# ANALYTICAL STUDY OF MICROSOMES AND ISOLATED SUBCELLULAR MEMBRANES FROM RAT LIVER

## III. Subfractionation of the Microsomal Fraction by Isopycnic and Differential Centrifugation in Density Gradients

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ABSTRACT

Rat liver microsomal fractions have been equilibrated in various types of linear density gradients. 15 fractions were collected and assayed for 27 constituents. As a result of this analysis microsomal constituents have been classified, in the order of increasing median density, into four groups labeled *a*, *b*, *c*, and *d*. Group *a* includes: monoamine oxidase, galactosyltransferase, 5′-nucleotidase, alkaline phosphodiesterase I, alkaline phosphatase, and cholesterol; group *b*: NADH cytochrome *c* reductase, NADPH cytochrome *c* reductase, aminopyrine demethylase, cytochrome $b_5$, and cytochrome P 450; group *c*: glucose 6-phosphatase, nucleoside diphosphatase, esterase, β-glucuronidase, and glucuronyltransferase; group *d*: RNA, membrane-bound ribosomes, and some enzymes probably adsorbed on ribosomes: fumarase, aldolase, and glutamine synthetase. Analysis of the microsomal fraction by differential centrifugation in density gradient has further dissociated group *a* into constituents which sediment more slowly (monoamine oxidase and galactosyltransferase) than those of groups *b* and *c*, and 5′-nucleotidase, alkaline phosphodiesterase I, alkaline phosphatase, and the bulk of cholesterol which sediment more rapidly (group *a2*). The microsomal monoamine oxidase is attributed, at least partially, to detached fragments of external mitochondrial membrane. Galactosyltransferase belongs to the Golgi complex. Group *a2* constituents are related to plasma membranes. Constituents of groups *b* and *c* and RNA belong to microsomal vesicles derived from the endoplasmic reticulum. These latter exhibit a noticeable biochemical heterogeneity and represent at the most 80% of microsomal protein, the rest being accounted for by particles bearing the constituents of groups *a* and some contaminating mitochondria, lysosomes, and peroxisomes. Attention is called to the operational meaning of microsomal subfractions and to their cytological complexity.

## INTRODUCTION

During the last two decades rat liver microsomes have been the matter of extensive subfractionation studies, performed in various laboratories, which aimed to establish the cellular localization of the numerous enzymes present in that complex subcellular fraction. The main contributions in this field are reviewed in the first paper of this series (8), which also presents the analytical manner by which we have approached this problem.

Analytical fractionation of rat liver microsomal fractions by isopycnic centrifugation in aqueous sucrose gradients was attempted in this laboratory following the resolution of mitochondrial fractions into three distinct populations of particles by the same approach (9, 10). The results of preliminary experiments were briefly alluded to (14, 19), but never published in detail because of some misgivings over the validity of the techniques used. It was feared that the particles might undergo significant damage in the course of the long period of centrifugation (18 h) at 39,000 rpm that had been considered necessary for near-equilibrium to be reached. Among other factors, these reservations provided an important incentive for the construction of an improved rotor (6) in which the centrifugation time could be reduced greatly.

The present paper provides a comprehensive account of all the results obtained on freshly isolated, untreated, microsomal fractions from rat liver. Analytical techniques (8) and the preparation and the composition of the microsomal fractions (4) have been described in previous papers of this series. Subsequent ones will report on the effects of various treatments of the microsomes (5). Some of the findings made in these experiments have since been reported at a symposium (2), as part of other publications (1, 7, 18, 39, 40, 44), and in abstract form (3).

## MATERIALS AND METHODS

### *Preparation of Microsomal Fractions*

Isopycnic centrifugation in sucrose-$H_2O$ gradients was carried out on P fractions separated exactly as described before (4) by centrifugation of a postmitochondrial supernate by an integrated force of $W = 3 \times 10^{10}$ rad$^2$ s$^{-1}$. The pellets were washed once; the suspension medium was 0.25 M sucrose buffered by 3 mM imidazole-HCl, pH 7.4. A threefold higher value of $W$ was adopted for the preparation of the fractions subjected to analysis by differential centrifugation, to yield P′ fractions (4) including even the smaller members of the particle populations. The same conditions were used for separating a postmicrosomal fraction from the postmicrosomal supernate.

When special gradients were used, washing and final resuspension were performed in the appropriate medium: buffered sucrose in $D_2O$, isoosmolal with 0.25 M sucrose in $H_2O$ (8.1 g sucrose/100 g $D_2O$) at 5°C for the sucrose-$D_2O$ gradients; buffered 0.5 M sucrose (19.2 g/100 g $H_2O$) for the Ficoll-0.5 M sucrose gradients. In the latter case, $W$ was increased by 40% to compensate for the greater viscosity of the suspending medium.

Some experiments were carried out with postmitochondrial supernates quickly prepared from complete homogenates (0.25 g liver per ml buffered 0.25 M sucrose) by a single centrifugation at 15,000 rpm ($W = 1.23$ or $0.81 \times 10^9$ rad$^2$ s$^{-1}$).

### *Subfractionation by Isopycnic Density Gradient Centrifugation*

Density equilibration was performed in the automatic rotor designed by Beaufay (6) to fulfill the particular requirements of centrifugation systems in which equilibrium is to be approached. This rotor will be subsequently referred to as E-40. Its main characteristics, mode of operation, and advantages have been described by Leighton et al. (26). An additional important quality of this rotor has been brought to light recently by the work of Wattiaux et al. (42, 43), showing that the hydrostatic pressure generated by the centrifugal force may cause extensive alterations of mitochondria and other subcellular particles in the course of density equilibration experiments. Because of the shortness of the radial distance over which the gradient extends, the hydrostatic pressure developed at a given position in the gradient is much lower in E-40 rotor than in swinging-bucket and in zonal rotors spinning at the same speed. As an example, at 39,000 rpm the pressure would attain 630, 380, and 98 kg/cm$^2$ at the periphery of the SW-39, zonal Ti-14 (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), and E-40 rotors, respectively, loaded with water.

For loading, we inject successively: (*a*) 10 ml of a P fraction containing the particles from 8 to 10 g of liver; (*b*) a 32-ml gradient, linear with respect to volume; (*c*) a 6-ml cushion. With these conditions, the microsome layer extends from 5.30 to 5.55 cm, and the gradient from 5.55 to 6.26 cm from the axis. The speed of the rotor was 5,000 rpm during injection of the microsomes, and was then raised to 8,000 rpm for the remainder of the filling procedure. The

TABLE I

*Composition of Solutions used in Density Gradient Centrifugation*

| Gradient | Solvent | Solution to which the varied solute is added | Solution | Varied solute | g per 100 g solvent | Density |
|---|---|---|---|---|---|---|
| *Density equilibration* | | | | | | |
| Sucrose-$H_2O$ | $H_2O$ | 3 mM imidazole-HCl buffer, pH 7.4 | Light | Sucrose | 30.0 | 1.100 |
| | | | Heavy | Sucrose | 109.0 | 1.250 |
| | | | Cushion | Sucrose | 206.0 | 1.340 |
| Sucrose-$D_2O$ | $D_2O$* | 3 mM imidazole-HCl buffer, pH 7.4 | Light | Sucrose | 17.8 | 1.163 |
| | | | Heavy | Sucrose | 113.0 | 1.330 |
| | | | Cushion | Sucrose | 185.0 | 1.380 |
| Ficoll-sucrose 0.50 M | $H_2O$ | 3 mM imidazole-HCl buffer, pH 7.4, supplemented with 19.2 g sucrose per 100 g solvent | Light | Ficoll | 13.2 | 1.100 |
| | | | Heavy | Ficoll | 51.1 | 1.171 |
| | | | Cushion | Ficoll | 79.5 | 1.214 |
| Ficoll-sucrose 0.25 M | $H_2O$ | 3 mM imidazole-HCl buffer, pH 7.4, supplemented with 9 g sucrose per 100 g solvent | Light | Ficoll | 12.1 | 1.070 |
| | | | Heavy | Ficoll | 53.7 | 1.160 |
| | | | Cushion | Ficoll | 72.8 | 1.193 |
| *Differential sedimentation* | | | | | | |
| Sucrose-$H_2O$ | $H_2O$ | 3 mM imidazole-HCl buffer, pH 7.4 | Light‡ | Sucrose | 9.0 | 1.034 |
| | | | Heavy‡ | Sucrose | 19.2 | 1.068 |
| | | | Cushion | | | |
| | | | Light | Sucrose | 45.9 | 1.140 |
| | | | Heavy | Sucrose | 178.1 | 1.320 |

* When $D_2O$ is used as solvent temperature is kept near 5°C.

‡ These solutions contain microsomes at a final concentration of 90 mg of tissue per ml.

gradients were made as described by Leighton et al. (26) from two solutions the composition of which is given in Table I.

