TISSUE FRACTIONATION

PAST AND PRESENT

CHRISTIAN DE DUVE

From The Rockefeller University, New York 10021, and the Université de Louvain, Belgium

THIS IS NOT a review. It is an essay, conceived in the first place as a tribute to one of the founders of modern biology. Accordingly, it dwells over the past and considers tissue fractionation mainly in its historical perspective. It does take a brief look at the present state of the art, mainly in order to give some sort of idea of what has been accomplished in the last 25 years thanks to the development of tissue fractionation. This survery is restricted to liver, the original object of the method. The bibliography is mainly historical, but includes references to a few recent reviews and research papers, through which much of the remaining literature can easily be traced back.

CONCEPTS

SIGNIFICANCE OF AN ANNIVERSARY

It is exactly 25 years since Albert Claude published in *The Journal of Experimental Medicine* two consecutive papers under the common title "Fractionation of Mammalian Liver Cells by Differential Centrifugation" (49, 50). He was summarizing in them the results of a long effort that started in 1937 with an attempt to isolate the Rous sarcoma virus by high-speed centrifugation (44), led to the discovery of microsomes (45), and came to its final fruition in the concept of enzyme distribution studies (48) which, under the impulse of Claude's pupils, George Hogeboom, Walter Schneider, and George Palade, was to renovate biology.

Indeed, we may well ask on the occasion of this anniversary what would be the present state of cell biology without cell fractionation. Even had the development of electron microscopy proceeded unchanged, what else would we have to contemplate today but a rich atlas of beautiful and tantalizing images, through which our imagination would be forever weaving the elusive threads of hypothetical mechanisms?

Molecular biology itself might have followed a very different course without Claude's contribution. It is sometimes forgotten by enthusiastic historians of *Escherichia coli* and phage biology how much the adventure that culminated in the unravelling of the mechanism of protein synthesis and in the deciphering of the genetic code owes

initially to the fractionation of mammalian cells. From the early "in vivo" investigations of Borsook et al. (33, 34) and Hultin (105) on animals injected with labeled amino acids, which pointed to the microsomes as the main sites of protein synthesis, to the "in vitro" dissection of the system by Zamecnik and his coworkers (120, 181) and by Palade and Siekevitz (134, 135, 160), which led to the isolation, morphological identification, and biochemical characterization of ribosomes, all the significant work until 1958 had been performed on mammalian tissues fractionated by differential centrifugation. Only later did the bacterial system come into its own.

Cell fractionation was violently criticized by some, and still is today by the advocates of the holistic approach, for whom nothing short of an intact living cell can be a valid object of biological investigation. The quarrel is an old one. In his Introduction to the Study of Experimental Medicine, Claude Bernard cites the following passage by Cuvier:

> All the parts of a living body are related; they can act only to the extent that they all act together; to attempt separating one from the whole is to return it to the order of dead substances, it is to alter entirely its essence.[1]

To this declaration Claude Bernard then opposes his own philosophy:

> In order to succeed in solving these various problems, one must so to speak progressively dismantle the organism, as one takes to pieces a machine in order to recognize and study all its works. . . . One must therefore resort to a successive analytical study of the phenomena of life.[2]

It is no exaggeration to state that cell fractionation has done for cell biology what Claude Bernard's analytical approach did for organismic biology a century ago. Admittedly, reduction must be followed by reconstruction, or "physiological synthesis" as Claude Bernard termed it, and the resulting picture must fit the original model. Cell physiology still has a long way to go in this respect, and its crude descriptive schemes yet bear little resemblance to the intricate processes they are intended to represent. But the validity and, indeed, necessity of the analytical approach cannot be denied. Before any attempt can be made to give an integrated view of living processes, their complexity must first be reduced to a number of soluble problems, in conformity with the famous Cartesian precept "to divide each of the difficulties to be examined in as many parts as would be possible and required for better solving them."[3]

This is what Claude Bernard and the founders of physiology did for the organism, and what Albert Claude and his followers have now done for the cell.

THE IMPORTANCE OF BEING QUANTITATIVE

The idea of breaking up cells and using a centrifuge to separate some of their parts for the purpose of analysis is an old one. Witness the nucleic acids, whose very name recalls the fact that they were first extracted from nuclei. The pioneer of this work was

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[1] *Toutes les parties d'un corps vivant sont liées; elles ne peuvent agir qu'autant qu'elles agissent toutes ensemble; vouloir en séparer une de la masse, c'est la reporter dans l'ordre des substances mortes, c'est en changer entièrement l'essence.* From G. Cuvier, Lettre à J.-C. Mertrud, cited by C. Bernard. *In* Introduction à l'Etude de la Médecine Expérimentale, Pt. 2, Chapt. 1.

[2] *Pour arriver à résoudre ces divers problèmes, il faut en quelque sorte décomposer successivement l'organisme, comme on démonte une machine pour en reconnaître et en étudier tous les rouages . . . Il faut donc recourir à une étude analytique successive des phénomènes de la vie.* C. Bernard *In* Introduction à l'Etude de la Médecine Expérimentale, Pt. 2, Chapt. 1.

[3] [*Le second précepte était*] *de diviser chacune des difficultés que j'examinerais en autant de parcelles qu'il se pourrait et qu'il serait requis pour les mieux résoudre,* R. Descartes *In* Le Discours de la Méthode, Pt. 2.

Friedrich Miescher (125), who exactly 100 years ago broke up pus and other cells by various methods, and then isolated the nuclei by centrifugation. In the 1930s, the same concern prompted Martin Behrens (24) to develop improved techniques using density gradients of nonaqueous solvents for the separation of nuclei, while Bensley and Hoerr (27) set out similarly to work out a method for the purification of mitochondria. At a symposium held in his honor in 1943, Bensley (26) summed up his philosophy with admirable clarity and conciseness. "It would seem to be an axiom of analytic chemistry," he writes, "to separate separable things before proceeding to their analysis," wisely adding: "The analysis of particulate components of cytoplasm is only as good as the species-purity of the population." Descartes or Bernard could not have put it better.

But if not novel, what then is Claude's contribution? Where is his merit and why is it so great? I believe it is the fact that he insisted on being quantitative, or, as expressed elsewhere (66, 68), that he substituted analytical for preparative fractionation. He first made this point when reporting the early enzyme distribution data he obtained in collaboration with Rollin Hotchkiss and George Hogeboom, at an American Association for the Advancement of Science conference in July 1944:

> In those experiments, special emphasis was attached to the quantitative aspects of the results and efforts were made, whenever possible, to express the enzymatic activity exhibited by each fraction in terms of the total activity possessed by the unfractionated liver extract (48).

What Miescher, Behrens, Bensley, and their followers had aimed at until then had been the purification of a given visible intracellular entity, for the purpose of subsequently determining its physical characteristics, chemical composition, enzymatic equipment, metabolic and other functional properties. To this approach Claude was now substituting one focused on the actual objects of the analyses, enzymes or other biochemical components, inquiring in which manner these entities were distributed between all the fractions separated from the homogenate.

It is not easy to see that this new approach represented a fundamental change with respect to the former one, nor how it could have had such far-reaching consequences for the development of biological knowledge. To many workers, even today, the choice between one and the other would seem to depend largely on one's interests or inclinations. If you were a cytologist, you would naturally turn to purification as a means of knowing more about a given subcellular organelle, whereas your biochemically oriented colleague would more readily resort to distribution experiments, for instance in the course of some enzyme studies. He may in doing so uncover some information relating to the organelle that you are studying. But to actually select his approach as a means of securing the information would appear to most a very roundabout and haphazard way of accomplishing a straightforward job.

There is a flaw in this reasoning, but it is not easily exposed. The difficulty resides in the fact that there is nothing intrinsically wrong in the preparative approach. To purify for the purpose of analysis is a standard procedure of chemistry, a perfectly respectable and legitimate goal. The question is not whether it is permissible or commendable, but whether it is feasible and how it can be controlled (66, 68). Certainly, in the early days of centrifugal fractionation, adequate purification of a subcellular organelle was quite unattainable technically, and there were practically no means of evaluating the purity of a preparation. This is how, for instance, nuclei came to be credited with such a wealth of cytoplasmic enzyme activities (see references 3, 81, 82,

158). Even today, with all our technical improvements, and with the vast advances in our knowledge, preparative fractionation remains a hazardous undertaking.

This, I believe, is what Albert Claude grasped intuitively when he insisted on quantitative recovery and analysis of all fractions, the hallmark of analytical fractionation. Schneider and Hogeboom championed the same idea, insisting repeatedly, sometimes in the face of strong opposition, on "the need of establishing balance sheets in which the summation of the activities of the tissue fractions is compared with that of the whole tissue" (155). Thanks to their efforts and those of their followers, a host of quantitative enzyme distribution studies were performed in the 1950s and early 1960s (for reviews, see references 71, 76, 142, 143), adding immeasurably to our knowledge of the biochemical organization of cells.

THE APPEAL OF PURITY

In spite of its hazards, purification has been pursued as a primary aim by numerous investigators. Claude himself has described the isolation of melanin granules (46), chromatin strands (56), zymogen granules (47), and particulate glycogen (50). He has even confided that he was first drawn to cell fractionation by a desire to purify the granules from eosinophil leucocytes, because "he had fallen in love with their shape and color" (53). Many others have been similarly attracted by the prospect of purifying what they saw. Their efforts have been largely successful, to the extent that almost every one of the entities that have been detected in living cells has now been obtained in purified form. In many cases, however, the achievement was, so to speak, premature in that adequate information for assessing the purity of the preparations was not available. As a result of this, our conquest of the cell has proceeded by a succession of leaps followed by partial retreat. The history of tissue fractionation is replete with examples of properties that were erroneously attributed to the object of an incomplete purification. Most often, the correction came from analytical fractionation experiments rather than from an improvement in the purification procedure. The latter came only later, after the contaminant was detected.

The manner in which knowledge is acquired is of course less important than the knowledge itself. On the other hand, past errors are better not repeated if they can be avoided. In this respect, the most useful lesson that can be drawn from past experience is that purification should be pursued with the safeguards of analytical fractionation. Claude's golden rules that all fractions must be analyzed and that an accurate balance sheet must be kept, should be obeyed, whether or not certain fractions are to be discarded. Only with the information secured in this manner can the significance of the data obtained on the purified fraction be assessed in a proper context.

TECHNIQUES

Tissue fractionation started as an art. It has now largely become a science. It is the purpose of this section to give a bird's eye view of this evolution, with special emphasis on recent developments. A more complete survey of the techniques and instruments of tissue fractionation can be found in the reviews by Anderson (8, 9) and Allfrey (2), and in earlier publications from our group (71, 72).

HOMOGENIZATION

In his choice of conditions for breaking cells, Claude was strongly influenced by his concern to minimize injury to the cell components, to the point even of sacrificing his

requirement for quantitative recovery. He used a very gentle grinding technique, "rubbing the cells against each other" with a mortar and pestle, and a "physiological" saline mixture as medium (49). As a result, his first low-speed sediment contained many intact cells and a considerable amount of agglutinated cytoplasmic particles, in addition to the nuclei of the broken cells. This sediment, which comprised almost 50% of the starting material, was discarded. The remaining "liver extract" was then fractionated quantitatively, but obviously was far from representative of the whole tissue.

Significant improvements to Claude's scheme were brought in 1948 by his collaborators at The Rockefeller Institute, Hogeboom, Schneider, and Palade (103), who adopted the Potter-Elvehjem homogenizer as grinding device and 0.88 M sucrose as medium. With these modifications, cell breakage was made practically complete and agglutination was largely avoided. The first sediment approximated much more closely to a "nuclear fraction" and was kept for analysis. However, the hypertonic sucrose solution, which was adopted by the authors because it preserved the elongated shape of the mitochondria, turned out to have several drawbacks and was used only infrequently afterwards. It was replaced with 0.25 M sucrose by Schneider (152), who first described the fractionation scheme that is still followed by most investigators today.

The problem of homogenization has received a great deal of attention over the last 20 years, and many new instruments, procedures, and suspension media have been designed with the aim of improving the efficiency and selectivity of tissue disruption, as well as the integrity and degree of dispersion of the components of the homogenate. On the whole, the new inventions have enjoyed little success, and the favorite instrument has remained the Potter-Elvehjem homogenizer, especially the kind consisting of a smooth-walled glass tube and a Teflon pestle. It is commercially available in a variety of sizes, easy to operate, and somehow gives a certain illusion of gentleness. Actually, it does not deserve the latter qualification, since it is really quite mutilating. For instance, the minimum level of damage in our liver homogenates has been of the order of 15% for the lysosomes and peroxisomes, while evidence of distinctly greater damage has been reported by other workers. The mitochondria do not fare much better. Criss (59) has recently obtained convincing evidence that some 10% of the mitochondria have their outer membrane torn off in a liver homogenate, an estimate which agrees with our own results.

