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

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ABSTRACT

Isopycnic equilibration and sedimentation rate studies of rat liver microsomes led previously to the assignment of microsomal constituents into group *a1* (monoamine oxidase), group *a2* (5′-nucleotidase, alkaline phosphodiesterase I, alkaline phosphatase and cholesterol), group *a3* (galactosyltransferase), group *b* (NADH cytochrome *c* reductase, NADPH cytochrome *c* reductase, aminopyrine demethylase, cytochrome b_5 and P 450), and group *c* (glucose 6-phosphatase, esterase, nucleoside diphosphatase, β-glucuronidase and glucuronyltransferase).

Confirmation and extension of the assignment into groups has been obtained by studying the differential effect of the reagents digitonin, EDTA, and PPi. Digitonin specifically affected the equilibrium density only of the group *a2* and (to a lesser extent) group *a3*, and not of groups *b* and *c* under conditions which preserved the structure-linked latency of nucleoside diphosphatase and galactosyltransferase. Within experimental error the rate of sedimentation of all microsomal constituents was unaffected. The morphological appearance under the electron microscope was indistinguishable from that of nondigitonin-treated microsomes, except that a few smooth membranes (< 10%) exhibited broken-looking profiles.

Treatment of microsomes with EDTA or PPi detached a substantial part of RNA and released protein in excess over the amount accountable for by detachment of ribosome constituents. This detachment was confirmed by electron microscopy. EDTA and PPi decreased markedly the equilibrium density and the density dispersion of groups *b* and *c*, due mainly to the uncoating of rough

elements. EDTA and PPi shifted slightly the distribution profiles of groups *a* towards lower densities, possibly as a result of the release of adsorbed proteins. The combination of EDTA and digitonin, used subsequently, rendered the average equilibrium density of group *a2* higher than that of groups *b* and *c*. Dense subfractions were thus enriched in constituents of group *a2* and showed mainly broken-looking vesicles under the electron microscope. The import of our results on the biochemical and enzymic properties of the subcellular components of the microsome fraction is discussed.

INTRODUCTION

The work reported here is a continuation of our studies on the properties and fractionation of rat liver microsomes. The state of this subject and the relevant contributions of other laboratories are summarized in the introductory paper of this series (6) which also justifies our approach to the question and describes the biochemical methods used. The procedure followed to prepare the microsome fraction and the biochemical properties of our microsomes were presented by Amar-Costesec et al. (4), whereas morphometric data were reported by Wibo et al. (33). Finally, a classification of several enzymic and chemical constituents[1] of rat liver microsomes into several groups was proposed according to both their distribution after density equilibration in various gradients and differential sedimentation in a shallow stabilizing gradient of sucrose (3, 7).

Group *a* comprises monoamine oxidase, galactosyltransferase, 5′-nucleotidase, alkaline phosphodiesterase I, alkaline phosphatase, and cholesterol. All of these constituents equilibrated in the low density range (1.08–1.20) and were distinguished into those which sedimented faster (group *a2:* 5′-nucleotidase, alkaline phosphodiesterase I, alkaline phosphatase, and cholesterol) than the bulk of microsomes, and those which sedimented more slowly (monoamine oxidase and galactosyltransferase). On the basis of the results presented here, and briefly alluded to before (7), a further distinction was proposed between monoamine oxidase (group *a1*) and galactosyltransferase (group *a3*).

Group *b* constituents, namely NADH and NADPH cytochrome *c* reductases, cytochromes b_5 and P 450, and aminopyrine demethylase displayed broad, asymmetrical density distributions, with a major peak in the low density range (mode $\simeq$ 1.14) and a tail extending up to the upper density limit (1.27) of the gradient. Constituents of group *c* behaved in a similar manner except for a slight but systematic higher frequency on the dense side of the distributions (i.e. their distributions were more skewed). Glucose 6-phosphatase, nucleoside diphosphatase, esterase, glucuronyltransferase and, to a large extent, microsomal β-glucuronidase activity belong to this group. Phospholipid and protein never dissociated markedly from constituents of groups *b* and *c*.