Unless otherwise stated, centrifugation was carried out for 3 h at 35,000 rpm, conditions which sufficed to bring the particles close to their equilibrium position, thanks to the high centrifugal force prevailing at the starting layer and to the short distance to be traveled. At the end of the run, some 15 fractions were delivered automatically into tubes kept at 0°–5°C, while the rotor decelerated slowly from 9,000 to 6,000 rpm. Essentially quantitative recovery of the rotor content was achieved in this operation. The tubes containing the microsomal subfractions were weighed and their contents thoroughly mixed. The fraction densities were then determined at 2°C (5°C when containing $D_2O$) by equilibration of a droplet in a gradient of *o*-dichlorobenzene and light petroleum ether (bp 80°–100°C), calibrated with KBr solutions of known densities (10).

As shown by Fig. 1, the shape of the density gradient was slightly altered at the end of centrifugation, due to diffusion which exerts pronounced effects when the concentration gradient is steep with respect to distance. This increased rate of diffusion, a consequence of the small radial height of the centrifugation cell, represents the price paid in the E-40 rotor for shortening equilibration time and decreasing the hydrostatic pressure.

## *Subfractionation by Density Gradient Differential Sedimentation*

In order to avoid the complications and the limitations of zonal sedimentation, we adopted the boundary technique (20). A special rotor S-25 was constructed similar to E-40 rotor and operated in the same way, but with a geometry better adapted to the requirements of analyses by differential sedimentation. The cell is ring shaped, as in the E-40 rotor, and has strictly radially oriented sidewalls, which is not true of commercial zonal rotors; it has the capacity of 135 ml and its radial dimensions extend from 4.71 to 8.10 cm from the axis.

The rotor was filled while running at 3,000 rpm, so as to produce the following initial conditions: (*a*) between radial distances 4.87 and 7.56 cm, 110 m. of a sucrose gradient extending linearly with re-

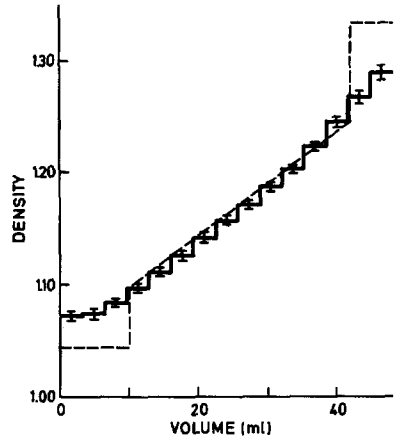


FIGURE 1 Shape of final density gradient after centrifuging 3 h in E-40 rotor. The thin dashed line indicates the density-volume relationship of the solutions injected in the rotor, from left to right: 10 ml of microsomes in 0.25 M sucrose (density = 1.044), 32 ml of linear sucrose gradient extending from 1.10 to 1.25, and 6 ml of cushion (density = 1.34). The thick staircase-like line gives the average densities of fractions ± standard deviation, obtained from 13 experiments. To average these experiments each density profile has been standardized by constructing 15 virtual fractions of identical volume = 3.2 ml. The density of a given reconstructed fraction was taken to be the mean ordinate (density) computed from the actual density profile within the limits of the reconstructed fraction on the abscissa.

spect to volume from 0.25 to 0.50 M, containing homogeneously dispersed microsomes from 10 g of liver; (*b*) between 7.56 and 8.10 cm a 10-ml linear sucrose gradient from density 1.14 to 1.32, supported by a 10-ml cushion of density 1.32. The microsome suspensions used for preparation of the microsome containing sucrose gradient were made up by mixing 1 vol of a P′ suspension (microsomes from 1 g of liver per ml of buffered 0.25 M sucrose) to 10 vol of buffered 0.25 and 0.525 M sucrose, respectively.

Centrifugation was carried out at 20,000 rpm under continuous recording of the speed to allow calculation of $W$, the time integral of the squared angular velocity. Between 15 and 20 fractions were recovered from the rotor decelerating between 8,000 and 5,000 rpm, and processed as in isopycnic equilibration experiments.

### *Analytical Methods*

All biochemical assays were performed as described in the first paper of this series (8). In some experiments, the fractions were also submitted to quantitative morphological analysis, as reported by Wibo et al. (44).

### *Presentation of Results*

All results are presented in the form of normalized histograms constructed (17) and averaged (11) as described previously, with the improvements introduced by Leighton et al. (26). As mentioned before, averaging causes some loss of resolution. This is inevitable since sampling between strictly reproducible boundaries of density or radial distance is impossible. The actual recoveries are given separately.

## RESULTS

### *Density Equilibration Experiments*

The results recorded in all experiments in which microsomal fractions were centrifuged for 3 h through a sucrose-$H_2O$ gradient are summarized in Table II by the averaged median density of each constituent. In Fig. 2 are shown the averaged density distribution patterns of chemical constituents and of four representative enzymes.

Phospholipids and proteins show similar highly skewed distribution patterns, with modes around density values of 1.14–1.15, but extending beyond 1.25. The two distributions are slightly shifted with respect to each other, so that the protein-to-phospholipid ratio increases progressively with increasing density. In contrast, the distribution of cholesterol is almost perfectly symmetrical; median and mode coincide, at about 1.14, and the distribution does not appreciably extend beyond a density value of 1.20. RNA peaks at a much higher density value, on the order of 1.21; its distribution pattern is highly skewed on the low density side, covering the whole range of the gradient from 1.10 to 1.27.

The enzyme distributions shown in Fig. 2 are typical of the four groups that have been distinguished previously (2, 3). The difference between the four behaviors is easily seen by reference to the 1.20 demarcation line: in order of increasing median density, 5′-nucleotidase (group *a*), NADH cytochrome *c* reductase (group *b*), glucose 6-phosphatase (group *c*), and fumarase (group *d*).

Characteristic of group *a* is a distribution pattern resembling that of cholesterol, symmetrical or only moderately asymmetrical, fairly narrow, and especially extending to only a very small extent beyond the limit of 1.20. This group includes, in addition to 5′-nucleotidase, galactosyltransferase, monoamine oxidase, alkaline phosphodiesterase I, and alkaline phosphatase. As il-

TABLE II

*Median Density and Recovery of Microsomal Constituents Equilibrated in Sucrose-$H_2O$ Gradients*

| Constituent | Percent in P* | No. of exps. | Median density‡ | Percent recovery‡ |
|---|---|---|---|---|
| Protein | 18.8 | 15 | 1.1637 ± 0.0060 | 102.7 ± 13.7 |
| Phospholipid | 49.1 | 9 | 1.1496 ± 0.0050 | 99.4 ± 5.2 |
| Galactosyltransferase | 80.2 | 2 | 1.1269 ± 0.0054 | 83.2 ± 0.1 |
| Monoamine oxidase | 21.1 | 14 | 1.1332 ± 0.0050 | 96.8 ± 12.8 |
| 5′-Nucleotidase | 46.3 | 13 | 1.1372 ± 0.0048 | 102.6 + 15.8 |
| Cholesterol | 58.0 | 7 | 1.1413 ± 0.0032 | 90.4 ± 21.2 |
| Alkaline phosphodiesterase I | 50.3 | 6 | 1.1445 ± 0.0051 | 102.9 ± 9.6 |
| Alkaline phosphatase | 34.9 | 5 | 1.1435 ± 0.0045 | 104.5 ± 9.3 |
| NADH cytochrome *c* reductase | 58.9 | 11 | 1.1488 ± 0.0058 | 98.4 ± 16.4 |
| Cytochrome $b_5$ | | 5 | 1.1496 ± 0.0041 | 99.1 ± 5.9 |
| Cytochrome P 450 | | 3 | 1.1533 ± 0.0024 | 99.3 ± 10.8 |
| NADPH cytochrome *c* reductase | 65.8 | 4 | 1.1536 ± 0.0021 | 94.4 ± 11.7 |
| Aminopyrine demethylase | 71.2 | 3 | 1.1527 ± 0.0013 | 93.0 ± 10.1 |
| Esterase | 68.6 | 5 | 1.1667 ± 0.0053 | 100.4 ± 11.4 |
| Nucleoside diphosphatase | 73.7 | 3 | 1.1656 ± 0.0035 | 97.0 ± 12.8 |
| Glucose 6-phosphatase | 72.8 | 11 | 1.1678 ± 0.0062 | 100.0 ± 11.3 |
| Glucuronyltransferase | | | | |
| Acceptor:4 methylumbelliferone | 78.1 | 2 | 1.1788 ± 0.0095 | 98.6 ± 9.3 |
| Acceptor:bilirubin | 69.1 | 1 | 1.1706 | 98.8 |
| β-Glucuronidase | 28.0 | 3 | 1.1625 ± 0.0059 | 108.3 ± 4.6 |
| Ribonucleic acid | 53.9 | 8 | 1.2007 ± 0.0071 | 99.3 ± 11.7 |
| Fumarase | 9.6 | 2 | 1.1993 ± 0.0062 | 79.2 ± 34.1 |
| Aldolase | 33.7 | 1 | 1.1833 | 113.0 |
| Glutamine synthetase | 27.7 | 2 | 1.1822 ± 0.0156 | 103.2 ± 13.1 |
| Acid phosphatase | 17.4 | 5 | 1.1569 ± 0.0057 | 95.5 ± 6.2 |
| *N*-Acetyl-β-glucosaminidase | 9.3 | 3 | 1.1873 ± 0.0032 | 123.5 ± 45.9 |
| Cytochrome oxidase | 4.6 | 3 | 1.1699 ± 0.0038 | 91.0 ± 13.0 |
| Catalase | 5.9 | 1 | 1.2199 | 84.5 |

* From Table I of reference 4. Percentages in P relate to the sum of the absolute values found in fractions N, ML, P, and S.