Such damage can be partly avoided by the use of less traumatic devices, for instance that described by Emanuel and Chaikoff (86), as shown on mammary gland by Greenbaum et al. (95), or the manually operated tube with spherical pestle-head devised by Dounce et al. (83), as observed on spleen by Bowers et al. (36) and on liver by Deter and de Duve (79).

It is a common experience that the greatest obstacle against "gentle" homogenization is the presence of a tough connective framework. Pressing the tissue through a perforated plate or screen, a widely used procedure first utilized by Claude (49) himself for removing the connective tissue framework, has been reported to cause considerable damage to certain organelles (102, 170). A simple preliminary mincing of the tissue is a more innocuous means of minimizing homogenization damage. Even better are the various techniques whereby the connective tissue structures are dissociated by means of chelating agents (5) or with enzymes (29, 139). Enzymic procedures have proved invaluable with such tough tissues as thyroid (99), cartilage (80),

and aorta (Fig. 4). They have the additional advantage that they yield a suspension of cells which can itself be fractionated if need be. Many methods have been described for this purpose (60, 124). On the other hand, free cells have been said to squeeze through relatively tight homogenizers, and to be more difficult to break up than pieces of solid tissue. According to Dingle and Barrett (80), this difficulty can be overcome by the simple device of embedding the cells in gelatine. Another possible drawback of the enzymic methods is that they may cause alterations of the cell surface.

FRACTIONATION PROCEDURES

Differential centrifugation of the conventional type was used by Claude and in all the early fractionation work. Each sediment was washed one to three times, an essential precaution in view of the low resolving power of the technique (71), and the washings were either added to the first supernatant or analyzed separately so that a complete balance sheet could be computed. The fractionation was monitored by microscope examination, and could almost be controlled by simple visual inspection thanks to the distinctly different colors and textures of the fractions. Claude (51) even went to the trouble of centrifuging pieces of whole tissue and demonstrating the correspondance between the fractions obtained centrifugally and the layers seen in the centrifuged cells. Other important developments were the demonstration by Hogeboom et al. (103) that Claude's large granule fraction was mostly made up of mitochondria, and, a few years later, that by Palade and Siekevitz (134) that the microsomes originate mainly from the endoplasmic reticulum. Finally, it soon became clear that certain enzymes were associated predominantly with a single fraction, for instance: nicotinamide adenine dinucleotide pyrophosphorylase with the nuclear fraction (156), cytochrome oxidase with the mitochondrial fraction (154), glucose 6-phosphatase with the microsomal fraction (98), and enzymes of glycolysis and related systems, such as aldolase (109), glucose 6-phosphate dehydrogenase (127) and phosphoglucomutase (98), with the final supernatant (for a more complete review of the early enzyme data, see reference 71).

All these findings served to put the four-fraction scheme on a firm experimental basis. But at the same time, they tended to obscure its essentially analytical nature. Except for a certain amount of inevitable cross-contamination, the procedure came to be regarded as a sort of happy combination of four preparative methods. The term "fraction" almost disappeared from the vocabulary, to be replaced by the words "nuclei," "mitochondria," "microsomes," and "cell sap." The distinction may appear a pedantic one, but it is easy to show that neglect of it lies behind many erroneous interpretations (66). Not that the danger was not recognized. As early as 1951, Potter et al. (137) complained: "The misuse of the words nuclei, mitochondria and microsomes is much more regrettable, although all of us are guilty in varying degrees."

The warning fell largely on deaf ears, and understandably so, since the field opened by Claude and his colleagues had proved such a bonanza that investigators were too busy collecting nuggets to stop reflecting on the finer aspects of their methodology and nomenclature. This is the time of the great upsurge of work on mitochondrial oxidations and phosphorylations, so aptly recounted by Lehninger (117). Microsomes came into their own a little later, after their role in protein synthesis (120, 160) and in drug metabolism (38) was discovered.

In the meanwhile, a few investigators had been experimenting with different fractionation and subfractionation schemes (11, 41, 74, 106, 113, 131, 132). One of these

experiments, by Novikoff et al. (131), deserves to be recalled for its almost prophetic insight. The workers isolated from 6 to 10 successive fractions and analyzed them for a number of different enzymes. As illustrated by Fig. 1, reproduced from their publication, it so happens that they were, quite unknowingly, tracing the fate of almost every distinct cytoplasmic entity that has since come to be recognized: mitochondria (succinoxidase), lysosomes (acid phosphatase), peroxisomes (urate oxidase), endoplasmic reticulum (esterase), and plasma membranes (5′-nucleotidase). These and other similar experiments were very important in drawing attention to the biochemical heterogeneity of the fractions, and pointed the way to the identification of new classes of cytoplasmic organelles (73, 74).

In 1953, a technical development of considerable importance took place, with the successful construction of high-speed swinging-bucket rotors which made possible separation by centrifugation through a density gradient. The history, theoretical basis, and practical applications of this powerful technique have been reviewed by Anderson (8, 9) and by de Duve et al. (72).

There are two distinct forms of density gradient centrifugation depending on

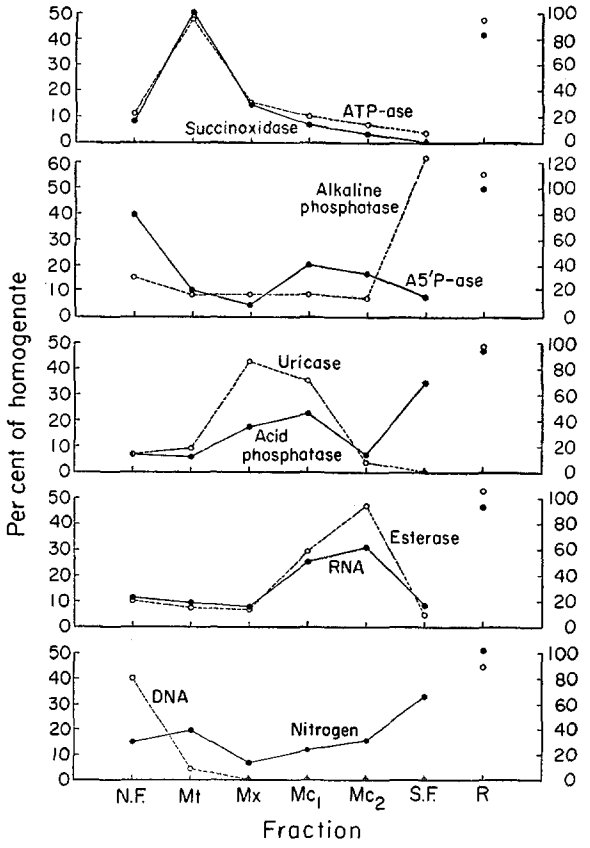


FIGURE 1 Distribution of enzymes between rat liver fractions, as reported in 1953 by Novikoff et al. (131). *N.F.*, nuclear fraction (10 min, 600 *g*, washed twice); *Mt*, mitochondrial fraction (10 min, 5200 *g*, washed twice); *Mx*, mixed fraction (10 min, 22,000 *g*, washed twice); Mc_1, first microsome fraction (69 min, 27,000 *g*, unwashed); Mc_2, second microsome fraction (69 min, 111,000 *g*, unwashed); *S.F.*, supernatant fluid; *R*, recovery.

whether the separation is based on differences in sedimentation coefficient (density gradient–differential sedimentation, kinetic-gradient centrifugation) or in buoyant density (isopycnic centrifugation, equilibrium-gradient centrifugation). Each type can be applied in two distinct ways depending on the starting conditions. In most cases, the material is layered on top (or below) the gradient, and centrifugation is termed zonal (rate-zonal or s-zonal for the kinetic type; isopycnic-zonal or ρ-zonal for the equilibrium type). However, the material may also be incorporated homogeneously within the gradient, as was done, for instance, by Kuff et al. (112) and by Beaufay et al. (20). In the case of a kinetic experiment, the analyses then focus on the shape and position of the sedimentation boundaries, as in conventional analytical centrifugation, though, of course, with a considerably greater choice of methods (for examples, see Figs. 5 and 15). This procedure avoids the serious limitations caused by drop sedimentation or density inversion (6, 28), but is of little value for preparative purposes. In isopycnic centrifugation, one should theoretically expect the final distribution of the particles to be unaffected by the initial conditions. In practice, however, this is not always so (22), because particles present initially in the lower part of the tube may suffer irreversible alterations as a result of their exposure to excessive osmotic and hydrostatic pressures. The latter effect, which has recently been recognized by Wattiaux et al. (177), is illustrated in Fig. 2. As can be seen, it can cause a complete disruption of mitochondria.

There is still a third way of classifying density-gradient centrifugation experiments, depending on whether a continuous or a discontinuous gradient is used. The discontinuous gradient is essentially a device for generating artificial bands. This may be a convenient way of compressing together for preparative purposes certain segments of the distributions observed in continuous gradients. But it is also a very dangerous procedure, in that it creates the illusion of clear-cut separation.

Density-gradient centrifugation in a continuous gradient is the analytical method "par excellence." It lends itself to an entirely objective assessment of the frequency-distribution curves of certain physical properties, such as density or sedimentation coefficient, from which in turn other characteristics of a population, including its size distribution, can be derived. Especially when performed with several media of different composition, it can provide a very extensive physical description of subcellular particles (21, 22, 67, 72). It is comparable in this respect to conventional analytical centrifugation, with the additional advantage that it can use any type of detection procedure, not just optical ones. It has for this reason become a major tool in the study of macromolecules as well. Hogeboom and Kuff were actually the first to apply this technique to proteins (101).

Density-gradient centrifugation has been further improved in recent years by the construction of automatic rotor assemblies that can be filled and emptied while running. Most of the difficulties and artifacts attending the use of swinging-bucket rotors are thereby obviated. The major pioneer in this area has been Norman Anderson, at the Oak Ridge National Laboratories, who has designed a number of different rotor types, several of which are now available commercially (9). Less well known, though of great practical value, is the rotor assembly developed by Henry Beaufay (18), with the help of the instrument shops at both Louvain and Rockefeller Universities. In Fig. 3, the Beaufay rotor is shown schematically, along with the Anderson B-XIV rotor.

In our laboratory, the B-XIV rotor has proved particularly useful for rate-zonal

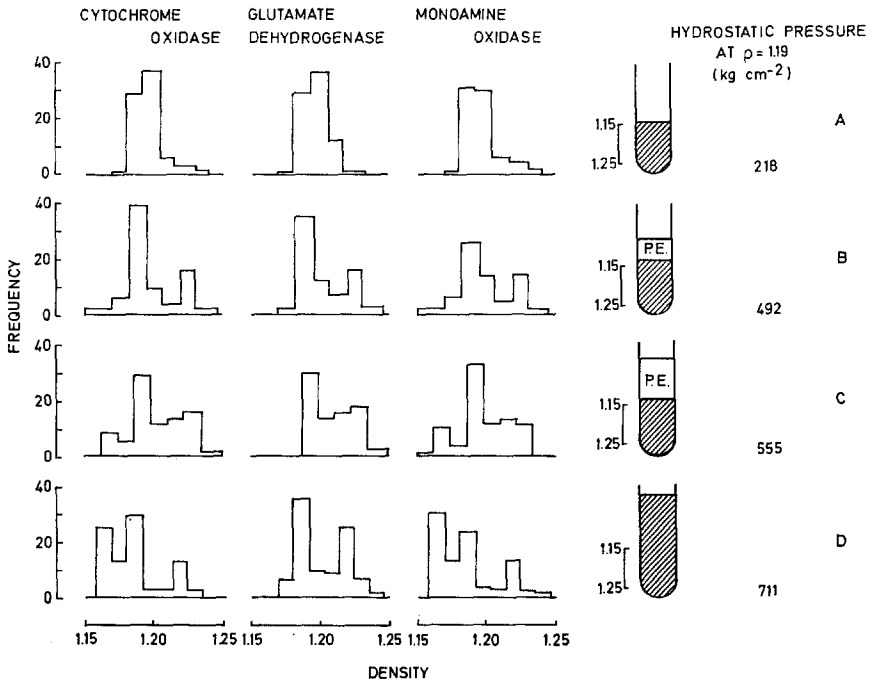


FIGURE 2 Damaging effect of hydrostatic pressure on mitochondria. Two steps are involved in the experiments depicted in this figure. First, the components of a rat-liver mitochondrial fraction were brought near their equilibrium position in a sucrose gradient by a 40 min centrifugation at 39,000 rpm, which generates a relatively moderate hydrostatic pressure. In the second step, shown schematically in the right-hand column, about 40% of the fluid was removed and replaced in *A* by air, in *B* by 1 ml of petroleum ether (*P.E.*) of density 0.66, in *C* by 2 ml of petroleum ether. No substitution was made in tube *D*. Upon recentrifugation for 90 min at 65,000 rpm, the hydrostatic pressures shown on the right were generated at the level of density 1.19 occupied by the mitochondria. In *A*, the three mitochondrial enzymes show reasonably unimodal density distributions around the normal equilibrium density value of 1.19. Almost no mitochondria have changed into the dense form of density 1.22 (22). In *B*, some mitochondria have suffered conversion to the dense form. This phenomenon is accentuated in *C*, where, in addition, some outer membranes (monoamine oxidase) and inner membranes (cytochrome oxidase) have been secondarily displaced to a density of 1.16–1.17, unaccompanied by soluble matrix proteins (glutamate dehydrogenase). In *D*, extensive flotation of mitochondrial membranes has occurred, leaving matrix glutamate dehydrogenase at 1.22 level. Results of Wattiaux et al. (177).

separations (12), whereas the Beaufay rotor is specially adapted to isopycnic centrifugation, for which it has almost four times the efficiency and three times the capacity of a fully loaded SW-39 rotor. An additional advantage of the Beaufay rotor, unanticipated in the original design, is that the hydrostatic pressure developed in it at high speed is much smaller than in other rotors. Damage of the kind recently described by Wattiaux et al. (177) (see Fig. 2) is thereby minimized. To this feature must be attributed the successful purification of peroxisomes (118), which is complicated in the SW-39 rotor by the movement of damaged mitochondria into the dense zone occupied by the peroxisomes.