RNA, which in our microsome preparations represents essentially the ribosomal RNA of rough vesicles (33), also equilibrated asymmetrically over the whole density range of the gradient and peaked at the density of 1.22. It was classified into a group *d* together with fumarase, aldolase, and glutamine synthetase which behaved in a somewhat similar way. Evidence was, however, put forward showing that these enzymes are soluble proteins adsorbed by microsomal vesicles.

The results previously reported are extremely encouraging in that they allowed classification of enzymes into groups, as well as tentative assignment of the groups to subcellular components of the microsome fraction. However, the subfractionation of rat liver microsomes failed to separate completely the microsomal entities, due mainly to the wide dispersion existing within the various populations of microsomal particles with respect to both density and size. To achieve better fractionation, to support the classification into groups, and to strengthen the cytological assignments, the microsomes were treated in various ways before

[1] As in the preceding papers (4, 6, 7), the term *component* designates a structural entity in intact cells or in tissue homogenates (for instance rough ER and rough microsomes), and the term *constituent* designates a biochemical entity (enzyme, cholesterol...). A *group* is a set comprising one or several constituents which behave similarly in all the fractionation systems used and can be distinguished from other constituents in at least one fractionation system (see the note added in proof, reference 7).

TABLE I

Composition of the Microsome Fractions Obtained after Various Treatments of Microsomes and Analyzed by Density Equilibration in Sucrose Gradient*

Constituent	Untreated microsomes	Microsomes treated with: Digitonin	PPi	EDTA	EDTA + digitonin
Protein	42.2 ± 7.4 (50)	40.3 ± 5.7 (6)	39.7	37.8 ± 0.6 (2)	30.7
Phospholipid	22.4 ± 3.2 (16)	22.5 ± 1.6 (3)	25.1	21.3	20.7
Cholesterol	1.45 ± 0.33 (18)	1.58 ± 0.22 (4)	1.55	1.90	1.30
Ribonucleic acid	4.69 ± 1.12 (32)	4.07 ± 1.57 (3)		3.27 ± 0.26 (2)	2.03

* Treatments are described under Materials and Methods. Values refer to 1 g fresh weight of liver. They are given as means ± SD, in milligrams for protein, RNA, and cholesterol, and in micromoles of organic phosphorus for phospholipid. The number of experiments is given in parentheses.

subfractionation in the hope that this would specifically modify the physical properties of a single or limited number of components. The results of digitonin, PPi,[2] and EDTA treatment are reported here. Preliminary accounts of this work have already appeared (1, 2, 5, 12, 28, 29, 34).

MATERIALS AND METHODS

Preparation and Treatment of Microsomes

GENERAL FEATURES: All operations were performed at 0–4°C. Except where otherwise stated, sucrose solutions were buffered at pH 7.4 with 3 mM imidazole-HCl. Rat liver homogenates were fractionated in 0.25 M sucrose as previously described (4) until unwashed microsome pellets were obtained. Treated and untreated microsomes were prepared identically from these microsome pellets, the designation "treated" meaning addition of digitonin, PPi, or EDTA at the appropriate step as described below. Depending on whether they were intended for analysis by isopycnic equilibration or by differential sedimentation in a density gradient respectively, microsomes were sedimented (7) at 40,000 rpm by an integrated force of $W = 3 \times 10^{10}$ rad$^2 \cdot$s^{-1} (P fraction) or 9×10^{10} rad$^{2} \cdot ^{-1}$ (P' fraction). The chemical composition of the treated and untreated preparations are compared in Table I. The enzymic activities of untreated microsomes are listed in Table II. Except for a partial inactivation of 5'-nucleotidase, caused by treatment of microsomes with EDTA, the enzymic activities of treated microsomes did not significantly differ from those obtained for untreated microsomes.

