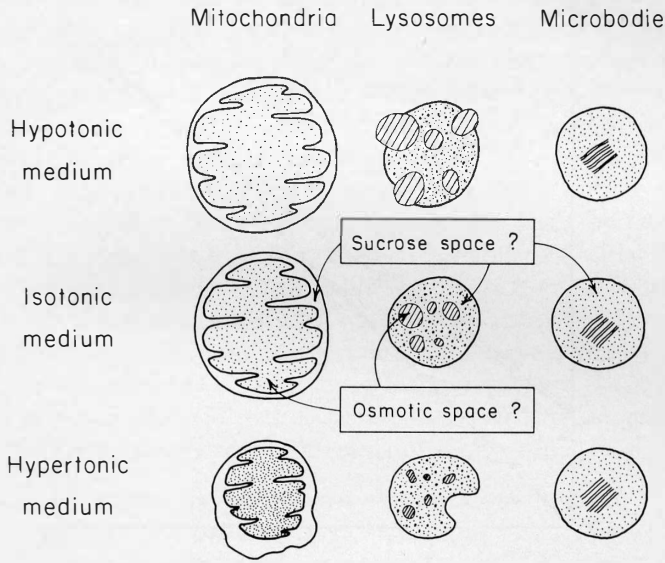


FIG. 9. Hypothetical diagram combining data of Fig. 8 with known morphological properties of particles.

As far as the mitochondria are concerned, the predicted properties agree surprisingly well with those which have been established by other, more direct methods. This is an encouraging result, which provides some measure of support to the approach.

Another finding which inspires us with some confidence relates to the physical characteristics of the microbodies. These are pictured in Figs. 8 and 9 as being devoid of an osmotic space and as containing a particularly large sucrose space, which actually provides them with 50 per cent more water per unit dry weight than is present in the other particles (see also Table I). For a particle

of this type, the equation shown in Fig. 8 ($\alpha = 0$) reduces to a simple linear relationship between the density of the particle (ρ_p) and that of the sucrose solution (ρ_m), with a slope determined by the size of the sucrose space. How well such a relationship is obeyed by all three enzymes located in the microbodies is

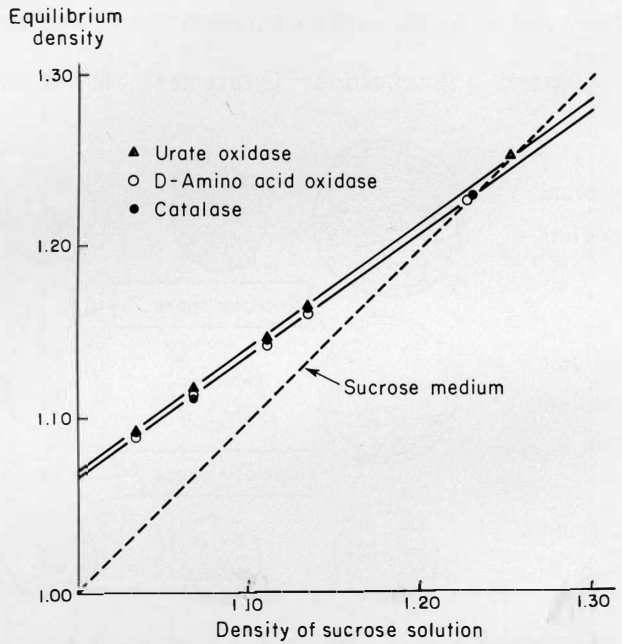


FIG. 10. Relationship between equilibrium density and density of sucrose solution in medium for three enzymes believed to be associated with microbodies. Results of Beaufay *et al.* (1964), as examined by Beaufay and Berthet (1963).

shown in Fig. 10. Here again, some independent support for this deduction is provided by the observation that the particles do not lose their catalase content when exposed to distilled water, whereas other treatments will easily detach the enzyme. For these reasons, we believe the microbodies to be surrounded by a membrane that is relatively permeable to small molecules, though not to large ones.

The data computed for the lysosomes are much less reliable, in view of the irregular shape of the distribution curves from

which they were calculated and of the large differences found between the two representative enzymes, acid phosphatase and acid deoxyribonuclease. I should add that we chose these enzymes because they show the largest differences. The other acid hydrolases of the group generally show distributions intermediate between those of acid phosphatase and of acid deoxyribonuclease. The results obtained agree with those of other experiments in showing the existence of an osmotic space, but are somewhat surprising in that they indicate that lysosomes may also contain a space accessible to sucrose. Since the particles have a single membrane, we must admit either that the calculations cannot be trusted in this case, or that the bag model at which we have arrived on the basis of other experiments (see below: Fig. 14) applies to smaller bags within the main bag, rather than to the whole particle. This view is not entirely incompatible with the morphological data. As shown in Fig. 5, small spherules of variable size are often seen within the pericanalicular dense bodies. When the latter are disrupted in distilled water, these spherules appear to form bleblike projections at the periphery of the particles (Fig. 11). It is conceivable that they actually represent the osmotic space in which the acid hydrolases are segregated, as suggested in Fig. 9. However, this possibility remains highly conjectural.