While centrifugation remains the main tool utilized in tissue fractionation, efforts continue to be made towards the development of other separation techniques. Phase

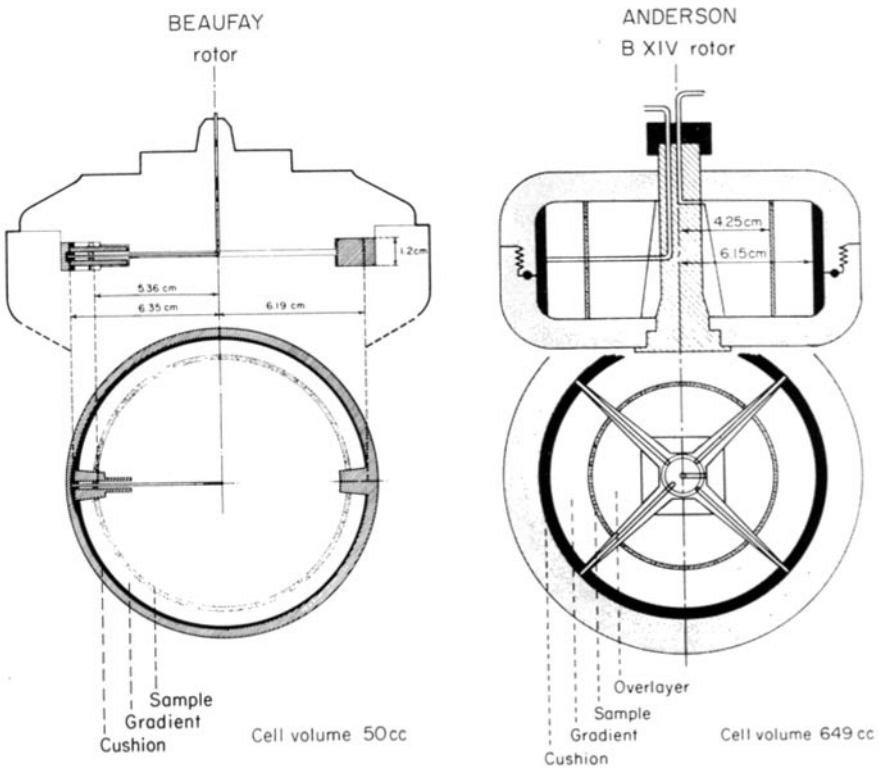


FIGURE 3 Schematic view of automatic rotors. The Beaufay rotor (18) has a ring-shaped cell which is filled and emptied through a single axial tube, the direction of flow being determined by the excess of the gas pressure prevailing in dead space of rotor over the hydrostatic pressure generated by centrifugation (flow is outward if excess is positive, inward in opposite case). Rotor is operated entirely from the outside, under uninterrupted high-vacuum and temperature control. Thanks to the high starting radial distance and the short path to be travelled, the efficiency is high and the hydrostatic pressure remains low, causing little damage (see Fig. 2). Graph shows initial conditions in the experiments of Leighton et al. (118). The Anderson B-XIV rotor (9) has separate tubes for inlet and outlet. It requires opening of the centrifuge chamber for the filling and emptying operations. The graph shows initial conditions in the experiments of Baggiolini et al. (12).

distribution, as worked out by Albertsson (1) is a very powerful method, but which so far has rarely been applied to subcellular particles. Electrophoresis, which has been attempted many times with little success, has recently given some encouraging results with the free-flow apparatus developed by Hannig (96, 97, 164). Differential filtration through membranes of defined pore-size has lately been used by several workers (58, 61, 91).

MARKER ENZYMES

Tissue fractionation was originally developed as a cytological method, with the microscope as supreme guide and chemical analysis as a subordinate tool. After it was found that certain enzymes were largely concentrated in one fraction, probably as a result of their association with a single subcellular component, it occurred to us to reverse the priorities and to let the enzymes dictate over the morphological observa-

tions, which in any case could hardly be trusted to be very discriminating with objects close to the resolving power of the then available instruments (71, 73, 74). This switch opened up a new interpretation of biochemical heterogeneity, and eventually led to the identification of lysosomes, and later of peroxisomes, as components of the mitochondrial fraction (22, 65, 70, 74, 75). It is at the origin of the now widely used practice of evaluating the composition of subcellular fractions by means of enzymic analyses.

Right at the start, it was pointed out that the use of an enzyme as marker for its host-particle rests on two postulates: The enzyme must belong to a single class of particles (single location), and its specific activity must be the same in the different particle subclasses separated by the fractionation procedure (biochemical homogeneity). It was also made clear that these were working hypotheses, to be accepted only insofar as they had not been disproved, and that they need not be true for every enzyme to be useful since all that was needed for biochemical monitoring of a fractionation was one reliable marker enzyme per class of particles.

These points, which have been discussed in detail elsewhere (66, 68, 71), should be kept in mind whenever enzymes are used as markers. One should appreciate also that the two postulates have slightly different connotations. Single location obviously is essential. On the other hand, biochemical homogeneity defines the relationship between the enzyme and its host-particle that is necessary to permit quantitative evaluation of the data. If it is satisfied, the distribution of the enzyme may be taken to represent also the distribution of the corresponding particle protein. If, in addition, the specific activity of the marker enzyme in the pure particle is known, the absolute amount of protein belonging to the particles can be computed. When marker enzymes are available for all known particles, such calculations may even serve to detect unknown particles, in the form of "unassigned protein" (118).

Another point concerning the marker enzyme method is that the postulates on which it rests are likely to be true only in first approximation. It is obviously rather improbable that every mitochondrion or lysosome has exactly the same composition, and heterogeneity within a given population is almost bound to emerge at some stage as more refined techniques become available. However, such heterogeneity may be difficult to detect with certainty, since other factors, such as partial inactivation of the enzymes, injury to the particles, or cellular heterogeneity and the consequent existence of more than one group of homologous particles, can account for observations indicative of heterogeneity within a particle population (118).

An interesting exception to the postulate of biochemical homogeneity (or application of it, if only the membrane is considered) is to be expected on theoretical grounds for enzymes associated with the membrane of a particle. The specific activity of such enzymes in the particles should decrease with increasing particle radius. This prediction could be verified by a rate-zonal study of monoamine oxidase, a marker for the outer mitochondrial membrane. Cytochrome oxidase, for which a slight shift in the predicted direction has been observed (165) is not a good test object in view of its fragility and attachment to a convoluted membrane.

As to the postulate of single location, there are already numerous examples of enzymes that seem not to obey it. In most such cases, however, it has been found that the different locations were occupied by different isozymes, so that strictly speaking the postulate remains valid (76). It must definitely be at fault for those enzymes and other specific constituents that travel through separable intracellular structures, as is

the case for many of the proteins manufactured by ribosomes attached to rough-surfaced vesicles of the endoplasmic reticulum. However, the amount of enzyme actually under way could be very small with respect to the amount present at the final destination.

It should finally be pointed out that certain marker enzymes are tissue specific. Glucose 6-phosphatase, for instance, is a good microsomal marker in liver, but is absent in many other cell types.

BIOCHEMICAL ASSAYS

As was clearly appreciated by Claude, tissue fractionation owes its main power to the fact that it renders the different parts of the cell accessible to the whole battery of analytical techniques contained in the biochemist's arsenal. In reviewing the literature of the last 25 years, one will find that these advantages have indeed been fully exploited. However, the very advances that have been made have also created problems of a practical nature, largely connected with the limited amounts of material available for analysis, and the large number of assays to be performed. As in many other areas, the general answers to these problems have been miniaturization and automation.

Micro-assays for enzymes obviously cannot be reviewed here. But as a general hint, it may be useful to point out that in a large number of cases a considerable increase in sensitivity can be gained simply by substituting a fluorometric for the usual photometric assay system. For instance, in the measurement of hydrolases, the fluorogenic derivatives of 4-methyl-umbelliferone (116) have proved of great value as substrates, as compared to the chromogenic nitrophenol derivatives. They are, however, much more sensitive to degradation, and some commercial samples have been found to give entirely unacceptable blanks owing to improper preservation.[4] As an illustration of what can be accomplished without any major technical effort, Fig. 4 shows some recent results obtained on fractions separated from the smooth-muscle cells of a single rabbit thoracic aorta. In the experiments of the type shown, the total quantity of cell protein layered on the gradient could be as little as 0.5 mg. Similar results have been obtained with liver.

Automation of enzyme assays has been greatly developed in recent years, mainly in the field of clinical chemistry. Automated assays have been worked out also for a number of marker enzymes of particular interest in tissue fractionation, especially in the laboratories of Anderson (9, 123), Roodyn (144), Tappel (37), and in our own (12, 118). The recent survey by Roodyn (144) will be found particularly useful.

MORPHOLOGICAL EXAMINATIONS

It is interesting that Claude's first contribution to electron microscopy, published in 1945, actually deals with isolated mitochondria (54). The particles were simply spread and dried and, as might be expected, little detail of their structure could be seen. Soon afterwards, Porter, Claude, and Fullam (136 *a*) described the existence of a "lace-like reticulum" in thinly spread cells in culture. Claude, Porter, and Pickels (55) later made the important suggestion that "this endoplasmic material of the electron micrograph is the small particle fraction of broken-up cells described by Claude."

[4] We have obtained excellent results with substrates manufactured by the Koch-Light Laboratories (Colinbrooke, Buckinghamshire, England), which also offer the widest available choice of compounds.