‡ Statistics refer to mean ± standard deviation.

lustrated in Fig. 3, slight differences in the distribution patterns of these various enzymes exist, reflecting the heterogeneity of group *a* (see below).

Group *b* contains a number of microsomal enzymes concerned with electron transport and drug metabolism: NADH cytochrome *c* reductase, NADPH cytochrome *c* reductase, cytochrome $b_5$, cytochrome P 450, and aminopyrine demethylase. Like the enzymes of group *a*, these enzymes have median densities distinctly lower than that of the proteins. However, their median densities are not so low as those of the enzymes of group *a*, about 1.15 as against 1.13–1.14, and their distribution patterns cover the whole density range of the gradient, extending well beyond 1.20. As illustrated in Fig. 4, when all enzymes of the group are measured in the same experiment, they show closely similar distribution patterns, indicating great homogeneity within the group.

Typical of group *c* is an almost bimodal distribution pattern, with a definite peak around 1.14, and a marked shoulder or sometimes even second peak near 1.20. The enzymes in this group have median densities equal to or slightly higher than that of the proteins. They include the characteristic microsomal hydrolases glucose 6-

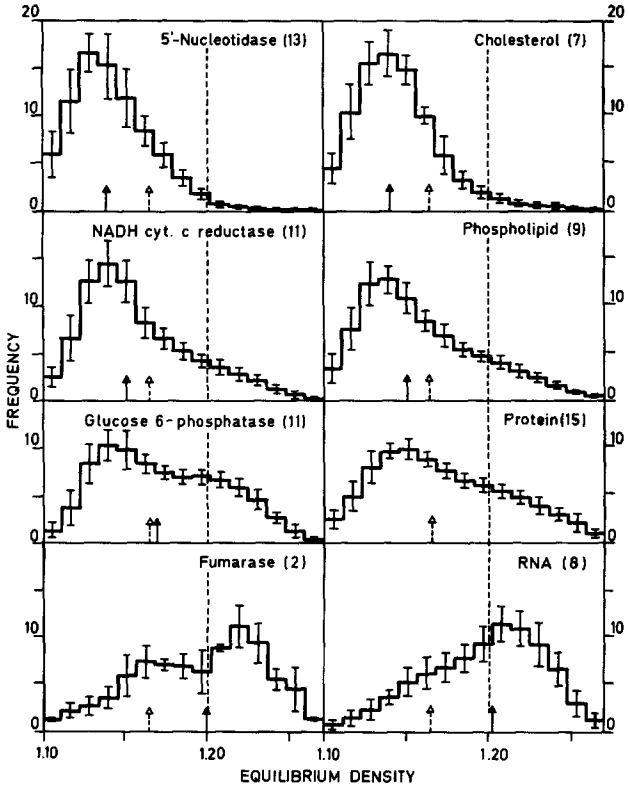


FIGURE 2 Density distribution of some characteristic constituents after isopycnic equilibration of microsomes in sucrose-$H_2O$ gradient. Frequency histograms are normalized and averaged. The represented portion of histograms, divided into 15 normalized fractions of identical density increment, extends from 1.10 to 1.27, and corresponds to more than 95% of constituents. Vertical lines through histogram bars represent standard deviations. Numbers in parentheses refer to number of experiments. Black arrows indicate the median density of constituent; white arrows, repeated on all graphs, indicate the median density of protein. Demarcation line at density 1.20 allows easier comparison of distribution profiles.

phosphatase, esterase, and nucleoside diphosphatase, as well as β-glucuronidase and glucuronyltransferase measured either with bilirubin or with 4-methylumbelliferone as acceptor. As shown in Fig. 5, all these enzymes have closely similar distribution patterns.

In group *d*, characterized by a distribution pattern similar to that of RNA, we find fumarase, and, allowance being made for some soluble activity in the upper part of the gradient, glutamine synthetase and aldolase (Fig. 6).

In Fig. 7 are shown a few distributions that do not fall into one of the four groups defined above. In the case of cytochrome oxidase and of catalase, we clearly see the density distributions of the host particles of these enzymes, mitochondria on one hand and peroxisomes on the other, which occur in the microsomal fraction as minor contaminants. Of the three acid hydrolases that were measured, only β-glucuronidase shows a microsomal type of distribution, characterized above as *c* type. The distributions of acid phosphatase and of *N*-acetyl-β-glucosaminidase differ from all other observed distributions as well as from each other.

In a number of other experiments, microsomal fractions were subfractionated under different conditions, summarized in Table III. Also, the possibility that the procedure used for the isolation of microsomes may cause some artificial injury or rearrangement of constituents was explored in several experiments carried out directly on a post-mitochondrial supernate. In one experiment, the very small microsomal fragments recovered by prolonged high-speed centrifugation from a post-microsomal supernate were subjected to analysis. Despite the numerous changes in experimental

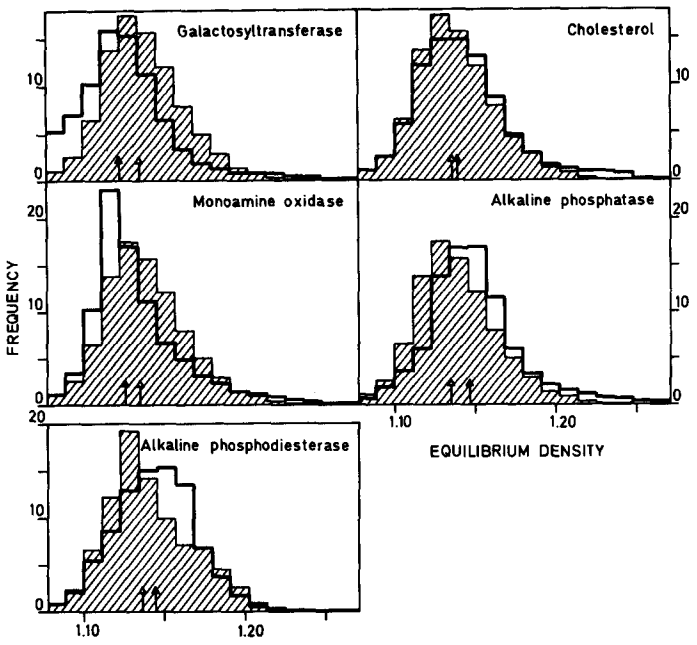


FIGURE 3 Density distribution of constituents of group *a* after isopycnic equilibration of microsomes in sucrose-$H_2O$ gradient. Results of two experiments are presented separately in two distinct graphs. Frequency histograms are normalized. The represented portion of histograms, divided into 17 fractions of identical density increment, extends from 1.0773 to 1.2700 and corresponds to more than 95% of each constituent. The distribution of 5′-nucleotidase is represented by the shaded area superimposed on each profile. Its median density is marked by the white arrow, whereas the median density of other constituents is marked by a black arrow.

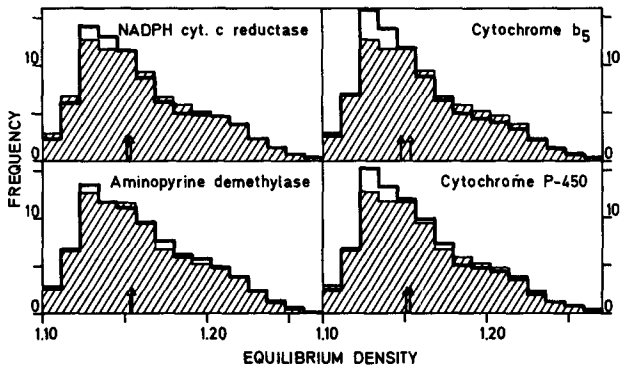


FIGURE 4 Density distribution of constituents of group *b* after isopycnic equilibration of microsomes in sucrose-$H_2O$ gradient. Frequency histograms are normalized. The represented portion of histograms, divided into 15 fractions of identical density increment, extends from 1.10 to 1.27 in density and corresponds to more than 95% of each constituent. The distribution of NADH cytochrome *c* reductase is represented by the shaded area superimposed on each profile. Its median density is marked by the white arrow, whereas the median density of other constituents is marked by a black arrow.

conditions, the main distinctions reported above were invariably observed. In particular, the enzymes of group *b* always showed a similar behavior, and so did those of group *c*, while the displacement of distribution pattern between the two groups remained noticeable. The existence of group *a*, as a separate but heterogeneous group, was also confirmed. For brevity's sake, these results will not be described in detail. Only some median density values are shown in Table III,