DIGITONIN-TREATED MICROSOMES: Unwashed microsome pellets were resuspended in 0.25 M sucrose by means of the tight pestle of a Dounce homogenizer, up to a final volume of 1 ml/g fresh weight of liver originally homogenized. 2.7 volumes of 0.25 M sucrose containing 0.235% (wt/vol) digitonin were added dropwise to the suspension with continuous stirring. After standing 15 min at 0°C, microsomes were washed once by centrifugation and resuspended in 0.25 M sucrose. For analysis by differential sedimentation in a density gradient, mi-

TABLE II

Enzymic Activities of Untreated Microsome Fractions

Enzyme	No. of exps.	Enzyme activity*
Galactosyltransferase	7	0.0083 ± 0.0023
5'-Nucleotidase	35	6.04 ± 2.26
Alkaline phosphodiesterase I	13	7.89 ± 2.53
Alkaline phosphatase	9	0.72 ± 0.36
Monoamine oxidase	47	0.103 ± 0.026
NADH cytochrome *c* reductase	40	62.2 ± 16.1
NADPH cytochrome *c* reductase	13	2.52 ± 0.90
Aminopyrine demethylase	3	0.052 ± 0.018
Cytochrome b_5	15	19.2 ± 3.0
Cytochrome P 450	3	21.7 ± 3.0
Esterase	17	178. ± 33.
Nucleoside diphosphatase	21	64.5 ± 13.1
Glucose 6-phosphatase	46	15.2 ± 3.9
Glucuronyltransferase	6	1.72 ± 0.40
β-Glucuronidase	9	0.30 ± 0.06

* Values refer to 1 g fresh weight of liver. They are given as means ± SD, in units for enzymes and in nanomoles for cytochromes. Glucuronyltransferase was assayed with 4-methylumbelliferone as acceptor.

[2] *Abbreviations used in this paper:* PPi, pyrophosphate; EDTA, ethylenediaminetetraacetate; W, time integral of the squared angular velocity ($W = \int_0^t \omega^2 dt$); DOC, deoxycholate; ER, endoplasmic reticulum.

crosomes were treated under the same conditions, but digitonin was added to a P′ fraction already washed once with 0.25 M sucrose. Treated microsomes were then subfractionated without any further washing.

PPI-TREATED MICROSOMES: Uwashed microsome pellets were resuspended (Dounce homogenizer, tight pestle) with 0.25 M sucrose containing 15 mM Na PPi at pH 8.2, up to a final volume of 2.9 ml/g liver from which microsomes derived. PPi was then washed out twice by centrifugation and resuspension of microsomes with 0.25 M sucrose.

EDTA-TREATED MICROSOMES: Unwashed microsome pellets were resuspended (Dounce homogenizer, tight pestle) with 0.25 M sucrose containing 50 mM EDTA at pH 7.4, up to a final volume of 3.7 ml/g liver originally homogenized. After centrifugation the pellets were treated once more in the same manner. EDTA was finally washed out by centrifugation and resuspension of microsomes in 0.25 M sucrose. The preparation of microsomes subjected to analysis by differential sedimentation in density gradient involved a single resuspension of the unwashed microsome pellets in EDTA-containing sucrose, followed by centrifugation and resuspension in 0.25 M sucrose.

MICROSOMES TREATED SUCCESSIVELY WITH EDTA AND DIGITONIN: Unwashed microsome pellets were resuspended in EDTA-containing sucrose (see above) and centrifuged by an integrated force of $W = 8 \times 10^{10}$ rad$^2 \cdot$s^{-1}. Digitonin was then added to resuspended microsomes, exactly as specified under "digitonin-treated microsomes." After centrifugation at the same integrated force the pellet was resuspended in 0.25 M sucrose.