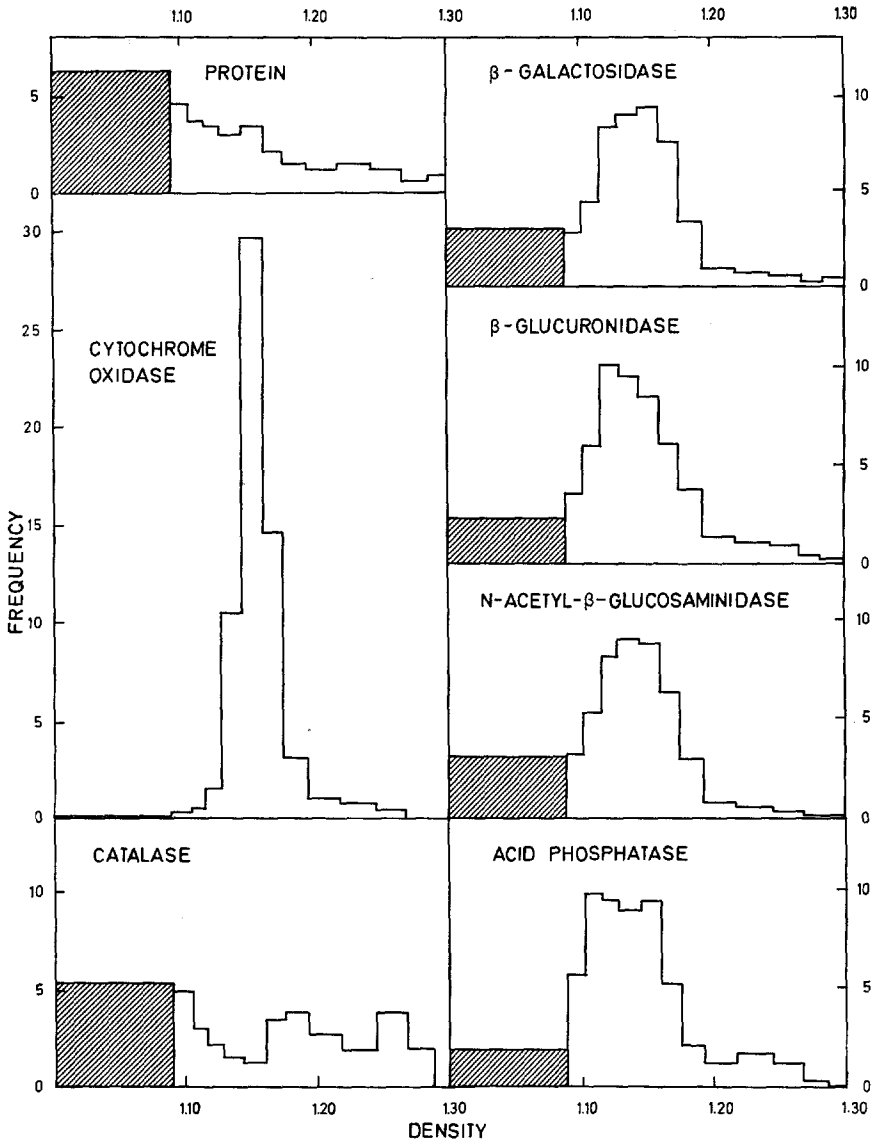


FIGURE 4 Fractionation of a postnuclear supernatant from rabbit aorta smooth-muscle cells by isopycnic centrifugation. The thoracic aorta of an adult rabbit was sectioned into rings and digested for 2½ hr at 30°C in Hank's solution containing 2 mg/ml of elastase, collagenase, and hyaluronidase. The cells were collected, washed, and disrupted in 0.25 M sucrose with a Dounce homogenizer. A sample of the postnuclear supernatant (600 g × 15 min) was layered over a linear sucrose gradient and centrifuged for 37 min at 35,000 rpm in the Beaufay rotor. Graph shows density-frequency distributions observed for various enzymes. The shaded blocks represent, over an arbitrary abscissa interval, the enzyme activities remaining in the sample layer in soluble form. Hydrolases were measured fluorometrically with 4-methylumbelliferone derivatives, cytochrome oxidase by a micro adaptation of the spectrophotometric method (118), using an expanded scale, and catalase by our usual procedure (118). Unpublished results of T. Peters and M. Müller.

It took almost 10 years before this hypothesis was actually verified, by Palade and Siekevitz (134, 135).

Since then, the examination of cell fractions in the electron microscope has become a routine procedure, and numerous methods have been described for processing the fractions and examining them either in thin sections or in negatively stained preparations. A survey of these procedures is beyond the scope of this paper.

In our laboratory, a special effort has been made by Pierre Baudhuin in order to make the morphological analysis of subcellular fractions quantitative and statistically valid. The particles are collected on Millipore filters in a manner ensuring true random sampling, an essential requirement in view of the extreme minuteness of the samples that are actually examined (16). A variety of measurements are then made by means of proven morphometric methods, allowing a complete evaluation of the contents of a fraction in terms of the number, size, and shape of the individual particles (13).

Thanks to these developments, it has now become possible to make direct quantitative comparisons between biochemical and morphological results (13, 15, 77, 78, 118, 179). As an example of this, Fig. 5 shows the cumulative distribution of sedimentation coefficients of rat liver mitochondria, as obtained from the measured sedimentation boundary of cytochrome oxidase in a sucrose gradient (79) and as computed from the size distribution determined by quantitative morphometry of the fractions (15). Another example of a similar correlative analysis, pertaining to microsomes, is shown below in Figs. 12 and 13.

PRESENTATION OF RESULTS

In their first reports on enzyme distributions, Claude and his colleagues (100, 153) took particular care to give the total activities found in each fraction, so that a complete balance sheet could be calculated, and abnormal losses or gains in enzyme activity be detected and, if possible, traced to their source. Specific activities were also reported as a means of assessing the relative concentration achieved in each fraction. The same practices were insisted upon subsequently by Hogeboom, Schneider, and many others. We proposed a graphical method whereby the relevant

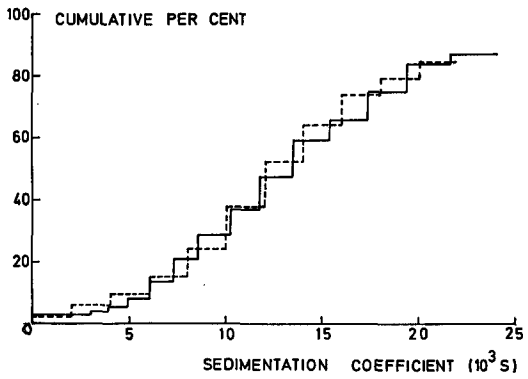


FIGURE 5 Cumulative distribution of the sedimentation coefficients of rat-liver mitochondria. The broken line represents the results obtained by Deter and de Duve (79) from measurements of the sedimentation boundary of cytochrome oxidase. The solid line is derived from the size distribution of mitochondria determined by quantitative morphometry by Baudhuin and Berthet (15). From reference 15.

information is conveniently summarized in histogram form (74). Results of density-gradient experiments can be represented similarly in the form of histograms or frequency distribution curves as a function of the parameter, sedimentation coefficient or density, on which the separation was based (see many of the figures in this paper). The distribution can be given also as a function of a related parameter, such as size, that can be derived from the sedimentation coefficient, as was first done by Thomson and Mikuta (171) and Kuff et al. (112). The procedures for such calculations have been described (21, 68, 72) and the analytical significance and importance of the resulting diagrams have been stressed repeatedly (66, 67, 68). Computer programs have been written and are available.

Not all workers, however, have accepted or followed the above rules. Fairly frequently, results of fractionation experiments are given exclusively in terms of specific activity. In our opinion, such information is incomplete, and it can be very misleading, especially when presented graphically against a scale other than the relative protein content of the fractions. The suggestion that there is more enzyme in the fractions with the highest specific activity is very strong, obscuring the fact that the fractions concerned could contain only small amounts of protein and little enzyme activity. Similarly, the absence of some marker enzyme in a purified fraction is relatively meaningless, if no evidence can be given that the recovery of this enzyme in the discarded fractions was satisfactory.

THE FOUR FRACTIONS

To review all the applications that have been made of tissue fractionation would have been quite beyond the scope of this essay. It would, in fact, have turned it into a survey of much that has been accomplished in cell biology over the last 20 years. It seemed, however, of interest, as an illustration of the distance that has been travelled since Albert Claude first fractionated mammalian liver, to take a look at our present knowledge of the composition of the four classical fractions. As will be seen, our inventory of the cell is almost complete, and we now know where to find most of the structural entities that have been recognized in the electron microscope. Our understanding of the functional properties of these entities, and especially of their interrelationships, of their assembly and breakdown, of their integration within the economy of the cell, is still very sketchy and superficial. But at least the stage is set, and we are now ready to enter the era of in-depth analysis.

THE NUCLEAR FRACTION

Two main cell components are significantly concentrated in the nuclear fraction: the nuclei themselves and fragments of the plasma membrane. It also contains blood cells if the organ has not been perfused, connective tissue fragments, as well as the cells and large debris that escaped adequate homogenization. In addition, it is generally contaminated quite heavily with mitochondria and other cytoplasmic components. The reason for this is not quite clear. As shown in Fig. 6, when the fraction is subjected to isopycnic centrifugation in a sucrose gradient, the cytoplasmic particles are resolved normally, offering no evidence of agglutination to the nuclei, which is the explanation generally given for the lack of purity of the nuclear fraction. However, the result shown does not exclude the possibility of loose binding, since particles entering the denser sucrose layers in association with the nuclei would become subjected to considerable buoyant forces that could detach them secondarily.

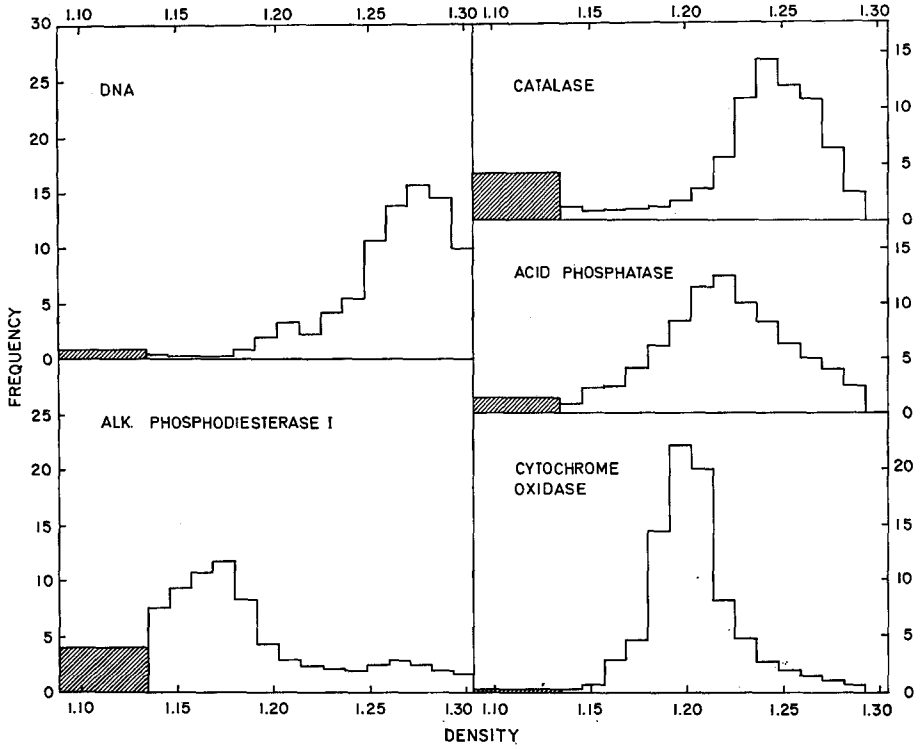


FIGURE 6 Subfractionation of nuclear fraction by isopycnic centrifugation through sucrose gradient containing 3 mM imidazole, pH 7.4. Centrifugation for 3 hr at 35,000 rpm in Beaufay rotor. Graphs show density-distribution histograms of DNA and of marker enzymes for plasma membranes (alkaline phosphodiesterase I), mitochondria (cytochrome oxidase), peroxisomes (catalase), and lysosomes (acid phosphatase). Shaded blocks represent, over an arbitrary abscissa interval, the enzyme activities remaining in the sample layer. Unpublished results of P. Baudhuin.

Nuclei. Numerous methods have been designed for the purification of nuclei (for literature, see references 2, 81, 82, 128, 140, 141, 148, 159). Of historical importance are the Behrens (24) technique using nonaqueous solvents, the sucrose-calcium method of Hogeboom, Schneider, and Striebich (104), and the dense sucrose procedure of Chauveau, which yielded the first preparation of nuclei shown to be reasonably free of cytoplasmic contaminants by assays for marker enzymes (42).

Although most workers now use aqueous solutions for the isolation of nuclei, there is as yet no consensus of opinion concerning the extent to which soluble constituents of the nuclear sap, including enzymes, are lost in such media by diffusion through the nuclear envelope. The permeability of nuclei remains a vexing question which seems not yet to have received a satisfactory solution (see reference 111). Certainly, when the nuclei are isolated in the presence of detergents, which dissolve the nuclear envelope, as has been done lately by a number of authors (see reference 148), losses of soluble proteins could easily occur. The main advantage of nonaqueous solvents is that such losses are avoided (even though the membrane is likewise destroyed), but the lack of cytoplasmic contamination of such nuclei has never been convincingly demonstrated.

Claude and Potter (56) were the first to subfractionate nuclei, from which they

separated chromatin threads. Nucleoli were first isolated from starfish oocytes by Vincent (176), and have since been purified from a variety of sources (see references 39, 148). Several recent papers have dealt with the isolation and analysis of the nuclear membrane (see reference 90). Fairly large differences in composition between nuclear membranes and microsomes have been noted by some workers, in surprising contrast with morphological and cytochemical observations. The separation of nuclear ribosomes and polysomes has also been accomplished (see reference 147).