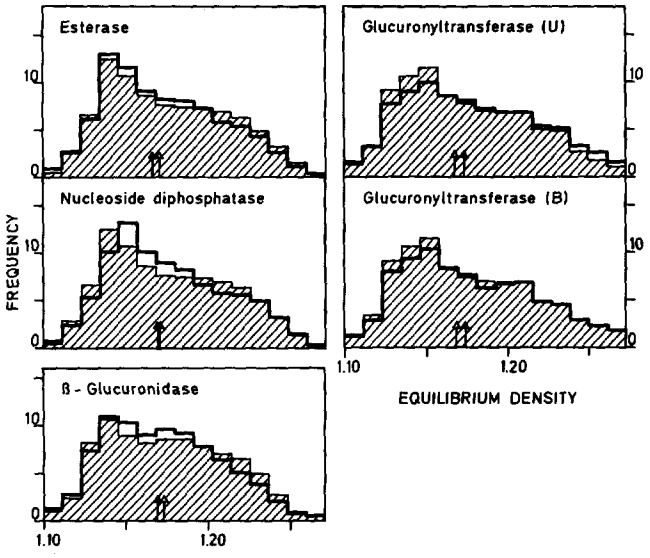


FIGURE 5 Density distribution of constituents of group *c* after isopycnic equilibration of microsomes in sucrose-$H_2O$ gradient. Results of three experiments are presented separately in three distinct graphs. Frequency histograms are normalized. The represented portion of histograms, divided into 15 fractions of identical density increment, extends from 1.10 to 1.27 in density and corresponds to more than 95% of each constituent. The distribution of glucose 6-phosphatase is represented by the shaded area superimposed on each profile. Its median density is marked by the white arrow, whereas the median density of other constituents is marked by a black arrow.

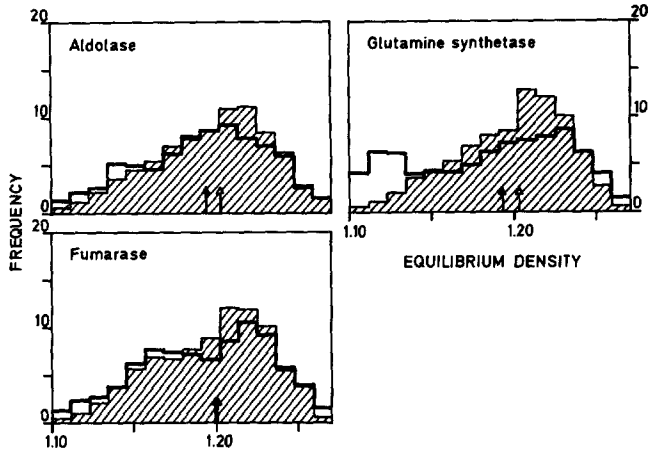


FIGURE 6 Density distribution of constituents of group *d* after isopycnic equilibration of microsomes in sucrose-$H_2O$ gradient. Results of three experiments are presented separately in three distinct graphs. Frequency histograms are normalized. The represented portion of histograms, divided into 15 fractions of identical density increment, extends from 1.10 to 1.27 in density and corresponds to more than 95% of each constituent. The distribution of RNA is represented by the shaded area superimposed on each profile. Its median density is marked by the white arrow, whereas the median density of other constituents is marked by a black arrow.

to illustrate the influence of experimental conditions on the position reached in the gradient by the various constituents.

As shown in Table III, prolonging the centrifugation time from 3 to 10 h increased all median density values by more than 0.01 density unit, suggesting that complete equilibrium was not achieved at the end of 3 h or that some change

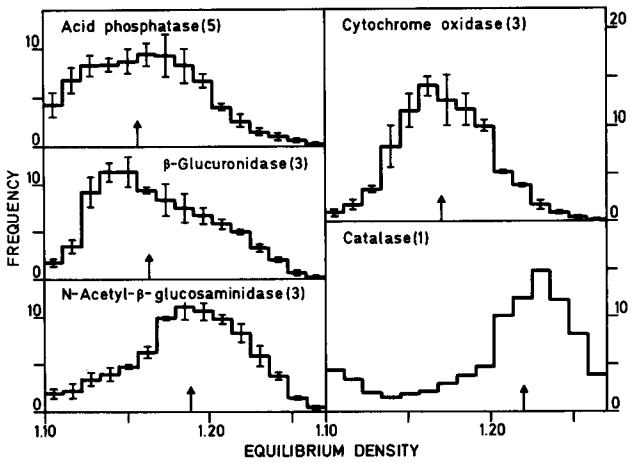


FIGURE 7 Density distribution of acid hydrolases, cytochrome oxidase, and catalase after isopycnic equilibration of microsomes in sucrose-$H_2O$ gradient. Black arrows refer to the median density of constituent. For explanation of the graphs, see Fig. 2.

TABLE III

*Median Density of Microsomal Constituents in Various Gradients*

| Constituent | Sucrose-$D_2O$ | Sucrose-$H_2O$ (10 h) | Ficoll-Sucrose 0.50 M | Ficoll-Sucrose 0.25 M |
|---|---|---|---|---|
| Protein | 1.2021 | 1.1774 | 1.1272 | 1.1059 ± 0.0081 (3) |
| Phospholipid | 1.1937 | 1.1650 | — | — |
| Monoamine oxidase | 1.1766 | 1.1460 | 1.1097 | 1.0912 ± 0.0047 (3) |
| 5′-Nucleotidase | 1.1775 | 1.1531 | 1.1038 | 1.0827 ± 0.0082 (2) |
| NADH cytochrome *c* reductase | 1.1884 | 1.1620 | 1.1158 | 1.0963 ± 0.0040 (3) |
| NADPH cytochrome *c* reductase | 1.1924 | 1.1651 | — | 1.0948 |
| Aminopyrine demethylase | — | 1.1710 | 1.1159 | 1.1012 ± 0.0089 (2) |
| Esterase | — | 1.1780 | 1.1217 | 1.1053 ± 0.0082 (3) |
| Nucleoside diphosphatase | 1.2019 | 1.1767 | 1.1194 | 1.1052 ± 0.0086 (3) |
| Glucose 6-phosphatase | 1.2022 | 1.1817 | 1.1209 | 1.1062 ± 0.0087 (3) |
| Ribonucleic acid | 1.2267 | 1.2196 | 1.1275 | 1.1140 ± 0.0091 (2) |
| Aldolase | 1.2336 | 1.2078 | — | — |
| Glutamine synthetase | 1.2189 | 1.1903 | 1.1245 | 1.1174 ± 0.0046 (3) |

Statistics refer to mean ± standard deviation; the number of experiments is given in parentheses.

of the physicochemical properties of microsomes results from longer exposure to high concentration of sucrose. Replacement of $H_2O$ by $D_2O$ as solvent in a sucrose gradient increased the median densities by some 0.04 density unit. Exchange of hydration water, and possibly a closer approach to equilibrium, thanks to the decrease in medium viscosity, are responsible for this increase. The influence of sucrose concentration on particle density is brought to light by the fractionations performed in Ficoll gradients. As illustrated in Fig. 8, the relationship between particle density and density of the sucrose solution approaches linearity for several constituents, as is to be expected on theoretical grounds (10, 16, 20) for particles permeable to sucrose, as microsomes have been reported to be (29). However, too much significance cannot be attached to this linearity, nor,

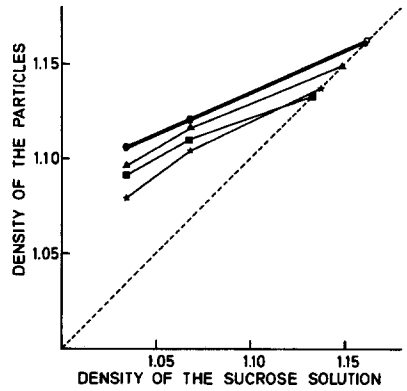


FIGURE 8 Median density of the particles bearing glucose 6-phosphatase (○), nucleoside diphosphatase (●), NADH cytochrome *c* reductase (▲), monoamine oxidase (■), and 5′-nucleotidase (★) as a function of the density of the sucrose solution in which they were suspended. The median densities are given in Tables II and III. The abscissa refers to the density of the corresponding sucrose solutions without Ficoll.

for that matter, to deviations from linearity, since complete density equilibrium was probably not reached in all experiments.

### *Differential Sedimentation Experiments*

As shown in Fig. 9 and Table IV, the three constituents of group *b*, NADH cytochrome *c* reductase, NADPH cytochrome *c* reductase, and cytochrome $b_5$, show identical sedimentation boundaries, which almost coincide with that of proteins. The two enzymes of group *c*, glucose 6-phosphatase, and nucleoside diphosphatase, sediment somewhat faster. The constituents of group *a* differ clearly from those of group *b* and *c*, but in addition separate into two subgroups, one represented by monoamine oxidase, which sediments distinctly more slowly than all other constituents, and the other including 5′-nucleotidase, alkaline phosphatase, alkaline phosphodiesterase I, as well as cholesterol, which show a particularly fast sedimentation rate. Fig. 10 and Table V show in addition that galactosyltransferase, another enzyme of group *a*, behaves like monoamine oxidase, and that β-glucuronidase follows glucose 6-phosphatase rather than acid phosphatase, justifying further the inclusion of β-glucuronidase in group *c* rather than in that of lysosomal contaminants.