VI. Characterization of Particles by Studies of Enzymatic Latency

Some simpler and earlier experiments show that one can already obtain a great deal of information concerning the structure of subcellular particles without any preliminary purification and with no other equipment than test tubes and pipettes. I am referring here to the experiments dealing with the so-called structure-linked latency of enzymes. This interesting phenomenon was actually brought under our eyes in our very first fractionation experiment; I remember how fascinating we found it at the time, and it certainly acted as the main bait which caused us to abandon our chosen itinerary and to follow the new enticing trail which opened on our roadside.

Briefly summarized, what we found at that time was that liver tissue freshly homogenized in isotonic sucrose has much less acid phosphatase activity than the same tissue homogenized in distilled

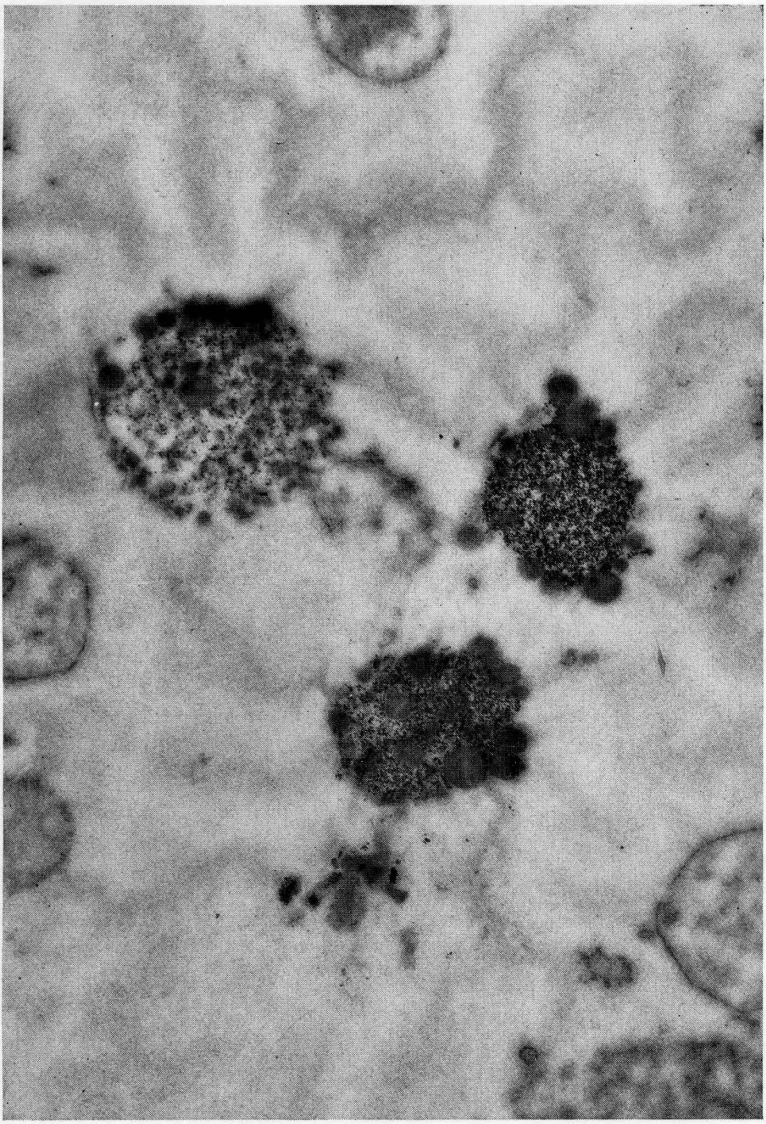


Fig. 11. Electron micrograph of pericanalicular dense bodies (lysosomes) in a thin section of a pellet obtained from a mitochondrial fraction from rat liver treated with distilled water. Unpublished observation of Baudhuin. Lead-stained. Magnification: × 60,000.