Plasma Membranes. The presence of large fragments of plasma membrane and bile canaliculi in the nuclear fraction was recognized already by early investigators (104, 131, 133), but no special attention was paid to this finding until Neville (130) first isolated such fragments. Since then, the plasma membrane has become a favorite object of study, and a considerable literature has already accumulated on the subject (see references 25 and 172).

Typical of enzymes associated with the plasma membrane, such as 5′-nucleotidase or alkaline phosphodiesterase I, is a nucleomicrosomal pattern of distribution. There has been some discussion as to the significance of the microsomal part of the enzymes, but it is now generally agreed that it belongs to plasma membrane fragments or to components closely related to the plasma membrane (see below).

It is somewhat puzzling that the highest purification ratio achieved so far for enzymes in purified plasma membrane preparations has been of the order of 25- to 30-fold, suggesting that the plasma membrane may account for as much as 3% of the total cell protein. This is 10 times the amount that would be found in a pellicle 10 nm thick surrounding a sphere of 20 μm diameter.[5] The extensive convolutions of the membrane undoubtedly account for a considerable part of this difference. It is also possible that some of the material originates, not from surface membranes, but from intracellular structures related to the plasma membrane, for instance endocytic vacuoles. Finally, it is likely that present preparations are still impure, as suggested by their content of variable amounts of enzymes predominantly localized in other parts of the cell, especially microsomal enzymes (25, 57, 172).

Plasma membranes are characterized by a high content in sphingomyelin and in cholesterol. There is some debate as to what proportion of the total cell cholesterol actually is associated with the plasma membrane. This point will be discussed below. A useful property of plasma membranes and related structures, almost certainly dependent on their content in cholesterol, is the "digitonin shift," i.e., an increase in equilibrium density (in a sucrose gradient) produced by exposure of the membranes to a low concentration of digitonin followed by washing (Fig. 7). This effect is attributed to the binding of digitonin by the membrane cholesterol. The structural changes associated with it are illustrated in Fig. 8.

Subfractionation of plasma membranes after mechanical comminution, following an approach similar to that of Dallman and coworkers (61), has been initiated in our laboratory by Denise Thinès. She has shown that after treatment of the membranes in an Ultra-Turrax blender (Janke and Kunkel, Staufen i.B., Germany), 5′-nucleotidase, alkaline phosphodiesterase I, and alkaline phosphomonoesterase segregate with fragments of different density and cholesterol content. This suggests that the plasma membrane is composed of biochemically distinct areas.

[5] For a spherical particle with a radius r and a membrane of thickness t, the ratio of membrane to total volume is given in first approximation by $3t/r$.

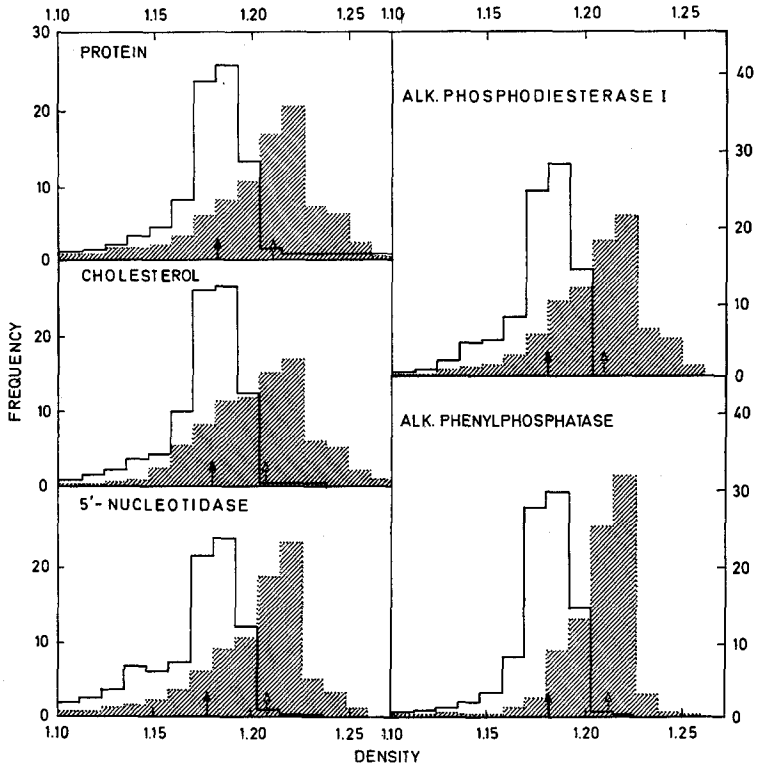
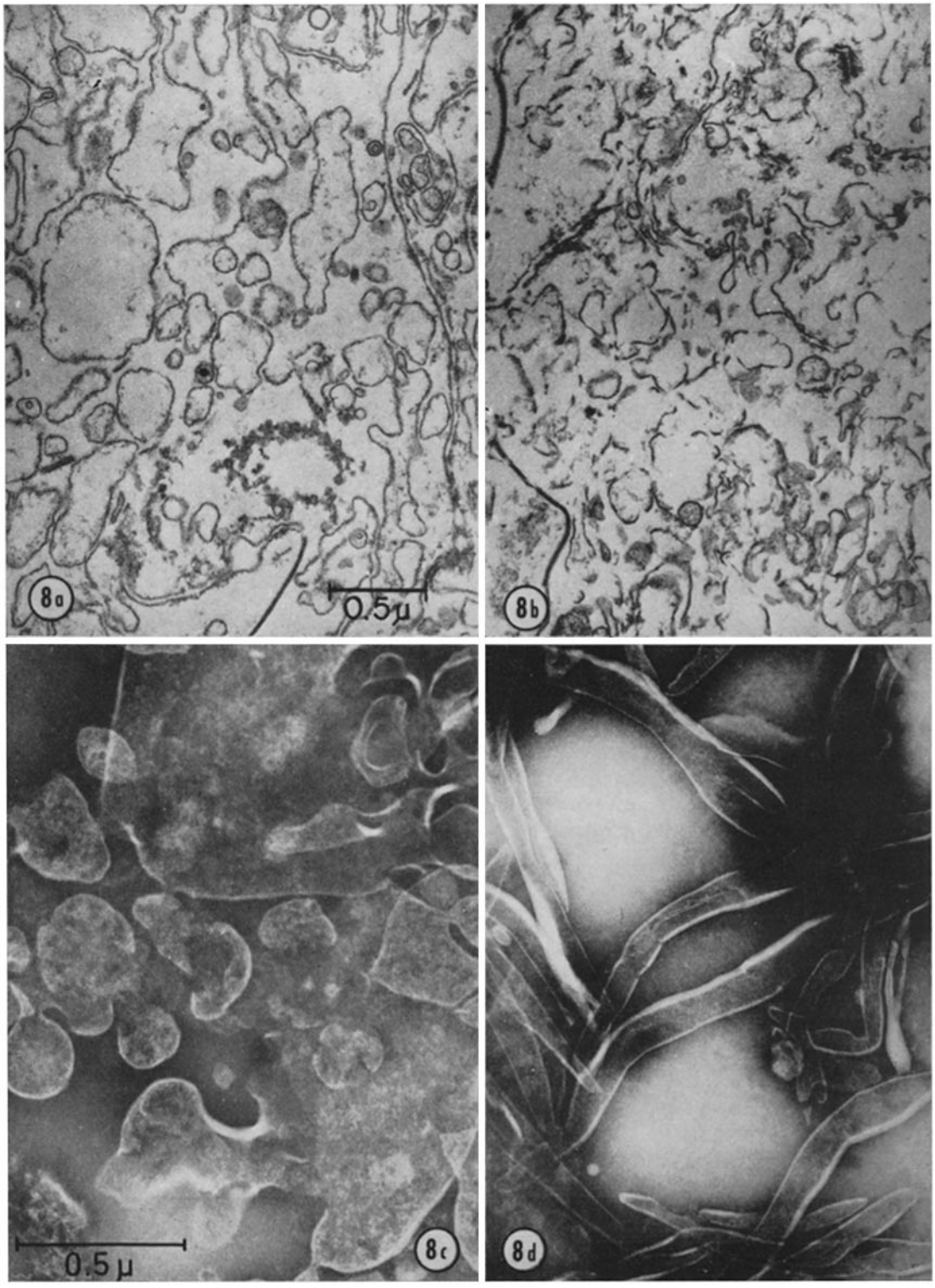


FIGURE 7 The digitonin shift. Plasma membranes prepared according to Song et al. (162) centrifuged for 30 min at 35,000 rpm in Beaufay rotor through sucrose gradient. Solid line, density distributions for control preparation; Shaded area, same preparation exposed to an amount of digitonin stoichiometrically equivalent to cholesterol content. Arrows indicate median densities. Results of Thinès et al. (169).

Other Components. As already mentioned, nuclear fractions usually contain fair amounts of enzymes associated predominantly with the cytoplasmic fractions. Results such as those shown in Fig. 6, together with data obtained on highly purified nuclei, indicate that many such enzymes belong entirely to cytoplasmic particles contaminating the nuclear fraction. The significance of soluble cytoplasmic enzymes found in the nuclear fraction is more difficult to appreciate. There is no doubt that nuclei can pick up proteins by adsorption. On the other hand, as mentioned above, there is also the possibility that enzymes occurring in the nuclear sap may be lost in the course of isolation as a result of leakage.

THE MITOCHONDRIAL FRACTION

Much of our work over the last 20 years has been concerned with the identification and characterization of two additional groups of cytoplasmic particles in the mitochondrial fraction: the lysosomes and the peroxisomes. The results of this work have been reviewed in detail elsewhere (65, 67, 70, 75) and will be alluded to only briefly here. Fig. 9 illustrates the separation of the three classes of particles that can be achieved by isopycnic density-gradient centrifugation after the density of lysosomes has been lowered artificially by a previous injection of Triton WR-1339 to the animals (118, 178).


8a
0.5μ
8b
0.5 μ
8c
8d

Mitochondria. For several years, Claude remained in doubt as to the identity of the large granules, which he first thought to be secretion granules and later considered a mixture of secretion granules and mitochondria (50). They were identified as mitochondria by Hogeboom et al. (103) on the basis of their elongated shape in 0.88 M sucrose and ability to stain with Janus green. Soon after, the role of mitochondria in cellular oxidations and phosphorylations was brought to light (93, 108, 109, 117, 157), and, in particular, the fundamental property of respiratory control and its relation to structural integrity were discovered (110, 114).

It is remarkable that, in spite of the unprecedented research effort sparked by these findings, no serious attempt has been made to design an adequate purification procedure for mitochondria, even after the presence of powerful contaminants in the

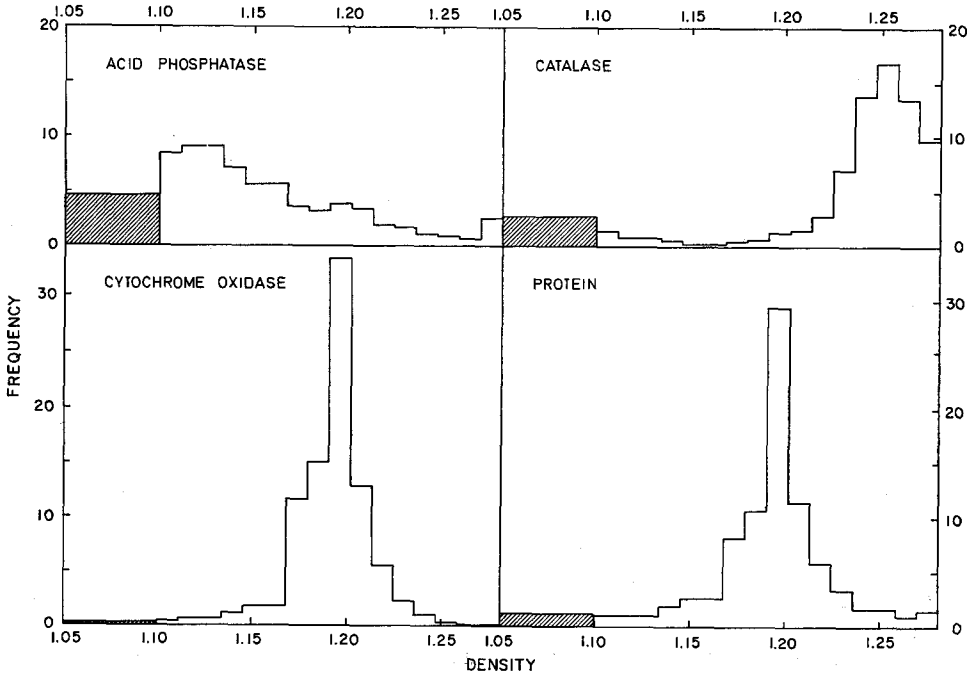


FIGURE 9 Subfractionation of mitochondrial fraction from liver of rat given an intravenous injection of 170 mg Triton WR-1339 4 days previously. Isopycnic centrifugation in Beaufay rotor according to Leighton et al. (118), but on M + L fraction. Graphs show density distributions of protein and of marker enzymes for lysosomes (acid phosphatase), mitochondria (cytochrome oxidase), and peroxisomes (catalase). First block represents, over an arbitrary abscissa interval, the enzyme activities remaining in the sample layer in soluble form. Experiment of C. Peeters-Joris.