## DISCUSSION

### *Experimental Design*

Numerous workers have reported on the subfractionation of rat liver microsomes (see reference 8). In spite of a fair amount of overlap with earlier findings, our results conserve unique features, thanks to the following characteristics of experimental design:

(*a*) Our microsomal fractions contain about 40 mg of protein per g liver and some 70% or more of the total hepatic content in typical microsomal marker enzymes such as glucose 6-phosphatase (4). This yield, which is more than twice that reported by most other investigators, provides some insurance against a gross sampling bias.

(*b*) A variety of fractionation systems have been applied, depending both on differences in density in various media and on differences in sedimentation coefficient. In each case, the experiments were performed in a purely analytical fashion, with due regard for quantitative recovery and without the introduction of misleading discontinuities. As has been repeatedly emphasized (15–18), these precautions ensure a maximal degree of resolution and offer the best chances of not confusing heterogeneity of fractions with heterogeneity of subcellular organelles.

(*c*) Up to 27 distinct constituents were measured, many of them in the same experiment, thus allowing a large number of meaningful comparisons to be made.

(*d*) In several experiments, extensive quantitative morphological analysis of the isolated fractions were performed as well, permitting a direct correlation between the biochemical and the morphological findings. This part of the work has been reported previously (44).

Although our main conclusions have been presented before, together with the essential supporting evidence (2, 40, 44), while subsequent publications in this series will provide additional elements of interpretation (5), the present account has nevertheless been deemed necessary to make available a comprehensive record of our findings and of the experimental basis of our views. However, in order to avoid undue repetition, we will restrict the present discussion to a few essential points.

### *Distribution of Chemical Constituents*

As clearly shown by Wibo et al. (44), the distribution of RNA reflects that of ribosomes, and the

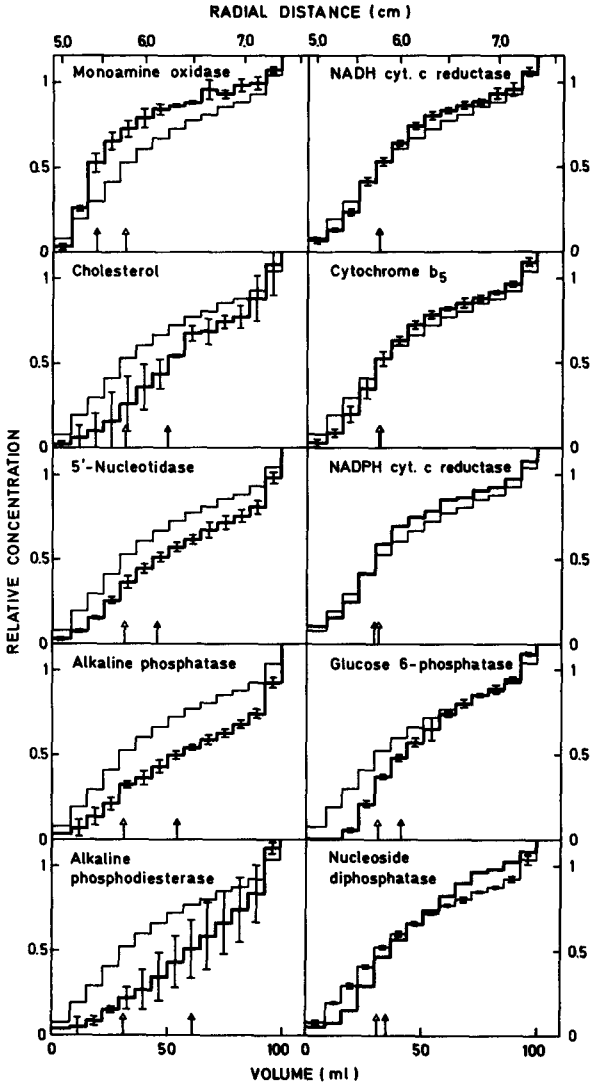


FIGURE 9 Sedimentation patterns of microsomal constituents after differential sedimentation in a density gradient. Three P′ fractions were centrifuged at 20,000 rpm ($W_{average} = 9.15 \times 10^9$ rad$^2$ s$^{-1}$). The profiles of sedimentation boundaries have been normalized and averaged. The volume increment is 7 ml per fraction except for the first (8 ml) and the last (11 ml) one. The relative concentration ($C/C_i$) is expressed by: $(110 \cdot Q)/(V \cdot \Sigma Q)$. In this relation Q is the amount of constituent present in the fraction, $\Sigma Q$ the recovered amount of constituent, V the volume of the fraction, and 110 the total volume in milliliters in which microsomes were initially suspended. The position in the gradient at which $C/C_i = 0.5$ is marked by a black arrow for the constituents and by a white arrow, repeated on all graphs, for protein. The thin lines reproduced on all graphs represent the distribution pattern of protein. Vertical lines through the bars refer to standard deviations of the relative concentrations. The upper abscissa gives the radial distance as a function of volume measured from the meniscus. Complementary data are reported in Table IV.

TABLE IV

*Complementary Data on Experiments of Fig. 9*

| Constituent | No. of exps. | Percent recovery | Amount recovered beyond 7.34 cm from axis* | Radial distance traveled at $C/C_i = 0.5$‡ |
|---|---|---|---|---|
| Protein | 3 | 99.5 ± 1.2 | 43.8 ± 0.4 | 8.8 ± 0.2 |
| Monoamine oxidase | 2 | 105.6 ± 14.6 | 33.2 ± 1.3 | 5.4 ± 0.5 |
| 5′-Nucleotidase | 2 | 102.5 ± 3.4 | 55.7 ± 1.7 | 12.7 ± 1.1 |
| Alkaline phosphodiesterase I | 2 | 60.0 ± 14.4 | 61.0 ± 8.9 | 16.2 ± 3.5 |
| Alkaline phosphatase | 2 | 99.3 ± 5.0 | 60.2 ± 0.9 | 14.6 ± 0.7 |
| Cholesterol | 2 | 93.2 ± 6.4 | 57.0 ± 0.6 | 13.5 ± 0.9 |
| NADH cytochrome *c* reductase | 3 | 97.8 ± 6.2 | 42.0 ± 0.6 | 8.7 ± 0.4 |
| NADPH cytochrome *c* reductase | 1 | 111.9 | 43.0 | 8.2 |
| Cytochrome $b_5$ | 3 | 97.4 ± 7.8 | 43.2 ± 1.3 | 8.9 ± 0.5 |
| Glucose 6-phosphatase | 2 | 102.7 ± 0.3 | 50.7 ± 0.2 | 11.3 ± 0.4 |
| Nucleoside diphosphatase | 1 | 106.7 | 44.3 | 9.8 |

Statistics refer to mean ± standard deviation.

* Amount of constituent recovered in the last fraction of Fig. 9 and in the cushion, expressed in percent of the total amount recovered.

‡ Expressed in millimeters from the meniscus and computed by linear interpolation, assuming that the average values of $C/C_i$ are attained in the middle of the fractions.

increase in RNA-to-protein ratio with increasing density reflects an increase in the number of ribosomes per vesicle or per unit of membrane surface area. We do not separate smooth and rough microsomes, but resolve smooth vesicles, which are concentrated largely in the fractions of lowest density, and a spectrum of what might be called increasingly rough vesicles. The increase in protein-to-phospholipid ratio with increasing density is not so easily interpreted. It is accounted for only partly by the contribution of ribosomal proteins, and could be due to differences in composition of the membranes or to differences in vesicle content or to both.

The distribution of cholesterol deserves special attention. In density equilibration experiments, it clearly parallels the distribution of group *a* enzymes. If we consider the fractions of density greater than 1.20, which on an average contain 26, 29, and 50% of the total microsomal protein, glucose 6-phosphatase, and RNA, respectively (Fig. 2), we find that they contain only 3.7% of the total cholesterol, as compared to 2.3% of 5′-nucleotidase, 1.2% of alkaline phosphodiesterase I, and 5.4% of alkaline phosphatase. Thus it is obvious that the rougher parts of the endoplasmic reticulum (ER) are essentially free of cholesterol. This conclusion may be extended to the remainder of the ER in view of the results of experiments (5, 40) which have shown that previous treatment of the microsomes with small amounts of digitonin causes a considerable shift to higher densities of the distribution patterns of cholesterol and of some group *a* enzymes, without at all affecting those of group *b* and *c* enzymes. These results are doubly meaningful, in that they show that the bulk of the microsomal cholesterol is physically independent from the structures bearing group *b* and *c* enzymes, and also that the latter structures do not contain appreciable amounts of cholesterol per unit weight, at least in a form accessible to digitonin binding. Our conclusion, which is at variance with the views of other authors (13, 23), will be further supported in the next paper of this series (5). The intracellular localization of cholesterol is examined below.