water. The particulate fractions separated from the homogenate by means of differential centrifugation also had a low acid phosphatase activity, but when the preparations had been allowed to age for a few days their activity was found to be greatly increased. The enzyme was largely present in the particulate fractions isolated from the fresh homogenate, but it was no longer sedimentable in the aged preparations. This of course, was the essential

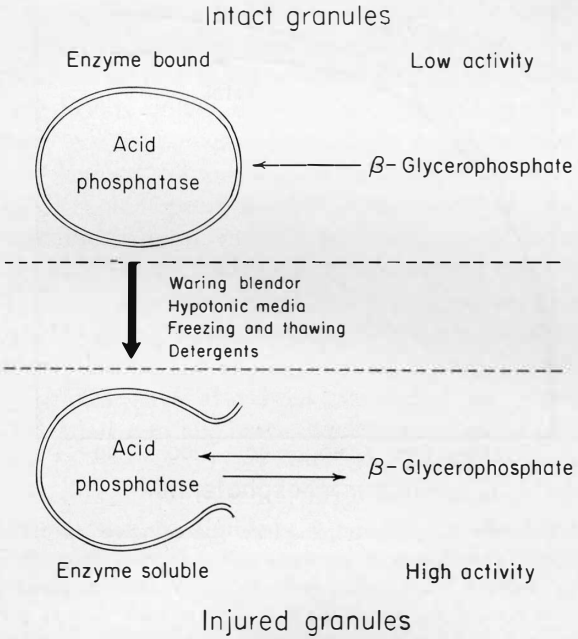


FIG. 12. Schematic model summarizing results and conclusions of Berthet *et al.* (1951) relative to latency of acid phosphatase in rat liver preparations.

clue, and it did not take many months to establish that the lack of activity, the so-called latency of the enzyme, was a direct consequence of its association with a structural entity. Various means were found to be effective in causing the simultaneous activation and solubilization of acid phosphatase and a detailed study of the phenomenon led to the interpretative model shown in Fig. 12 (Berthet, Berthet, Appelmans, and de Duve, 1951).

According to this model, the particles are considered to be sac-like structures surrounded by a membrane; the enzyme is believed to be present inside in perfectly active and soluble form, and its apparent lack of activity is attributed to the impermeability of the particle membrane to β-glycerophosphate, the substrate used in

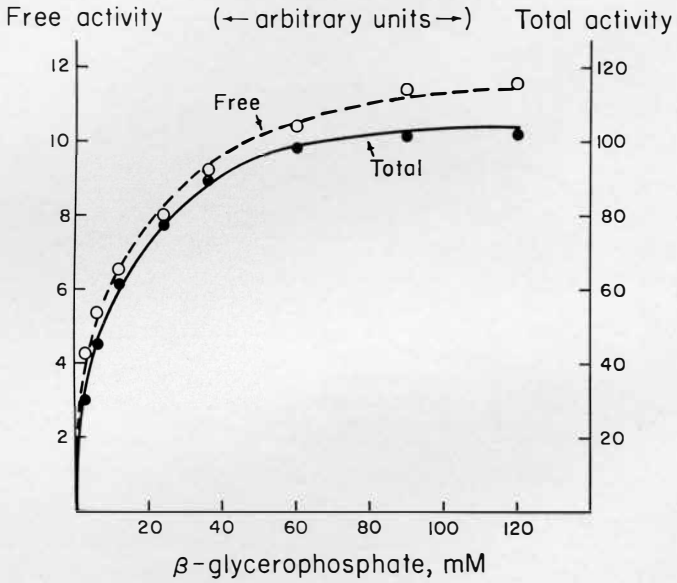


FIG. 13. Influence of β-glycerophosphate concentration on free and total activities of acid phosphatase in a mitochondrial fraction from rat liver. Note that the free enzyme behaves like fully accessible enzyme. There is no evidence that the masked enzyme is more reactive at high than at low substrate concentration, indicating that the membrane is essentially impermeable to β-glycerophosphate. Results of Appelmans and de Duve (1955).

the enzyme assay. This contention could be supported in two ways. It was found that β-glycerophosphate was as good an osmotic protector as sucrose, a role which it could fulfill only if it were unable to penetrate within the particle. It was also found that no relative increase in activity could be brought about by raising the concentration of β-glycerophosphate in the incubation medium, an observation which suggested an almost complete lack of permeability of the membrane to this ester (Fig. 13).