FIGURE 8 Influence of digitonin treatment on morphology of isolated plasma membranes. *a* and *c*, control preparation; *b* and *d*, preparation exposed to digitonin as in Fig. 7; *a* and *b*, ultrathin section; *c* and *d*, negative staining with potassium phosphotungstate. In section, the membranes exposed to digitonin seem to be broken or fenestrated, but no indication of this is seen in negative staining. Note the rigid appearance of membranes induced by digitonin in negatively stained preparation. Results of Wibo et al. (180). Fig. 8 *a–b*, × 30,000; Fig. 8 *c–d*, × 52,000.

mitochondrial fraction was demonstrated. Certainly, there is a surprising contrast between the mediocre quality of the mitochondrial preparations that serve for the study of oxidative phosphorylation, in no way different from the crude fractions that were isolated more than 20 years ago, and the increasing sophistication and sensitivity of the probes with which their functional properties are being scrutinized.

One way of obtaining pure mitochondria is shown by Fig. 9. The functional state of the particles after such isolation has not been tested. They appear relatively normal in the electron microscope (118), but could nevertheless have suffered some damage due to osmotic dehydration. In any case, the procedure is too laborious and time consuming for routine work. On the basis of present information on the distribution of sedimentation coefficients of mitochondria, lysosomes, and peroxisomes (13, 20), the simplest method for obtaining relatively pure mitochondria would seem to be rate-zonal centrifugation. With a suitably designed procedure, one should be able to collect about 50% of the mitochondria almost free of contaminants. The largest particles would be selected in this way, causing a somewhat biased sampling. But at least the possible interferences by lysosomes or peroxisomes in oxidative phosphorylation could be explored.

Interesting developments have happened in recent years thanks to the finding that the outer mitochondrial membrane can be ruptured selectively by exposure of the mitochondria to a mild osmotic shock (136), eventually followed by a brief period of sonication (163), or to a low concentration of digitonin (150). After such treatments, it is easy to separate fairly pure outer membranes, the soluble components enclosed between the two membranes, and the rest of the mitochondrial bodies. These, in turn, can be further subfractionated into inner membrane and matrix content (40). Experiments of this sort, of which an example is shown in Fig. 10, have paved the way towards studies of the distribution of enzymes within the mitochondria (14, 17, 87, 94, 150, 151, 163). Further dissection of the inner mitochondrial membrane can be achieved by more disruptive treatments (92, 138). Other intramitochondrial entities that are now evoking considerable interest are the mitochondrial DNA (129) and ribosomes (35), which many workers tend to regard as evolutionary remnants of the genetic apparatus of distant bacterial ancestors.

Lysosomes. As discussed in detail by Beaufay (19), lysosomes generally form highly polydisperse populations and are, for this reason, very difficult to purify with satisfactory yield. The main effort in this direction has been made by Tappel and his associates, who have succeeded in obtaining preparations of relatively pure lysosomes from liver (149) and other tissues, but with a very low yield. In our laboratory, the method of Trouet (173), taking advantage of pretreatment of the animals with Triton WR-1339 (178) (see Fig. 9), has been used extensively. An analogous procedure, based on dextran loading of the lysosomes has also been employed occasionally (167). These methods have the disadvantage that the lysosomes that are isolated have an abnormal content and could be altered in other properties as well. In addition, the preparations could contain significant amounts of endocytic vacuoles (phagosomes). An interesting electrophoretic method for purifying rat liver lysosomes has recently been described by Stahn et al. (164). Unfortunately, the yield is again very low, of the order of 6%.

A fair amount of work has been performed on the "membranes" separated from disrupted lysosomes by high-speed centrifugation. As found by Tappel and his co-

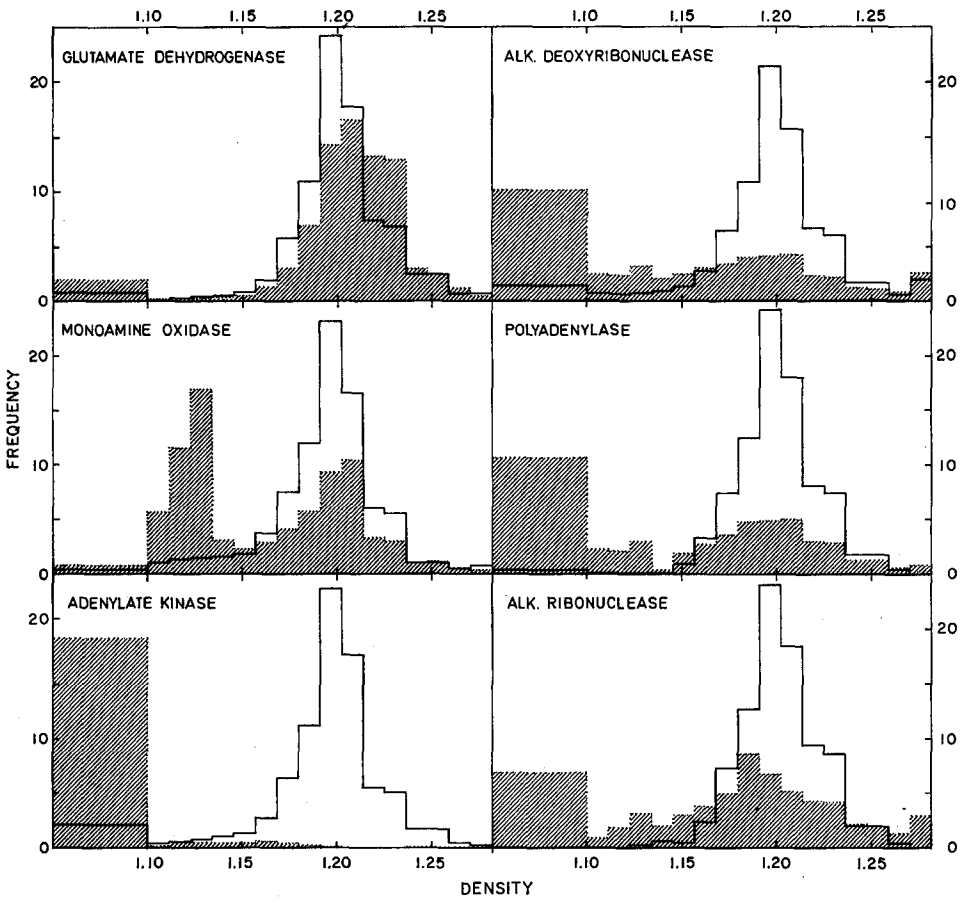


FIGURE 10 Subfractionation of mitochondria disrupted by mild osmotic shock. Centrifugation for 35 min at 35,000 rpm in Beaufay rotor. Solid line, density distributions of enzymes in control preparation; shaded area, similar enzyme distributions for preparation exposed to 0.02 M phosphate buffer pH 7.4 + 0.02% serum albumin according to Parsons et al. (136) and dispersed with tight Dounce homogenizer. First block, until density 1.10, represents over an arbitrary abscissa interval, the enzyme activities remaining in the sample layer. Complete solubilization of adenylate kinase (intermembrane space) and detachment of nearly 50% of monoamine oxidase (outer membrane) is accomplished with little release of glutamate dehydrogenase (matrix). The mitochondrial nucleases are released with adenylate kinase, but are partly retained by adsorption. They are completely solubilized in the presence of 0.3 M KCl, without additional release of glutamate dehydrogenase (14, 17). Alkaline ribonuclease refers to a thermolabile enzyme, one of several species participating in the RNase activity of liver (14). Unpublished results of C. Peeters-Joris, J. Bartholeyns, and P. Baudhuin.

workers (23, 122, 166), after repeated freezing and thawing of the particles, some enzymes, for instance β-glucosidase and acid lipase, remain largely bound to the sedimentable fraction, whereas many others are largely released in soluble form. Acid phosphatase is unequally distributed between the two fractions, apparently in the form of two distinct isozymes (161). The significance of these observations is not easily assessed, since some of the bound activities can be partly detached by simple washing with solutions, and cannot therefore be considered truly membrane bound. Furthermore, the insoluble fraction contains about 50% of the total lysosomal proteins, in-

stead of the expected 10–15 % (see above: footnote 5), and must therefore contain considerable amounts of nonmembrane material, presumably residues of one sort or another.

Comparison of insoluble fractions separated from Triton- or dextran-filled lysosomes with plasma membranes has revealed failrly strong similarities in lipid composition (107, 167), but also very distinct differences in antigenic (174) and enzymic (107) content. This is surprising, in view of the evidence indicating that secondary lysosomes derive a substantial part of their membranes from the plasma membrane through the mediation of endocytic vacuoles.

Peroxisomes. These particles, which are known in the morphological literature as microbodies, have been obtained in a state of about 95 % purity, as verified both by quantitative morphological examinations and by biochemical assays (118). The technique takes advantage of the effect of the injection of Triton WR-1339 for removal of the lysosomes by flotation (see Fig. 9). Enzyme assays have revealed a high degree of biochemical homogeneity in peroxisomes (22, 118). Some start has been made in the biochemical dissection of these particles. Their membrane has not yet been isolated; but the dense crystalloid core has been separated from the soluble components of the matrix and shown to contain urate oxidase (119, 175). The other known peroxisomal enzymes are part of the soluble matrix (119).

Other Components. Except for small amounts of microsomal and other membrane material, no other component has been recognized in mitochondrial fractions from rat liver. In particular, no trace has been found of Claude's hypothetical secretory granules. The situation is, of course, different in glandular cells containing typical secretory granules. These sediment either with the mitochondria or before them, and have been purified from a number of different tissues.

THE MICROSOMAL FRACTION

Unlike the other words of our jargon, the term "microsome" is operational and refers simply to a subcellular fraction isolated by high-speed centrifugation from a "post-mitochondrial supernatant." In practice, it is often used as a synonym of "fragmented endoplasmic reticulum," but this is a dangerous oversimplification, as shown by the variety of distribution patterns that can be recognized after subfractionation of microsomes by density equilibration in a sucrose gradient (Fig. 11).

Endoplasmic Reticulum. That microsomes originate mainly from the endoplasmic reticulum was suspected already by Claude (55), and firmly established by Palade and Siekevitz (134). As seen in tissue sections, the endoplasmic reticulum is made up of smooth-surfaced parts and of rough-surfaced parts, linked together by transitional elements. It is generally accepted that there is considerable continuity throughout the whole system, and that the microsomal vesicles form by a pinching-off process in the course of homogenization. According to this concept, the microsome fraction should be expected to consist largely of a mixture of rough-surfaced and smooth-surfaced vesicles, with some hybrid vesicles originating from the transitional elements. This view, in turn, has prompted a number of investigators to develop methods for the separation of rough and smooth microsomes (see reference 63) and for the further subfractionation of the two fractions (61, 62, 64, 91).