### *Distribution of Group a Enzymes*

This group, which has been defined on the basis of similarities in density distribution, can be subdivided into two groups on the basis of the results of differential sedimentation. Group *a2*, characterized by a higher sedimentation rate than that of the total microsomal proteins, comprises 5′-nucleotidase, alkaline phosphatase, and alkaline

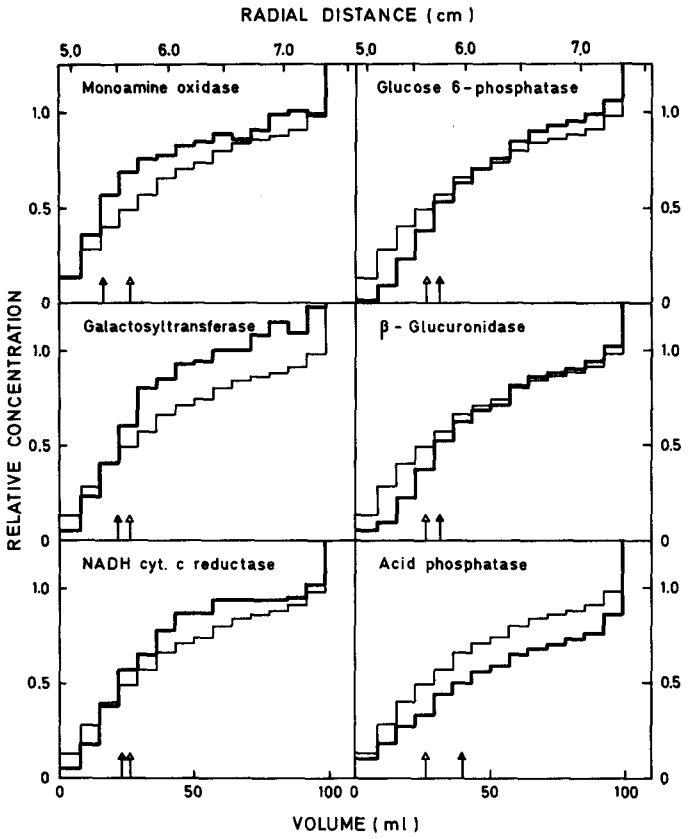


FIGURE 10 Sedimentation patterns of microsomal constituents after differential sedimentation in a density gradient. The experimental procedure was the same as described in Fig. 9 except that $W = 6.76 \times 10^9$ rad$^2$ s$^{-1}$. The results are presented in the same manner as in Fig. 9. Complementary data are reported in Table V.

TABLE V

*Complementary Data on Experiment of Fig. 10*

| Constituent | Percent recovery | Amount recovered beyond 7.34 cm from axis* | Radial distance traveled at $C/C_i$ = 0.5‡ |
|---|---|---|---|
| Protein | 104.2 | 41.1 | 7.6 |
| Monoamine oxidase | 94.4 | 32.2 | 4.8 |
| Galactosyltransferase | 92.6 | 27.6 | 6.4 |
| NADH cytochrome *c* reductase | 98.3 | 35.8 | 6.7 |
| Glucose 6-phosphatase | 95.1 | 42.5 | 8.9 |
| β-Glucuronidase | 120.7 | 44.8 | 9.0 |
| Acid phosphatase | 82.1 | 53.1 | 11.1 |

* Amount of constituent recovered in the last fraction of Fig. 10, and in the cushion, expressed in percent of the total amount recovered.

‡ Expressed in millimeters from the meniscus and computed by linear interpolation, assuming that the average values of $C/C_i$ are attained in the middle of the fractions.

phosphodiesterase I, three enzymes which are typically associated with plasma membranes (for a review see 21), and which show a considerable digitonin shift both in microsomes (5, 40) and in plasma membranes (1, 18). Sedimenting more slowly than the average proteins are monoamine oxidase and galactosyltransferase. There is good evidence that the similarities between these two enzymes are fortuitous and that they are bound to different structures. Galactosyltransferase is well established as a specific constituent of the Golgi apparatus (22, 28, 34) which is devoid of monoamine oxidase and displays a significant digitonin shift (Wibo, unpublished observations). Contrary to galactosyltransferase, monoamine oxidase is not shifted by digitonin treatment of microsomes (5, 40). Thus we must consider two additional subgroups, *a1* and *a3*, represented in our experiments by monoamine oxidase and galactosyltransferase, respectively.

Group *a2* is clearly related to the plasma membrane, but in what capacity is not entirely clear. It is rather remarkable that the enzymes in this group all show a nucleomicrosomal type of distribution upon conventional centrifugal fractionation (4). Biochemically comparable membrane fractions have been purified from both nuclear and microsomal fractions (41). The simplest hypothesis is to assume that upon homogenization, plasma membranes break up into large fragments, made up mostly of bile canaliculi and adjoining elements recovered with the nuclear fraction, and into pieces of much smaller size which come down with the microsomes. But there could be a contribution also of plasma membrane precursors or derivatives, including secretory and pinocytic vesicles.

Group *a3* obviously belongs to parts of the Golgi apparatus, which can be recognized morphologically in the low density fractions (44).

As to group *a1*, it is most likely related to mitochondrial outer membranes, which are known to contain the large part of monoamine oxidase (35). There are, however, some difficulties to this interpretation. The centrifugal behavior of the microsomal monoamine oxidase indicates attachment to particles of low density and low sedimentation coefficient. These properties, together with the entirely different behavior of the microsomal cytochrome oxidase, preclude association of the enzyme with intact mitochondria. Fragments of stripped-off outer mitochondrial membranes are a possibility, consistent with the density distribution of monoamine oxidase, which is similar to that of purified outer membranes (18). Moreover the supernatant fraction contains about as much (25% of the total activity) adenylate kinase, an enzyme located between the two mitochondrial membranes (36), as there is monoamine oxidase in the microsomal fraction. There is, however, evidence that adenylate kinase is represented by different isozymes in mitochondria and in the supernatant fraction (12). Also, when mitochondrial outer membranes are stripped off artificially, they are obtained in the form of large ghosts (31), which should sediment faster than does the microsomal monoamine oxidase. Finally, a recent quantitative morphometric study by Wibo (unpublished results) has shown that only 11% of the mitochondria lose their outer membrane upon homogenization. The matter therefore remains open.

The distribution of cholesterol between these three subgroups presents an interesting problem. Much of it undoubtedly belongs to plasma membranes, which are known to be very rich in cholesterol (21, 39), and to show a marked digitonin shift (1, 18). If we assume that all the cholesterol and group *a2* enzyme activities of the nuclear fraction are associated with plasma membranes, and that the plasma membrane fragments present in the microsomal fraction have the same composition as those in the nuclear fraction, we may estimate that about two-thirds of the microsomal cholesterol accompanies group *a2* enzymes in plasma membrane elements. Golgi components (group *a3*) account for an additional part (24), 10% according to unpublished data of Wibo, whereas group *a1* is probably cholesterol-free, since monoamine oxidase is not shifted by digitonin (5, 40). This leaves some microsomal cholesterol unaccounted for (about one-fourth), an argument used by Glaumann and Dallner (23) as proof that significant amounts of cholesterol must be located in the ER proper. For the reasons stated above, we consider such a view unacceptable. In our opinion, either the subcellular elements related to the plasma membrane, present in the microsomal fraction, contain more cholesterol than those recovered with the nuclear fraction, or the microsomes contain another unidentified cholesterol-rich entity of relative low density. The first possibility seems quite plausible, since the two groups of fragments probably originate from different parts of the cell membrane. It is ap-

parently contradicted by the result of Touster et al. (41) who have shown that the cholesterol-to-protein ratio is almost identical in plasma membrane preparations derived from the N or from the P fraction. However, the enzymatic analysis of these preparations indicates that the plasma membranes obtained from microsomes were more heavily contaminated by ER. Further, a noticeable contamination of these membranes by Golgi elements may be suspected in view of our analytical results.

### *Distribution of Groups b and c Enzymes*

Most of the enzymes in groups *b* and *c* are what may be called typical microsomal enzymes, which occur in our microsomal fractions to the extent of 65–75% of the total activity of the liver. Exceptions are, in group *b*, NADH cytochrome *c* reductase, of which about 20% is mitochondrial, and in group *c*, β-glucuronidase, which is largely lysosomal (4). In the case of β-glucuronidase, the existence of a true microsomal component (see reference 4) is confirmed by our results.