Since then, the phenomenon of structure-linked latency has been observed for numerous enzymes and investigated with a great variety of methods. It has been found to be a general property of all the acid hydrolases that have been attributed to the lysosomes on the basis of centrifugation experiments. These enzymes all behave in a closely similar fashion with respect to all disrupting agents that have been tried, and this parallelism has provided powerful support to the contention that they are all present together within miniature osmometers. Indeed, it is extremely difficult to conceive how so many different proteins can be unmasked to almost exactly the same degree under so many different conditions unless they are all shielded together by the same barrier. We were even able to obtain some information concerning the chemical composition of this barrier by showing that it could be disrupted specifically by proteases and phospholipases. Our present biochemical concept of lysosomes is shown schematically in Fig. 14. As I have mentioned earlier, this model may possibly apply to subunits within the particle rather than to the whole particle. More detailed observations are needed to settle this point.

Latency studies have also been performed on mitochondrial enzymes (Bendall and de Duve, 1960) and, more recently, on the microbody catalase. These investigations have revealed interesting differences. For instance, as illustrated in Fig. 15, approximately ten times more digitonin is needed for the complete unmasking of catalase from the microbodies than for that of acid phosphatase from the lysosomes. Not shown on this graph is the release of glutamate dehydrogenase from the mitochondria, which required even larger concentrations of digitonin. These and other similar observations have provided independent confirmation of the localization of these enzymes in different particles. They also furnish a variety of interesting means of probing the surface of subcellular particles and detecting small differences as well as similarities between them.

The latency of catalase is in itself a surprising phenomenon. As I have mentioned before, there are good indications that the membrane of the microbodies is permeable to sucrose. If so, it is certainly unlikely to be impermeable to hydrogen peroxide.

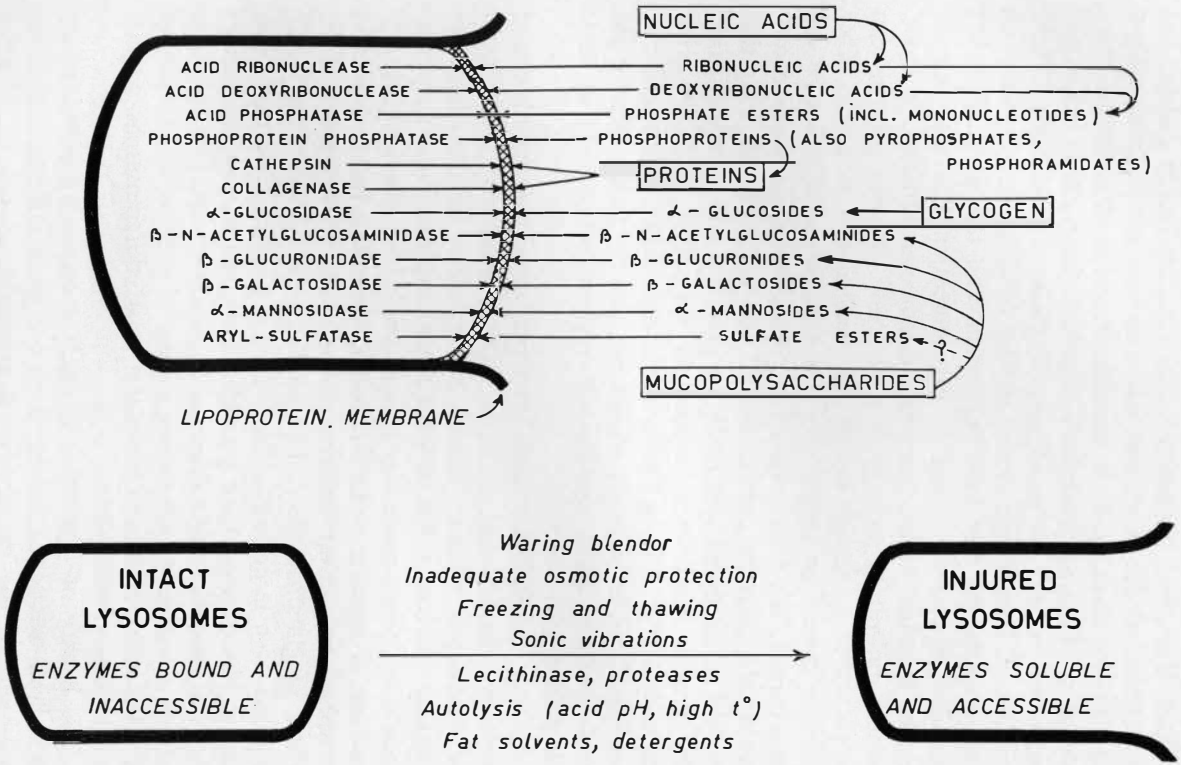


FIG. 14. Schematic model summarizing data on rat liver lysosomes. From de Duve (1963).