On the whole, the subfractionation of microsomes has proved rather disappointing

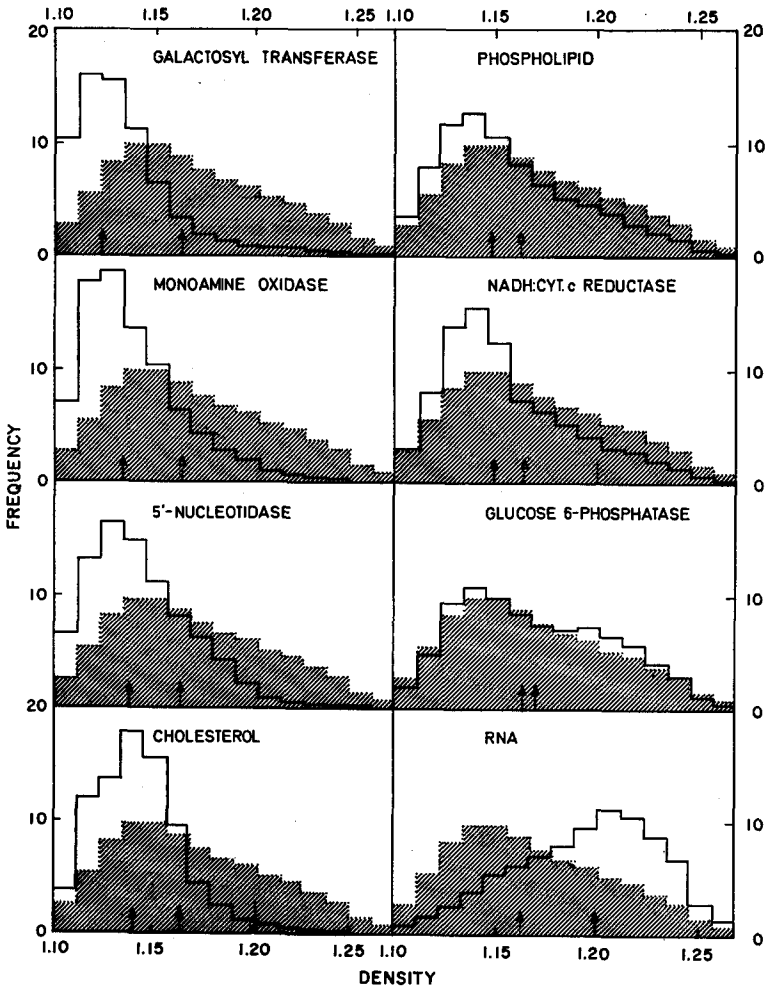


FIGURE 11 Subfractionation of microsomal fraction by isopycnic centrifugation through sucrose gradient containing 3 mM imidazole, pH 7.4. Centrifugation for 3 hr at 35,000 rpm in Beaufay rotor. Graphs show density distributions of cholesterol, phospholipids, RNA, and marker enzymes for different cytological components of microsomal fraction. Shaded area repeated on each graph is density distribution of protein. Arrows show median densities. Results of Amar-Costesec et al. (4) and unpublished results of M. Wibo.

to those who were hoping to find evidence of functional differentiation within the endoplasmic reticulum. Every one of the membrane-bound enzymes found in the rough fraction has turned out to be present also in the smooth fraction, often in comparable concentration. The converse was not true, but, as will be shown below, this is because the smooth fraction contains other components besides smooth endoplasmic reticulum vesicles. There are conflicting reports concerning the ability of the two fractions to metabolize various drugs. Some workers have reported considerable differences (see reference 89), but their results are not easily evaluated in view of the complex nature of the reactions involved and of the inducible nature of the enzymes.

The problem of the heterogeneity of the microsomal fraction has been approached somewhat differently in our laboratory (4, 168, 179). Microsomal fractions were iso-

lated with as high a yield as possible, in contrast with the general practice that accepts fairly large losses for the sake of expediency and purity, and they were then subdivided into some 15 subfractions by various forms of density-gradient centrifugation. The experiments were done in an essentially analytical fashion, and the fractions were subjected to a variety of biochemical assays and quantitative morphological measurements. Considerable advantage was derived in these experiments from the use of the Beaufay (18) rotor, which brings the particles very near their equilibrium position in a sucrose gradient in only 3 hr of centrifugation. Representative results, showing only one typical enzyme per group, are presented in Fig. 11.

One of the most important findings made in these experiments relates to the distribution of RNA. As shown in Fig. 11, it overlaps considerably that of protein, and seems, at first sight, to suggest a very poor separation between rough and smooth microsomes. Quantitative counts of the ribosomes showed that the RNA was essentially ribosomal, even in the fractions of low density (Fig. 12). Furthermore, it was found, contrary to what might have been expected on the basis of current ideas, that the increase in ribosome content with increasing density of the subfractions does not reflect an increase in the ratio of the number of rough to that of smooth vesicles, but is due to an increase of the average number of ribosomes per vesicle throughout the population (see Figs. 12 and 13). In other words, these results demonstrate that there is no clear-cut distinction between smooth and rough microsomes. Rather is there a continuous

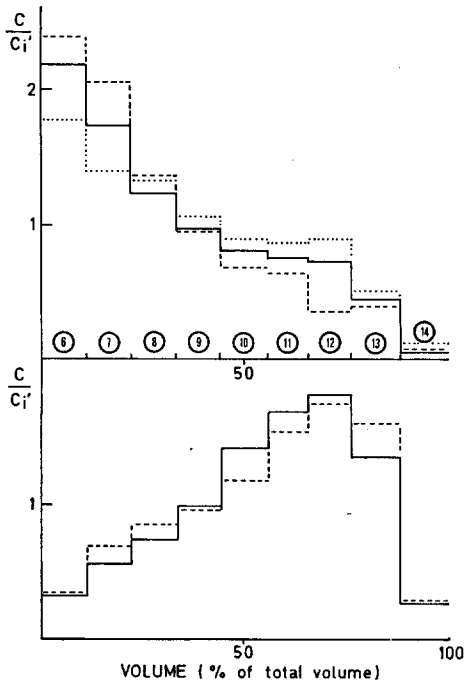


FIGURE 12 Correlation between biochemical and morphometric determinations on microsomal subfractions separated as in Fig. 11. Average densities of subfractions were: 6: 1.130; 7: 1.146; 8: 1.165; 9: 1.175; 10: 1.188; 11: 1.204; 12: 1.219; 13: 1.238; 14: 1.258. Appearance of fractions 6, 9, 11, and 13 is illustrated in Fig. 13. Upper graph: normalized distributions of membrane surface area (solid line), phospholipids (broken line), and nonribosomal proteins (dotted line). Lower graph: normalized distributions of ribosomes (solid line) and RNA (broken line). Results of Wibo et al. (179).

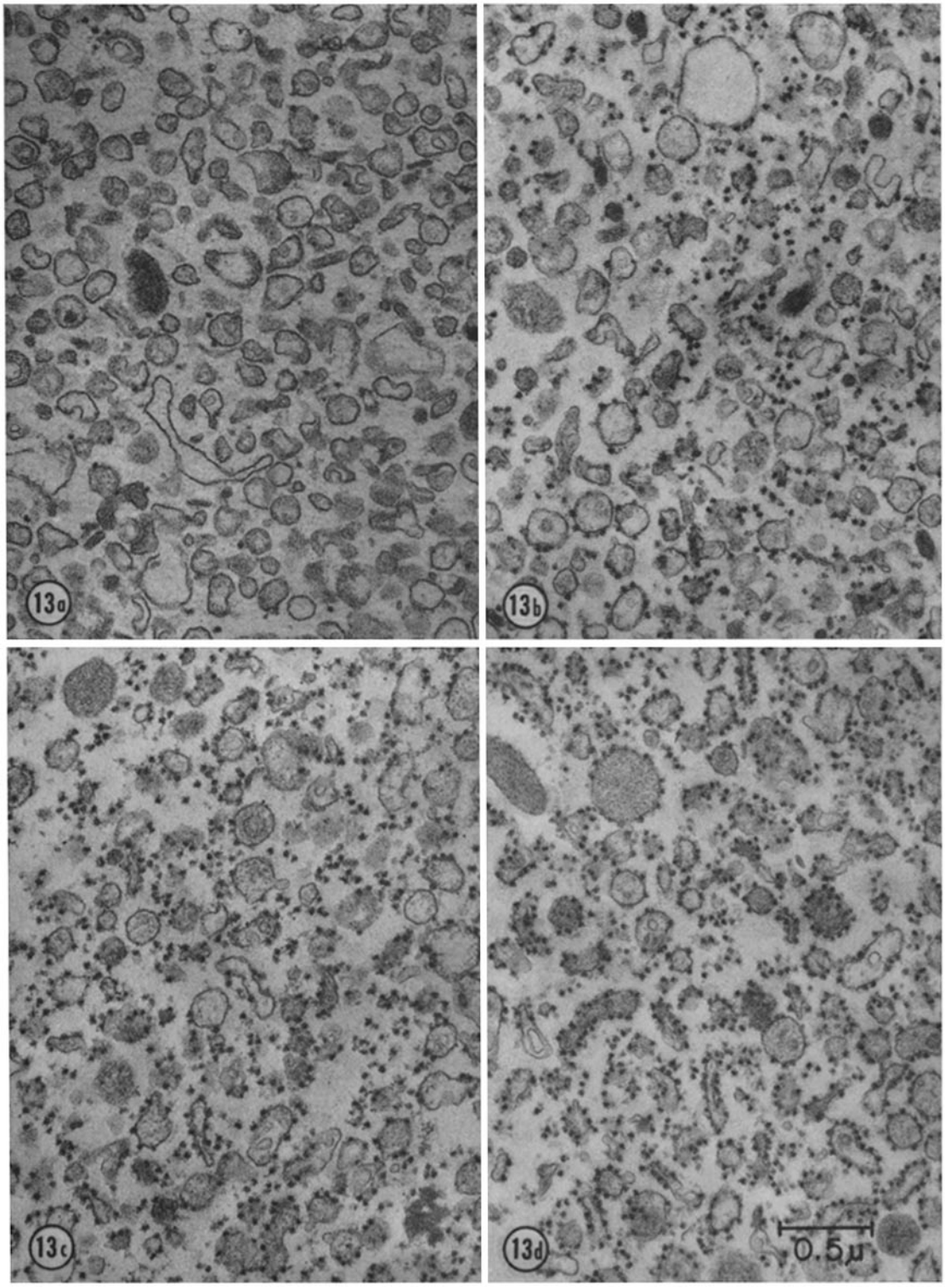


FIGURE 13 Appearance of four microsomal subfractions separated according to Fig. 11 and described biochemically in Fig. 12. *a*, subfraction 6, density 1.130; *b*, subfraction 9, density 1.175; *c*, subfraction 11, density 1.204; *d*, subfraction 13, density 1.238. Note that average number of ribosomes per vesicle increases with increasing density. × 29,000.

spectrum of vesicles ranging from those that are ribosome-free to those that are fully charged with ribosomes. A detailed examination of tissue sections would probably corroborate this view, showing great variability in the frequency of ribosomes along the membranes of the endoplasmic reticulum.

Considered in the light of the above data, the finding that the smooth and rough subfractions differ little in their content in many enzymes simply suggests that little biochemical change in the membranes may accompany the attachment or detachment of ribosomes. However, some subtle differences are encountered. In the first place, as shown in Fig. 12, the phospholipid-to-protein ratio of the membranes decreases with increasing density. This trend is real, but it could be due to a decreasing contamination of the subfractions by non–endoplasmic reticulum membranes having a higher phospholipid content. At the enzyme level, there is a small but distinct difference between the distribution pattern of glucose 6-phosphatase, which follows closely that of the proteins, and that of nicotinamide adenine dinucleotide:cytochrome *c* reductase, which parallels that of the phospholipids. Actually these two patterns define two groups of enzymes. Accompanying glucose 6-phosphatase are nucleoside diphosphatase, esterase, and glucuronyl transferase, whereas the group with nicotinamide adenine dinucleotide:cytochrome *c* reductase includes essentially other oxidizing systems, for instance nicotinamide adenine dinucleotide phosphate:cytochrome *c* reductase, cytochrome b_5, cytochrome P-450, and aminopyrine demethylase. These two groups could possibly define distinct parts of the endoplasmic reticulum, or distinct "patches" present throughout the system, or a structurally more random form of heterogeneity.

Plasma Membranes. As already mentioned, 5′-nucleotidase and other plasma membrane markers have a nucleo-microsomal distribution, with about half of the total activity of the tissue sedimenting with the microsomal fraction. Upon subfractionation of the microsomes by isopycnic centrifugation, these enzymes tend to be concentrated in the subfractions of low density, showing a distribution pattern very different from those of enzymes associated with the endoplasmic reticulum (see Fig. 11). When microsomes are exposed to a low concentration of digitonin, washed, and subfractionated as in Fig. 11, the plasma membrane markers are selectively shifted towards a region of higher density (Fig. 14), whereas the true endoplasmic reticulum enzymes are not (168). Purified plasma membranes behave in the same way (Fig. 7). Finally, as also found by Glaumann and Dallner (91), the plasma membrane markers sediment more rapidly than the main microsomal enzymes, upon differential sedimentation. Recently, practically identical preparations of plasma membranes have been separated by Touster et al. (172) from nuclear and from microsomal fractions.

It seems quite clear therefore, and most authors now agree with this view, that the plasma membrane markers of the microsomal fraction are associated with true plasma membrane fragments, which, on the basis of their centrifugal behavior, appear to be less dense but larger than the average endoplasmic reticulum vesicles. Furthermore, it is easily calculated from the specific activity of the enzymes in purified plasma membranes that these fragments could make up as much as 10% of the total microsomal protein.