Analysis by Isopycnic Density Gradient Centrifugation and by Differential Sedimentation in Density Gradient

TECHNIQUE: Operations were conducted exactly as described previously (7). Care was always taken to ensure that experiments on treated and untreated microsomes from the same homogenate were performed identically. Density equilibration was achieved in sucrose-H_2O gradients extending linearly with respect to volume from 1.10 to 1.25 in density (density of cushion = 1.34). The boundary technique, in which the initial concentration of particles was constant (microsomes obtained from 91 mg liver/ml) throughout a 0.25–0.50 M sucrose gradient, was adopted for analysis by differential sedimentation in density gradient. To achieve the standard initial conditions in experiments performed on digitonin-treated microsomes, the two microsome suspensions used for preparation of the sucrose gradient were made up by mixing 1 vol of the P′ suspension (microsomes from 0.27 g liver/ml in 0.25 M sucrose containing 0.17% digitonin) to 1.98 vol of 0.25 M and 0.627 M sucrose, respectively.

PRESENTATION OF RESULTS: To facilitate comparisons between different experiments, the results of isopycnic centrifugation are presented as previously (7), under the form of normalized histograms, averaged when necessary. The diagrams are plotted on a density (ρ) scale, expressed in g$\cdot$cm^{-3} and divided into 15 or 17 equal sections of density increment $\Delta\rho = 0.0113$. The ordinate gives the frequency within the corresponding span of density. The frequency is then $\Delta Q/(\Sigma Q \cdot \Delta\rho)$, where ΔQ is the absolute amount of constituent present within the section and ΣQ the sum of the absolute amounts found on all the microsomal subfractions. The dimension of the frequency scale is cm$^3 \cdot$g^{-1}. The surface area of each section of the diagram, which is the product, frequency $\times$ density increment, is thus equal to $\Delta Q/\Sigma Q$ and gives the fractional amount of constituent present within the section. The total surface area of each histogram should be equal to 1; however, it is slightly lower in the presentation adopted here, a small amount of constituent (less than 5%) being present beyond the density limits of the abscissa. The construction of normalized density distribution histograms was carried out as described in detail by Leighton et al. (19).

Similarly, the results of differential centrifugation are presented in the form of normalized sedimentation profiles, averaged whenever necessary. The diagrams are plotted on a volume (V) scale, expressed in milliliters, and divided, from the meniscus to the periphery, into a first section of 8 ml, 13 sections of 7 ml, and a last section of 11 ml. The ordinate gives the relative concentration C/C_i within the corresponding volume element, C being the final concentration of the constituent ($\Delta Q/\Delta$), and C_i the initial concentration ($\Sigma Q/110$) allowance made for recovery. C/C_i is thus computed as $(110 \cdot \Delta Q)/(\Delta V \cdot \Sigma Q)$, where ΔV is the actual volume of the section, and 110 the total volume in which microsomes were initially suspended. The surface area corresponding to each section is now $110 \cdot \Delta Q/\Sigma Q$. It gives the fractional amount of constituent, $\Delta Q/\Sigma Q$, after division by 110, the gradient volume expressed in milliliters. The construction of normalized sedimentation diagrams was carried out after smoothing the experimental plots in a manner similar to that recommended by Leighton et al. (19) for normalization of density distributions. The relative concentration is not reported for the last section, which begins at 7.34 cm from the axis, because it contains particles packed upon the cushion.

To evaluate more quantitatively the differences between treated and untreated microsomes, associated tables give the median densities (density equilibration experiments), or the radial distances for $C/C_i = 0.5$ and the amounts recovered beyond 7.34 cm from the axis (differential centrifugation in density gradient). Since the behavior of untreated microsomes has always been found similar to that reported before (7), averaged data concerning untreated microsomes are used here as reference to show the influence of digitonin, PPi, and EDTA on the physical properties of microsomes.

Biochemical and Morphological Methods

These methods have been described previously (6, 33). Whenever necessary, $MgCl_2$ was added in excess over EDTA for determination of Mg^{++}-dependent enzymes. This, however, did not fully restore activity of 5'-nucleotidase.

Chemicals

Digitonin was a product from Merck A. G., Darmstadt, W. Germany. According to the Merck Index, published by Merck and Co., Inc., Rahway, N.J., the commercial product contains 70–80% digitonin, 10–20% gitonin and tigonin which also form a complex with cholesterol, and 5–15% minor saponins. Molar concentrations of digitonin were computed assuming that the product was pure.