Also, neither of catalase's two companion enzymes displays any latency, and it would seem at first sight that in this case we are dealing not with a permeability barrier, but with a more specific inhibition of the bound enzyme. This is certainly a possibility. It appears from what we know of the amount of catalase and of microbodies present in the liver that catalase must represent at least 50 per cent of the total protein of the microbodies and that it must be present in these particles at an enormous concentration,

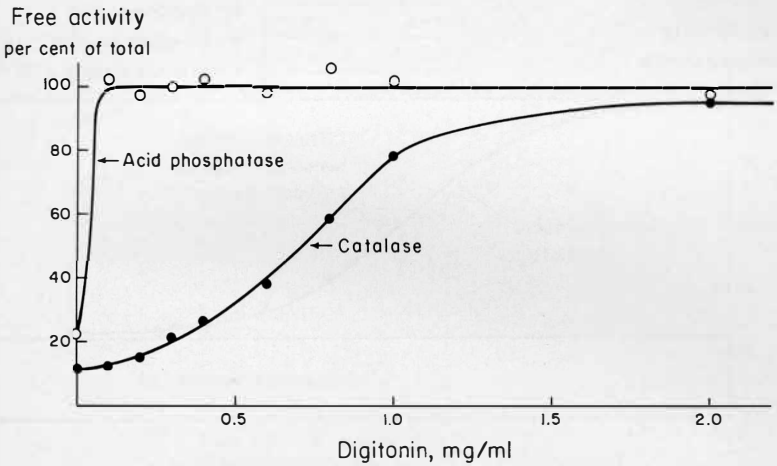


FIG. 15. Influence of digitonin concentration on latency of acid phosphatase and of catalase in a mitochondrial fraction from rat liver. Unpublished results of Baudhuin.

of the order of 10 per cent, or more. It would not be surprising if the enzyme did not display full activity under such conditions. However, the very activity of the enzyme suggested to us that even in this case one might be dealing with a membrane-dependent latency. Here, possibly, was a situation where the enzyme is so active that diffusion of the substrate through the membrane becomes rate limiting. The usual way of testing for a possibility of this kind is to increase the outside concentration of substrate above saturation levels in order to try and overcome the diffusion barrier, as was done for instance in the experiment on acid phos-

phatase depicted in Fig. 13. However, a device of this sort would be ineffective in the case of catalase which happens to display first-order kinetics. In such a case, a diffusion barrier, if it existed, would exert the same dampening effect on the reaction rate at all substrate concentrations. Another way of finding out is to decrease the enzyme activity. This experiment has recently been done by Baudhuin, who has measured the free and total catalase activity of a particulate preparation from rat liver after preincubation

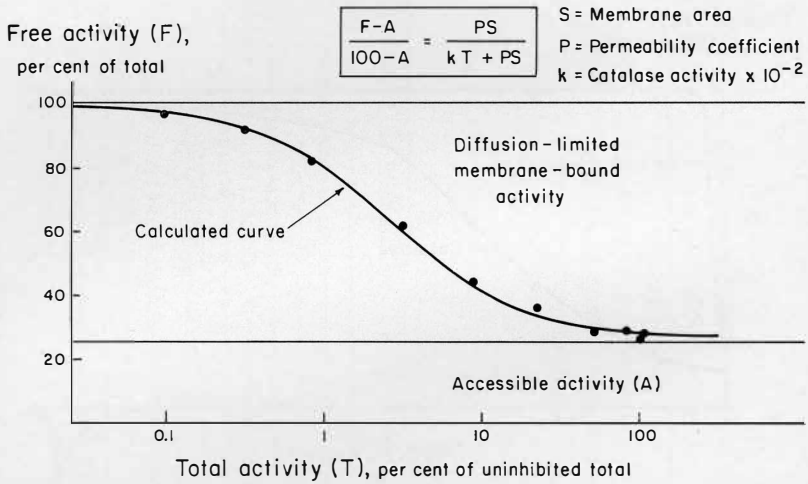
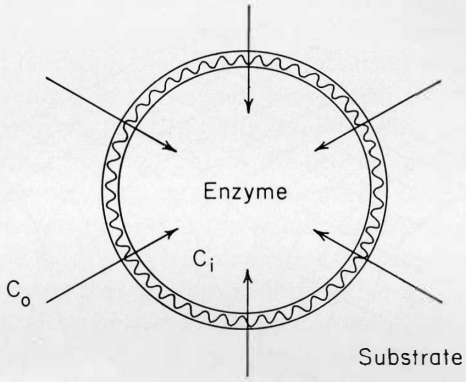