The distribution of microsomal cholesterol resembles very much that of 5′-nucleotidase (4) and is likewise shifted by digitonin treatment (168). This has been taken to indicate that most, if not all, of the microsomal cholesterol is associated with the plasma membrane component, or at least that there is little if any cholesterol in the endoplasmic reticulum proper. This view is confirmed by the total lack of digitonin

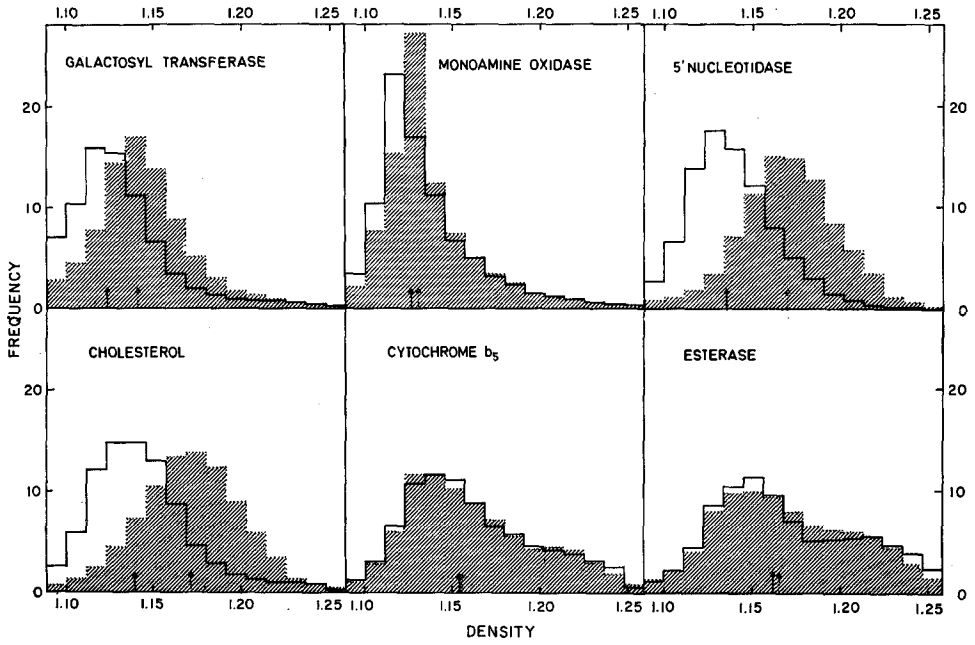


FIGURE 14 Digitonin shift of components of microsomal fraction. Subfractionation as in Fig. 11, before (solid line) and after (shaded area) exposure to digitonin as in experiment of Fig. 7. Graphs represent distributions, arrows indicate median densities. Note large shift of 5′-nucleotidase, slightly smaller for cholesterol, distinctly smaller but significant for galactosyl transferase, negligible for other enzymes. Results of Thinès-Sempoux et al. (168) and unpublished results of M. Wibo.

shift of true endoplasmic reticulum markers, since the increase in density induced by digitonin is probably due to binding of the digitonin by membrane cholesterol.

Glaumann and Dallner (91) have taken issue with this conclusion, arguing that the cholesterol to 5′-nucleotidase ratio is much higher in microsomes than in purified plasma membranes, indicating that a considerable fraction of the microsomal cholesterol must be associated with nonplasma membrane material. As will be shown, Golgi membranes probably account for part of this excess cholesterol but certainly not for all of it. Thus the objection would appear to be a valid one. It is, however, disturbing that the cholesterol-to-5′-nucleotidase ratio is not very different in the nuclear and in the microsomal fraction. Average values of this ratio, as determined in the Louvain laboratory, are 1.4 times higher in the microsomal than in the nuclear fraction. Therefore, either a large part of the cholesterol content of the nuclear fraction belongs to a component different from plasma membranes, or considerable losses in cholesterol accompany the purification of plasma membranes by the method used by Glaumann and Dallner (91). There is a distinct possibility that the second explanation may be the right one. Both Coleman et al. (57) and Touster et al. (172) have purified plasma membranes by methods that do not, like that of Neville (130) and its modifications, involve perfusion of the liver and homogenization in hypotonic bicarbonate. Their preparations, which show a particularly high degree of purity according to the specific activity of 5′-nucleotidase, are 2.5 times richer in phospholipid and 2.5–3.0 times richer in cholesterol, on a protein basis, than that of Glaumann and Dallner (91), which, on the other hand, compares favorably in terms of lipid content with

those isolated in other laboratories, including our own, where some variant of the procedure of Neville is used (25). Unfortunately, neither Coleman et al. (57) nor Touster et al. (172) give the total cholesterol content of the liver of their animals. But it can be calculated from their results, assuming biochemical homogeneity of the plasma membrane, that this structure should contain close to 2 mg of cholesterol and 6 mg of phospholipids, per gram of fresh liver. This corresponds to 72% of the cholesterol content and 15% of the phospholipid content of the livers of our animals.

Golgi Membranes. Purification of Golgi membranes from liver has been accomplished more or less simultaneously by Fleischer et al. (88) and by Morré et al. (43, 126). An alternative procedure has been described recently by Ehrenreich et al. (85). Particularly impressive are the images obtained by negative staining, which clearly show the flattened cisternae of the Golgi apparatus, partly filled with lipoprotein particles and surrounded by a corona of intertwined tubules, confirming the structure so beautifully reconstructed from ultra-thin sections in a recent paper by Claude (52).

Of major importance has been the discovery that *N*-acetylglucosamine:β-galactosyl transferase is specifically associated with the Golgi membranes (88). The enzyme is purified 40- to 50-fold in purified Golgi fractions (85, 88) and 110-fold in the membranes separated therefrom (85). It would appear on this basis that Golgi membranes account for a little less than 1% of the total cell protein.

In Louvain, Maurice Wibo has measured galactosyl transferase with ovalbumin as acceptor. The enzyme is recovered largely with the microsome fraction. After subfractionation of the microsomes, it shows a distribution similar to those of 5′-nucleotidase and monoamine oxidase (Fig. 11). However, in microsomes pretreated with digitonin, the three distributions separate, with galactosyl transferase occupying an intermediate position (Fig. 14). We tend to conclude from this behavior that the three enzymes are attached to three distinct groups of membranes, having about the same density distribution in sucrose but differing in their cholesterol content. The possibility that Golgi membranes may contain significant amounts of cholesterol but less than plasma membranes is interesting in view of the functions attributed to the Golgi system in secretion.

Mitochondrial Outer Membranes. As shown in Fig. 11, the microsomal monoamine oxidase–activity bands in the region of low density, together with the plasma membrane and Golgi markers. However, dissociations between monoamine oxidase and 5′-nucleotidase have been produced in other systems (4), and by digitonin treatment (168). As shown in Fig. 14, this treatment also differentiates between monoamine oxidase and galactosyl transferase. Presumably, therefore, monoamine oxidase belongs to yet another component of the microsomal fraction.

This component could be related in some way to the outer membrane of mitochondria. It has been mentioned above that a significant fraction of the mitochondria have their outer membrane torn off in the course of homogenization (59), and it seems probable that these membranes would be recovered mostly with the microsomal fraction. It is, however, doubtful that the total amount of microsomal monoamine oxidase, which is of the order of 20% of the total activity of the liver, can be accounted for in this manner.

Ribosomes. These particles were first separated from the microsomal membranes by

the rather drastic procedure of dissolving the latter in deoxycholate (120, 160). Gentler methods have since been devised (31, 145, 146). The function of these membrane-bound ribosomes in the synthesis of secretory proteins has been thoroughly documented.

Particulate Glycogen. When the livers of fed rats are fractionated, much of the glycogen collects in the form of a transparent pellet at the bottom of the microsomal sediment. Part of it also contaminates the mitochondrial fraction. This particulate glycogen was first isolated by Lazarow (115) and by Claude (50). It has been investigated in detail by Drochmans (84). As a rule, workers prefer to use fasted animals to avoid the complications introduced in the fractionation by the presence of glycogen.

Other Components. Microsomal fractions usually contain a few small mitochondria and mitochondrial debris, and roughly 20% of the total lysosomes and peroxisomes, together with the cores released from injured peroxisomes. In experiments of the type illustrated in Fig. 11, marker enzymes for these various particles band more or less like their counterpart in the mitochondrial fraction. So far no clear evidence of the presence of a precursor of larger cytoplasmic particles or of a link between them and the endoplasmic reticulum has been obtained. Such a link has been postulated on morphological grounds, at least for lysosomes and peroxisomes, but its identification may require the association of specific isotopic labeling with refined fractionation techniques.

Some enzymes occuring predominantly in the soluble fraction are found in variable amounts in the microsomal fraction. Examples are fumarase, aldolase, and glutamine synthetase. Upon subfractionation of the microsomes, the distribution of these enzymes tends to follow that of RNA (4). For this and other reasons, they are believed to be adsorbed to ribosomes. Whether this attachment is of any physiological significance cannot be decided at the present time.

THE SUPERNATANT FRACTION

Except for small amounts of particulate material that have failed to sediment with the microsomes or have been detached from the pellet upon decantation, the supernatant fraction contains essentially the components of the cell sap, minus what has been lost through adsorption or entrainment, plus what has been released from damaged particles. It also contains blood plasma if the tissue has not been perfused. Subfractionation of this fraction would seem to be essentially the concern of biochemistry, except for the fact that there is still a considerable gap between the minimum sedimentation coefficient of the objects recovered completely in the microsome fraction (190S in 0.25 M sucrose at 0°C (11), or about 400S in water at 20°C) and the range of sedimentation coefficients of individual macromolecules. There is room, for one thing, for free ribosomes and polysomes, and these have indeed been separated from the supernatant fraction (30, 32). There is also considerable room for multienzyme particles comparable, for instance, to the fatty acid–oxidizing particle isolated from yeast by Lynen (121).

In the case of liver, the evidence available so far indicates that if multienzyme aggregates are present in the cells, they either are not numerous or dissociate in the homogenate. It has been shown by Anderson (7, 10) that the gap of sedimentation coefficients alluded to above is a true void and that virtually no protein sediments in this range. Various attempts in our laboratory to detect any indication of the existence of the often postulated glycolytic particle have been entirely negative (69). As shown in

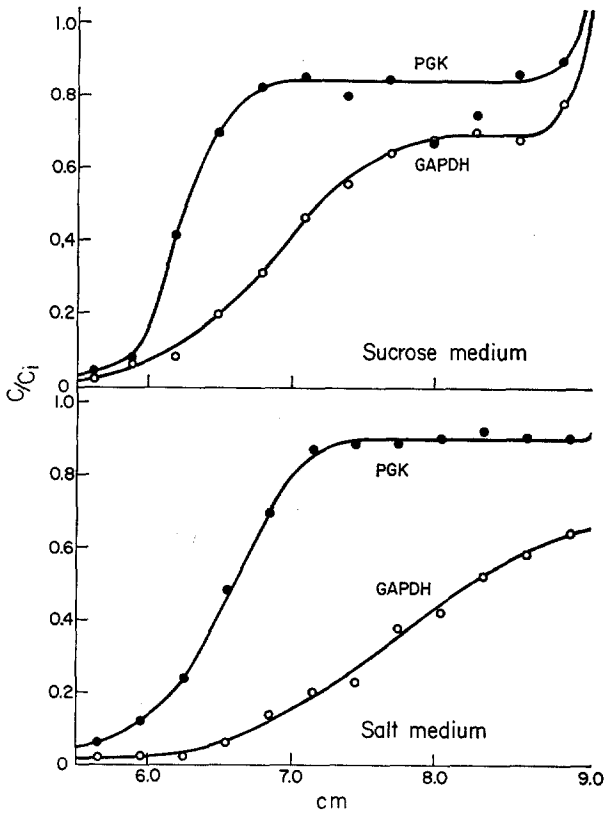


FIGURE 15 Sedimentation behavior of phosphoglycerate kinase (*PGK*) and glyceraldehyde phosphate dehydrogenase (*GAPDH*). Samples of the same rat liver were homogenized in either 0.25 M sucrose or in a modified Krebs-Henseleit solution, freed of particulate components by centrifugation for 1 hr at 40,000 rpm, and then centrifuged for 13.5 hr at 39,000 rpm. Graphs show, as a function of radial distance, ratio of enzyme concentration C to average enzyme concentration C_i. The $s_{20,w}$ values derived from these results were 3.15S and 3.28S for PGK, 5.85S and 6.30S for GAPDH, in sucrose and salt medium respectively, as against 3.20S for crystalline yeast PGK, and 7.0S–7.7S for crystalline rabbit muscle GAPDH. Data from de Duve (69).

Fig. 15, glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase, two enzymes which would be particularly likely to be linked in a particle of this sort, were found to sediment entirely independently and at a rate showing that they are molecularly dispersed.

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