RESULTS

Modification of Microsomes by Digitonin Treatment

Subfractionation of microsomes distinguished cholesterol from constituents attributed to endoplasmic reticulum (ER) (7). It was thus advisable to examine the effects of digitonin, which forms an insoluble equimolar complex with cholesterol, upon the physical characteristics of microsomal entities. The latency of nucleoside diphosphatase (13), an enzyme probably located on the inner surface of vesicles deriving from ER (18), was used as the criterion for testing the structural integrity of digitonin-treated ER components of microsomes. The experiment presented in Fig. 1 shows that when microsomes were incubated with digitonin at various concentrations, the structure-linked latency of nucleoside diphosphatase was retained up to 2.35 mg digitonin/ml, but higher concentrations overcame the latency and released partially the enzyme into the suspending medium (not shown). These observations are valid only for the concentration of microsomes used (microsomes obtained from 0.27 g fresh weight of liver/ml). Indeed, the digitonin concentration from which nucleoside diphosphatase became unmasked decreased together with the concentration of microsomes (not shown).

In view of these data, the conditions specified under Materials and Methods (1.7 mg digitonin/ml and /0.27 g liver) were adopted for the treatment of microsomes. They correspond to a slight molar excess of digitonin over the microsomal cholesterol (less than 1.2/1 since digitonin preparations are not pure). The biochemical composition of digitonin-treated microsomes did not differ significantly from that of normal microsomes (Table I). Electron microscopy (Fig. 2) also revealed little alteration of microsomes by digitonin treatment. Most microsomal profiles, in particular all profiles of rough microsomes, appear quite normal. However, a few large smooth elements, amounting to about 9–10% of the total surface area of microsomal membranes, seem to have been broken or fenestrated.

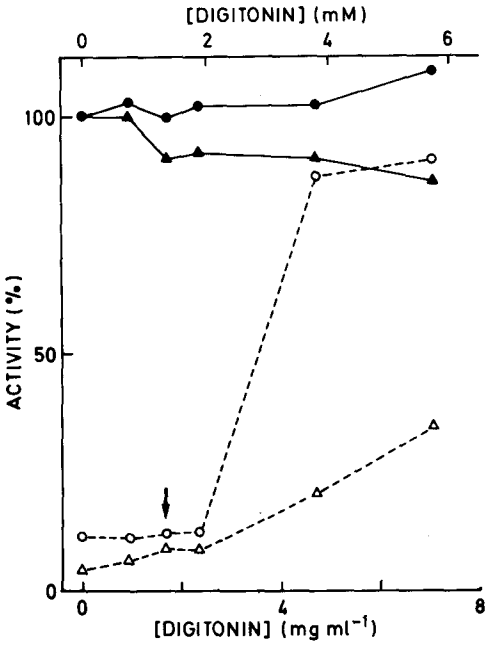

FIGURE 1 Structural latency of nucleoside diphosphatase and galactosyltransferase in microsomes treated with various amounts of digitonin. To suspensions containing per milliliter the microsomes obtained from 1 g liver, were added 2.7 vol of ice-cold 0.25 M sucrose supplemented with digitonin to obtain the final concentrations reported (abscissa). Nucleoside diphosphatase (circles) and galactosyltransferase (triangles) were then assayed for free (○, △) and total (●, ▲) activity. The methods previously described (6) were followed, except that the incubation medium was made 0.25 M in sucrose (free and total activities), and that free activity was assayed with omission of Na DOC or Triton X-100. Activities (ordinate) are expressed in percent of the total activity in absence of digitonin. The arrow indicates the final concentration of digitonin when microsomes are treated as described under Materials and Methods.