FIG. 16. Influence of enzyme activity on latency of catalase in a mitochondrial fraction from rat liver. Inhibition of enzyme was accomplished with increasing concentrations of potassium cyanide. Unpublished results of Baudhuin.

with increasing concentrations of cyanide, which enabled him to inhibit the enzyme by as much as three orders of magnitude. As shown in Fig. 16, decreasing the enzyme activity does indeed supress the latency. Moreover, the shape of the experimental curve is exactly that predicted for the very simple model shown in Fig. 17. It is possible on the basis of these results to estimate the permeability coefficient of the membrane to hydrogen peroxide. It is of the order of 0.2 cm. min^{-1}. For a spherule of the size of a microbody, this corresponds to a time of half equilibration with the outside medium of only 7 milliseconds. In other words, it is

a very high permeability coefficient, quite compatible with our previous deduction that the membrane of the microbodies is permeable to sucrose. Only with an enzyme as tremendously active and concentrated as catalase happens to be in these particles, could one expect to observe latency under such conditions.



Total activity

$$T = V \frac{C_o}{K + C_o}$$

Free activity (steady state)

$$F = PS(C_o - C_i) = V \frac{C_i}{K + C_i}$$

Kinetics of Latent Enzymes

FIG. 17. Model for theoretical analysis of enzyme latency (see Fig. 18). Note that for enzymes showing first-order kinetics (catalase), the rate equations take the form $= (V/K)C$, with V/K as the first-order velocity constant. P = permeability coefficient; S = surface area of particle membrane.

In the model of Fig. 17, passage through the membrane is taken to be the major rate-limiting step in the supply of substrate to the enzyme; diffusion in the outside medium and within the particle is considered sufficiently rapid to maintain a practically homogeneous concentration of substrate on both sides of the

membrane. This may not be true when the reaction rate is extremely fast. Berthet and Baudhuin have recently worked out a more complete theory in which the diffusion rate is taken into account. The function at which they have arrived can also be fitted to the results of Fig. 16. When this is done, it is found that diffusion through the membrane is the limiting step and that

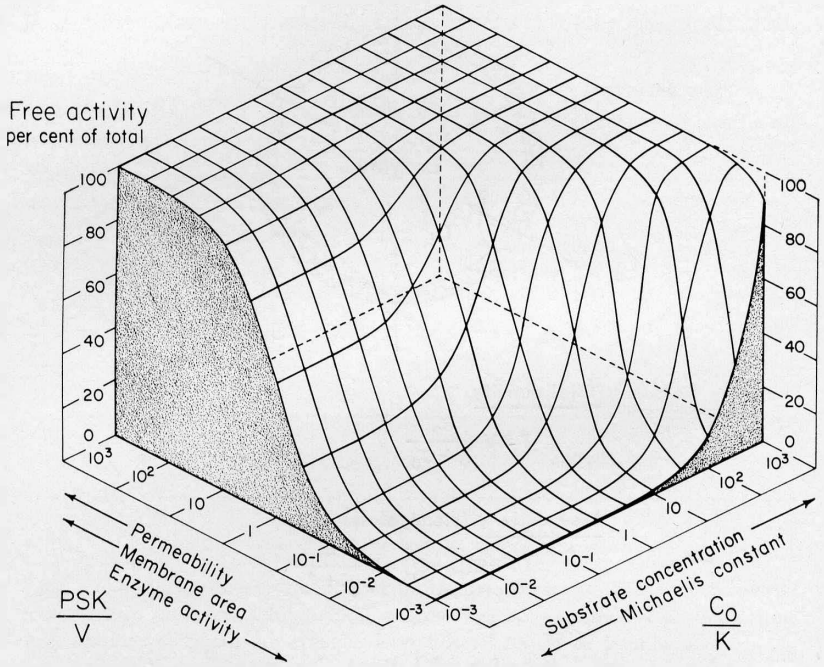


FIG. 18. Graph illustrating latency of membrane-bound enzyme as a function of the various parameters shown. The curves were calculated by means of the equations shown in Fig. 17.

diffusion inside and outside the particles may be neglected. Since one is dealing here with a relatively extreme situation, it seems that most if not all other cases of this kind of latency can be accounted for on the basis of the simple model of Fig. 17.