On the basis of their distribution patterns, in relation to those of protein, phospholipid, and RNA, the enzymes of both group *b* and group *c* appear to be associated with true elements of the ER. Further support for this contention will be given in subsequent publications. Nevertheless, the distinction between the two groups is real and has been verified in all centrifugation systems tried. Two alternative explanations could account for it. Either the two groups are associated with different parts of the ER, those containing group *c* being on an average richer in ribosomes than those containing group *b*. Or both groups are associated with the same membrane elements, but in such a manner as to produce an increase of the group *c*-to-group *b* ratio with increasing ribosome load. The latter type of heterogeneity could be intrinsic to the ER in each cell, or it could be a consequence of correlated differences in group *c*-to-group *b* ratio and in ribosome load between different cells. As will be reported in greater detail later, we feel that the first alternative can be ruled out on the basis, on one hand, of cytochemical results, especially those of Leskes et al. (27), showing the widespread distribution of glucose 6-phosphatase throughout the ER, and, on the other, of recent cytoimmunological determinations (32), showing that practically every true ER vesicle in the microsomal fraction contains cytochrome $b_5$. As to the type of heterogeneity involved within or between individual cells, we have no information at the present time.

### *Distribution of Group d Enzymes*

This group should include all the activities associated with ribosomes, since the distribution of RNA coincides with that of ribosomes (44). It is, however, unlikely that the microsomal fumarase, which shows the same kind of distribution pattern, is a true constituent of ribosomes. This enzyme, which is located partly in the mitochondria and partly in the cytosol, is found in addition in the microsomal fraction in amounts that seem to vary with the medium composition and with the number of washings (4); it is easily adsorbed by microsomes, but not by mitochondria (25). We have found that the microsomal enzyme is released in soluble form by washing with 0.25 M sucrose containing 5 mM Na pyrophosphate (5) and by treatment with ribonuclease. For these reasons, it appears likely that adsorbed soluble enzyme is responsible for the microsomal fumarase activity. The *d* type of distribution observed for this enzyme probably indicates that ribosomal RNA is the adsorbing material.

We are inclined to propose a similar interpretation for the microsomal occurrence and distribution patterns of glutamine synthetase and of aldolase, with the difference that these enzymes are more easily detached from the ribosomes and occur partly in soluble form in the microsomal suspension. It must be remembered that in the E-40 rotor soluble proteins sediment significantly in 3 h at 35,000 rpm. Thus, the patterns seen for glutamine synthetase and aldolase could reflect the combination of a *d* type pattern of adsorbed enzyme and of a sedimenting zone of soluble enzyme. Supporting this interpretation is the fact that both enzymes can be readily detached from the microsomes by washing with isotonic salt solution. Adsorption of glutamine synthetase to brain microsomes (37, 38) and of aldolase to nuclear DNA (33) has been reported.

### *Distribution of Large Granule Enzymes*

According to its distribution pattern, the small amount of cytochrome oxidase found in the microsomal fraction clearly belongs to small mitochondria or to mitochondrial fragments, in which it is presumably associated with other mito-

chondrial enzymes, including some of the activities measured here, for instance monoamine oxidase, NADH cytochrome *c* reductase, and fumarase. It is easily calculated that only a small part of these microsomal activities can belong to mitochondria, and subtracting the mitochondrial contribution does not appreciably alter their distribution pattern.

We may similarly conclude from the distribution pattern of catalase that this enzyme occurs in the microsomal fraction largely in association with contaminating peroxisomes, and to a small extent in soluble form.

As to the acid hydrolases, we have already seen that β-glucuronidase may be considered as truly microsomal, although the presence of some β-glucuronidase activity in contaminating lysosomes seems probable. On the other hand, acid phosphatase and *N*-acetyl-β-glucosaminidase probably are present mostly as contaminants. The extent to which lysosomal heterogeneity, adsorption artefacts, and the occurrence of minor microsomal activities account for the difference in distribution pattern between the two enzymes has not been investigated.

## CONCLUSION

As was first shown by the pioneering work of Palade and Siekevitz (30), the main component of the microsomal fraction of rat liver is represented by pieces of the ER, which fragments upon homogenization into self-sealing small vesicles. But the microsomal fraction contains in addition a number of minor components, including most of the Golgi apparatus, about half the plasma membranes and related materials, fragments derived from, or related to, mitochondrial outer membranes, and small numbers of mitochondria, lysosomes, and peroxisomes. Thanks to the use of a purely analytical form of centrifugal fractionation in various systems, we have been able to assign many of the enzyme activities found in the microsomal fraction to one, or exceptionally, more of these components. Their relative contributions to the total proteins of the fraction can be estimated from the ratios of the relative specific activities of typical marker enzymes in the fraction and in purified preparations. Such a computation leads to the following partition of proteins: plasma membrane, 7–8%; mitochondria, 6%; Golgi complex, 4–5%; external mitochondrial membranes, 3%; lysosomes, 1%; and peroxysomes, 1%. Microsomal elements deriving from ER account thus for no more than 77% of microsomal protein. It should be noted that this estimation applies only to microsomal fractions as isolated in this work.

Our results emphasize once again the importance of characterizing particle populations by their complete frequency distribution diagrams, as a function of a continuously varied parameter. Subfractions separated by artificially generated discontinuities, on the other hand, rarely contain the totality of a given population to the exclusion of the others, and the results of their analysis are correspondingly difficult to interpret.

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*Note added in proof:* It appears from various comments from reviewers and other readers of our manuscripts that, despite detailed descriptions of the *analytical approach* (15–18), a confusion is likely to subsist between what we call here a *group*, and a subcellular *fraction*.

A *fraction* is any entity separated by fractionation. In most instances fractions are *mixtures* of subcellular components. For instance, a mitochondrial fraction is a mixture of mitochondria, lysosomes, and peroxisomes, together with smaller amounts of other components.

To assess the true composition of fractions we resort to subfractionation. Ideally this technique should separate the different components of the fraction and allow their identification. In practice, this rarely occurs and in some cases turns out not to be feasible with available methods. It does, however, happen fairly frequently that subfractionation leads to a certain degree of *partial* separation of different components. To take again the example of a mitochondrial fraction, most subfractions separated from it by density gradient fractionation are still mixtures of mitochondria, lysosomes, and peroxisomes. But

they do not all contain these components in the same proportion. A consideration of the manner in which different enzymes are distributed between the subfractions will reveal that they fall within three main *groups*. One such group will include cytochrome oxidase, succinate dehydrogenase, glutamate dehydrogenase, and many other enzymes truly associated with mitochondria. A second group will contain a variety of acid hydrolases. A third one catalase and a number of oxidases. These groups are distinguished by the characteristics (mode[s], median, shape, etc . . .) of their distribution patterns between the subfractions.

When the existence of such groups is uncovered, the *tentative* interpretation of the finding according to the "postulate of biochemical homogeneity" is that each group identifies a distinct intracellular entity. A "robot picture" of this hypothetical entity in terms of the statistical distribution of its size, density, osmotic content, etc . . . , is progressively derived from the distribution patterns observed for the enzymes of the corresponding group.

The existence of an intracellular entity behind the group is considered increasingly probable, the more numerous the fractionation conditions under which the group goes on behaving as a single distinct group. It is taken as established only after complete separation of the group has been achieved and the underlying intracellular component identified, or when confirmation is obtained by other means.

In short, a group defines enzymes or other biochemical markers showing a common characteristic behavior, different from that of other groups, upon fractionation by various methods. It is a tentative biochemical definition of a subcellular component assumed to be the specific bearer of the biochemical constituents in the group.

The present fractionation studies have led to the identification of a number of such *groups*, which were recognized by their centrifugal behavior and tentatively identified with distinct classes of cytomembranes. The *fractions* themselves we believe to be impure and mixtures of these different classes of membranes in varying proportion. In this respect, our attitude differs from that of many other investigators who in comparable work tend to identify the *fractions* as such with distinct intracellular entities. In our opinion, this difference is neither minor nor purely semantic. At issue is a point of *analytical rigor* which not infrequently is of decisive importance with respect to the interpretation of the results.