This has encouraged me to make a more complete theoretical study of this model. The results are shown in the three-dimensional graph of Fig. 18. The vertical ordinate gives the activity

of the membrane-shielded enzyme in percentage of its activity after destruction of the membrane. Five variables determine the position on the horizontal plane: the substrate concentration and the Michaelis constant of the enzyme (right-hand axis); the surface area of the membrane, its permeability coefficient, and the total activity of the enzyme (left-hand axis). The two edges of this graph correspond very much to the two experimental situations which have been analyzed. The left-hand edge applies to a substrate concentration very much lower than the Michaelis constant, in other words to a condition under which any enzyme will display first-order kinetics. This is the case of catalase, and we see that the latency decreases as the permeability and surface area of the membrane increase and as the activity of the enzyme decreases. The right-hand edge or its prolongation to even lower values of $(PSK)/V$, corresponds to the acid phosphatase situation where we raised the substrate concentration to more than ten times the Michaelis constant without any appreciable decrease in latency (Fig. 13).

The interest of the graph of Fig. 18 is that it shows that two enzymes shielded by the same membrane will not necessarily display the same degree of latency. Such we believe to be the case for the microbodies, where catalase is largely latent owing to its very high activity and first-order kinetics, whereas urate oxidase and D-amino acid oxidase do not show any latency, presumably because their activity is much lower, their kinetics are of the Michaelis-Menten type, and they are assayed at relatively high substrate concentration. A similar situation has not been encountered so far for the lysosomes, possibly because their membrane has a very low degree of permeability to all substrates, but it is not an impossible one and it could very well occur under conditions where, for instance, the permeability of the membrane is increased as a result of some injury or of some other treatment. It is also interesting to point out that the latency decreases as the surface area of the membrane increases. This means that when an enzyme is present at the same concentration in a collection of particles of different size, the smaller particles may, with a suitable combination of variables, show a lower degree of latency than the larger ones. Finally, it is well to keep in mind the im-

portance of the substrate concentration. Enzymes that do not show any latency under the conditions of an *in vitro* assay may very well do so in the intact cell, where substrates are often present at very low concentration.

VII. Functional Characterization of Particles

To complete this brief survey of what biochemical techniques can tell us about subcellular components, I would now like to consider some biological aspects. Here again, as in the other aspects that I have discussed, one can learn a great deal by simply letting the enzymes speak for themselves. They are the most important inmates of these submicroscopic dwellings which we attempt to search. Let only a few of them once be known to us and we begin to appreciate the significance of their abode in the economy of this complex city, the cell. Sometimes even, as it happens in our society, the most secretive of them may betray their activities through their associations.

Take the example of the lysosomes. Acid phosphatase, the enzyme which first led to their recognition, belongs to an obscure category of potential trouble makers, many of which, to our Lippman-trained eyes, appear to have little excuse for their existence. It was gratifying to find such a noisome object safely behind bars, but the observed association shed little light on the significance either of the prison or of the prisoner. When its companions slowly revealed themselves one by one, some sort of a pattern began to emerge. They were all acid hydrolases, sharing with acid phosphatase the ability to break down at a slightly acid pH important cell constituents, not only phosphate esters, but proteins, nucleic acids, glycosides. All equally destructive and dangerous, all rendered equally harmless by the same segregation phenomenon. Even this negative aspect was of interest, since it helped to explain how cells can safely harbor such potential criminals, a problem which has puzzled biologists for a long time. It also led us to assign an important protective function to the lysosomal membrane and to suggest a new mechanism of cell injury through rupture of this membrane.

However, we never thought of lysosomes as being merely dormant killers or undertakers, waiting for an opportunity to destroy

their host cells or to remove their mortal remains. Our speculations also took on a more positive direction. Spurred no doubt by teleological considerations, we looked for some useful role for these particles. From what the enzymes had told us, there was only one possible answer to this question: acid digestion. Now it has been known since the days of Metchnikoff that a number of cells carry out acid digestion in connection with the normal process of phagocytosis, and the more recent discovery of pinocytosis and micropinocytosis has shown that the ability to engulf is fairly general and not restricted to a few types of cells. The hypothesis that lysosomes are functionally connected with such processes was therefore not an unreasonable one.

It has been possible to support this hypothesis to some extent by means of purely biochemical experiments: by showing, for instance, that engulfed material is associated with the same subcellular fractions as the lysosomal hydrolases. However, I must concede here that biochemistry has its limitations and that this kind of problem is much better approached by morphological techniques. By a strange coincidence, the very enzyme which started our quest happens to belong to this small group which, thanks to the pioneering work of the late George Gomori, can be located in tissue sections through their precipitated reaction product; it shares with very few others the ability to lend itself to visualization in the electron microscope.

In themselves, the observed staining patterns might have given us little insight into the function of the stained structures. Much more work of this kind has been devoted to alkaline phosphatase, and the enzyme nevertheless continues to baffle our imagination. In the case of acid phosphatase, on the other hand, we are now helped by our knowledge of its associates, the other acid hydrolases. Of course, we do not know whether the association is a constant one, in all cells and under all circumstances; but it has already been found sufficiently frequently to serve as a useful working hypothesis in the interpretation of cytochemical staining patterns for acid phosphatase.

I have already recalled the early biochemical experiments on the localization of this enzyme which were carried out more than ten years ago by Alex Novikoff and his co-workers on this side

of the Atlantic and by our own group on the other side; and I have mentioned the slight difference of opinion which came up at that time with respect to the interpretation of the results. The exchange of correspondence and subsequent personal contacts which resulted from this friendly controversy have started a most happy and fruitful association which has continued ever since. In 1955, when the lysosome concept was first proposed, Novikoff helped us to identify the new particles in the electron microscope, and he has since—I must say very much against my own inclinations, which in those days were rather snobbishly wedded to biochemistry—pursued with great persistence and success the study of lysosomes by means of the cytochemical staining method for acid phosphatase. He has been joined in this pursuit by a number of other prominent workers, particularly S. J. Holt and F. Miller, and their investigations have greatly extended and clarified our knowledge of the role of lysosomes, not only in intracellular digestion, but also in a variety of autolytic processes.

Many other approaches have contributed to this, and I cannot possibly do justice to them without taxing your patience. However, I cannot resist citing, to illustrate how powerful the right combination of morphological and biochemical methods can become in the hands of imaginative investigators, the work of W. Straus on kidney droplets, that of J. Hirsch and Z. Cohn on leucocyte and macrophage granules and that of H. Fell, L. Thomas, G. Weissman and their co-workers on the remarkable antagonistic effects of vitamin A and hydrocortisone on cartilage, skin, and other tissues.

Enzymes can indeed be very eloquent, but for this, it seems that some sort of critical mass of information must be reached. The microbodies, with only three enzymes identified so far, still withhold their secret. They contain urate oxidase, but can hardly play a very important role in the catabolism of purines since many of the enzymes known to be involved in this catabolism are not associated with it in the microbodies but are present in the cell sap. They contain D-amino acid oxidase, a mysterious enzyme in itself, and which again is not accompanied by other enzymes involved in amino acid metabolism, many of which are known to be localized in the mitochondria and in the cell sap. Finally they

contain enormous amounts of catalase, of which we still do not know whether it acts physiologically as a true catalase or as a peroxidase. The only apparent link between these three somewhat unlikely roommates is hydrogen peroxide, which is made by the oxidases and utilized by catalase. But, it is a tenuous and rather unconvincing link, and we are beginning to wonder whether we may not be mistaken in trying to think of these particles as metabolic units. For there is another clue available to us, undoubtedly a very significant one if we could only interpret it correctly, namely, the high turnover of catalase. As shown quite convincingly by several groups of workers (Price *et al.,* 1962; Roodyn *et al.*, 1962; Higashi and Peters, 1962; Peters and Higashi, 1963), the whole content of the liver in catalase, meaning the major part at least of the protein content of the microbodies, is renewed in a little less than two days. One would certainly like to know where it goes and how it disappears. Perhaps it needs only one more enzyme to make the significance of the microbodies clear to us.

VIII. Concluding Remarks

In conclusion, I can only repeat what I hope you have by now recognized as the leitmotiv of this talk. Let the enzymes speak, listen to them, and they will tell you a great deal: about the size and shape of their intracellular locus, about the envelopes which shroud them, about their companions, and eventually, if it was not known before, about their role in the machinery of life. To this I should add, even though I have been mostly a listener myself: also look at them, and they will tell you a great deal more.

But whether we use our eyes or our ears, there is one thing more that is required of us, a belief in the natural order and beauty of things. To some, this belief may have its roots in religious faith; to others it may be the rational counterpart of an aesthetic or poetic emotion; to others again, it may be a simple factual recognition of the internal logic of selective evolution. Whichever its origin, this belief is essential to attune our ears so that they may detect an elusive rhythm or harmony behind the noise which so often obscures the messages we receive from nature, to sharpen our eyes so that they may recognize a pattern

in the blurred images which are projected on the screens of our microscopes. Sometimes the harmony is an illusory one, the pattern a mere mirage. But even this phantasm of our imagination may turn out not to be without fruit. If pursued with enough humility, intellectual honesty, and experimental rigorousness, it may yet lead us a little nearer to our ultimate but remote goal as scientists, the knowledge of ourselves and of the world around us.

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