## REFERENCES


1. Amar-Costesec, A. 1973. Etude analytique des membranes subcellulaires par centrifugation en gradient de densité. Application à la fraction microsomiale de foie de rat. *Spectra 2000.* **4**:137.
2. Amar-Costesec, A., H. Beaufay, E. Feytmans, D. Thines-Sempoux, and J. Berthet. 1969. Subfractionation of rat liver microsomes. *In* Microsomes and Drug Oxidations. J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts, and G. J. Mannering, editors. Academic Press, Inc., New York. 41.
3. Amar-Costesec, A., H. Beaufay, D. Thines-Sempoux, and J. Berthet. 1968. Heterogeneity of the membranes of the microsomal fraction. *Excerpta Med. Int. Congr. Ser.* **166**:38.
4. Amar-Costesec, A., H. Beaufay, M. Wibo, D. Thines-Sempoux, E. Feytmans, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparation and composition of the microsomal fraction. *J. Cell Biol.* **61**:201.
5. Amar-Costesec, A., M. Wibo, D. Thines-Sempoux, H. Beaufay, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. IV. Biochemical, physical, and morphological modifications of microsomal components induced by digitonin, EDTA, and pyrophosphate. *J. Cell Biol.* Submitted for publication.
6. Beaufay, H. 1966. La centrifugation en gradient de densité. Applications à l'étude des organites subcellulaires. Thèse d'Agrégation de l'Enseignement Supérieur, Université Catholique de Louvain, Louvain, Belgium. Ceuterick S. A., Louvain, Belgium. 1–132.
7. Beaufay, H. 1971. Le fractionnement des membranes cellulaires. *Bull. Acad. R. Med. Belg.* 7e série. **11**:815.
8. Beaufay, H., A. Amar-Costesec, E. Feytmans, D. Thines-Sempoux, M. Wibo, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. I. Biochemical methods. *J. Cell Biol.* **61**:188.
9. Beaufay, H., D. S. Bendall, P. Baudhuin, R. Wattiaux, and C. de Duve. 1959. Tissue fractionation studies. 13. Analysis of mitochondrial fractions from rat liver by density gradient centrifuging. *Biochem. J.* **73**:628.
10. Beaufay, H., P. Jacques, P. Baudhuin, O. Z. Sellinger, J. Berthet, and C. de Duve. 1964. Tissue fractionation studies. 18 Resolution of mitochondrial fractions from rat liver into three distinct populations of cytoplasmic particles by means of density equilibration in various gradients. *Biochem. J.* **92**:184.
11. Bowers, W. E., and C. de Duve. 1967. Lysosomes in lymphoid tissue. II. Intracellular distribution of acid hydrolases. *J. Cell Biol.* **32**:339.

12. CRISS, W. E., V. SAPICO, and G. LITWACK. 1970. Rat liver adenosine triphosphate: adenosine monophosphate phosphotransferase activity. I. Purification and physical and kinetic characterization of adenylate kinase III. *J. Biol. Chem.* **245**:6346.
13. DALLNER, G., and L. ERNSTER. 1968. Subfractionation and composition of microsomal membranes: a review. *J. Histochem. Cytochem.* **16**:611.
14. DE DUVE, C. 1960. La localisation des enzymes dans les éléments figurés des cellules. *Bull. Soc. Chim. Biol.* **42**:11.
15. DE DUVE, C. 1964. Principles of tissue fractionation. *J. Theor. Biol.* **6**:33.
16. DE DUVE, C. 1965. The separation and characterization of subcellular particles. *Harvey Lect.* Ser. 59. 49.
17. DE DUVE, C. 1967. General principles. *In* Enzyme Cytology. D. B. Roodyn, editor. Academic Press Inc. Ltd., London. 1.
18. DE DUVE, C. 1971. Tissue fractionation. Past and present. *J. Cell Biol.* **50**:20 D.
19. DE DUVE, C., H. BEAUFAY, P. JACQUES, Y. RAHMAN-LI, O. Z. SELLINGER, R. WATTIAUX, and S. DE CONINCK. 1960 Intracellular localization of catalase and of some oxidases in rat liver. *Biochim. Biophys. Acta.* **40**:186.
20. DE DUVE, C., J. BERTHET, and H. BEAUFAY. 1959. Gradient centrifugation of cell particles. Theory and applications. *Prog. Biophys. Biophys. Chem.* **9**:325.
21. DEPIERRE, J. W., and M. L. KARNOVSKY. 1973. Plasma membranes of mammalian cells. A review of methods for their characterization and isolation. *J. Cell Biol.* **56**:275.
22. FLEISCHER, B., S. FLEISCHER, and H. OZAWA. 1969. Isolation and characterization of Golgi membranes from bovine liver. *J. Cell Biol.* **43**:59.
23. GLAUMANN, H., and G. DALLNER. 1970. Subfractionation of smooth microsomes from rat liver. *J. Cell Biol.* **47**:34.
24. KEENAN, T. W., and D. J. MORRE. 1970. Phospholipid class and fatty acid composition of Golgi apparatus isolated from rat liver and comparison with other cell fractions. *Biochemistry.* **9**:19.
25. KUFF, E. L. 1954. The distribution of fumarase activity in mouse liver homogenates. *J. Biol. Chem.* **207**:361.
26. LEIGHTON, F., B. POOLE, H. BEAUFAY, P. BAUDHUIN, J. W. COFFEY, S. FOWLER, and C. DE DUVE. 1968. The large-scale separation of peroxysomes, mitochondria, and lysosomes from the livers of rats injected with Triton WR-1339. Improved isolation procedures, automated analysis, biochemical, and morphological properties of fractions. *J. Cell Biol.* **37**:482.
27. LESKES, A., P. SIEKEVITZ, and G. E. PALADE. 1971. Differentiation of endoplasmic reticulum in hepatocytes. I. Glucose-6-phosphatase distribution *in situ*. *J. Cell Biol.* **49**:264.
28. MORRE, D. J., L. M. MERLIN, and T. W. KEENAN. 1969. Localization of glycosyl transferase activities in a Golgi apparatus-rich fraction isolated from rat liver. *Biochem. Biophys. Res. Commun.* **37**:813.
29. NILSSON, R., E. PETERSON, and G. DALLNER. 1973. Permeability of microsomal membranes isolated from rat liver. *J. Cell Biol.* **56**:762.
30. PALADE, G. E., and P. SIEKEVITZ. 1956. Liver microsomes. An integrated morphological and biochemical study. *J. Biophys. Biochem. Cytol.* **2**:171.
31. PARSONS, D. F., G. R. WILLIAMS, and B. CHANCE. 1966. Characteristics of isolated and purified preparations of the outer and inner membranes of mitochondria. *Ann. N. Y. Acad. Sci.* **137**:643.
32. REMACLE, J., S. FOWLER, H. BEAUFAY, and J. BERTHET. 1974. Ultrastructural localization of cytochrome $b_5$ on rat liver microsomes by means of hybrid antibodies labeled with ferritin. *J. Cell Biol.* **61**:237.
33. ROODYN, D. B. 1957. The binding of aldolase to isolated nuclei. *Biochim. Biophys. Acta.* **25**:129.
34. SCHACHTER, H., I. JABBAL, R. L. HUDGIN, L. PINTERIC, E. J. MCGUIRE, and S. ROSEMAN. 1970. Intracellular localization of liver sugar nucleotide glycoprotein glycosyltransferases in a Golgi-rich fraction. *J. Biol. Chem.* **245**:1090.
35. SCHNAITMAN, C., V. G. ERWIN, and J. W. GREENAWALT. 1967. The submitochondrial localization of monoamine oxidase. An enzymatic marker for the outer membrane of rat liver mitochondria. *J. Cell Biol.* **32**:719.
36. SCHNAITMAN, C., and J. W. GREENAWALT. 1968. Enzymatic properties of the inner and outer membranes of rat liver mitochondria. *J. Cell Biol.* **38**:158.
37. SELLINGER, O. Z., and F. DE BALBIAN VERSTER. 1962. Glutamine synthetase of rat cerebral cortex: intracellular distribution and structural latency. *J. Biol. Chem.* **237**:2836.
38. SELLINGER, O. Z., F. DE BALBIAN VERSTER, R. S. SULLIVAN, and C. LAMAR, JR. 1966. Morphological and biochemical correlates of cerebral microsomes. II. A study of the structural association of glutamine synthetase, ATPase and an esterase acting on *o*-nitrophenyl acetate. *J. Neurochem.* **13**:501.
39. THINES-SEMPOUX, D. 1972. A comparison be-

tween the lysosomal and the plasma membrane. *In* Lysosomes in Biology and Pathology. J. T. Dingle, editor. North-Holland Publishing Co., Amsterdam. 3:278.

40. THINES-SEMPOUX, D., A. AMAR-COSTESEC, H. BEAUFAY, and J. BERTHET. 1969. The association of cholesterol, 5'-nucleotidase and alkaline phosphodiesterase I with a distinct group of microsomal particles. *J. Cell Biol.* **43**:189.
41. TOUSTER, O., N. N. ARONSON, J. T. DULANEY, and H. HENDRICKSON. 1970. Isolation of rat liver plasma membranes. Use of nucleotide pyrophosphatase and phosphodiesterase I as marker enzymes. *J. Cell Biol.* **47**:604.
42. WATTIAUX, R., and S. WATTIAUX-DE CONINCK. 1970. Distribution of mitochondrial enzymes after isopycnic centrifugation of a rat liver mitochondrial fraction in a sucrose gradient: influence of the speed of centrifugation. *Biochem. Biophys. Res. Commun.* **40**:1185.
43. WATTIAUX, R., S. WATTIAUX-DE CONINCK, and M. F. RONVEAUX-DUPAL. 1971. Deterioration of rat-liver mitochondria during centrifugation in a sucrose gradient. *Eur. J. Biochem.* **22**:31.
44. WIBO, M., A. AMAR-COSTESEC, J. BERTHET, and H. BEAUFAY. 1971. Electron microscope examination of subcellular fractions. III. Quantitative analysis of the microsomal fraction isolated from rat liver. *J. Cell Biol.* **51**:52.