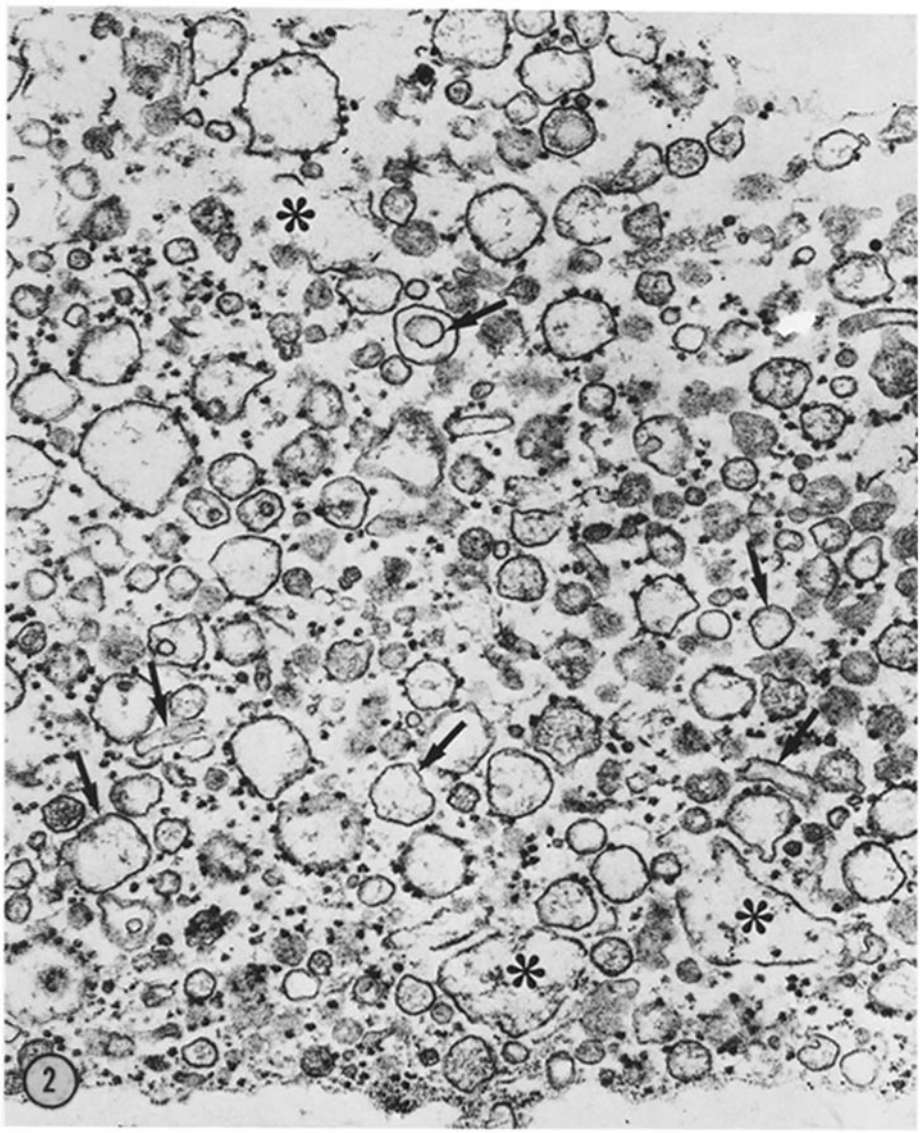

FIGURE 2 Morphology of digitonin-treated microsomes. Unwashed P fraction treated with digitonin as described under Materials and Methods. All the rough elements and the majority of smooth profiles (arrows) look unaffected by digitonin. Some smooth, usually large, elements present a broken appearance (asterisks). × 50,000.

The density distributions of various constituents of digitonin-treated microsomes after equilibration in linear sucrose-H_2O gradient are represented in Figs. 3 and 4. Median densities and recoveries are listed in Table III. In Fig. 3, comparison is made with untreated microsomes. It can be seen that the treatment with digitonin had no influence on the density distribution of NADH cytochrome *c* reductase and glucose 6-phosphatase. The density distribution profiles of these latter enzymes, taken as models of groups *b* and *c* respectively, were almost superimposable upon those of untreated microsomes. As shown by Fig. 4, NADPH cytochrome *c* reductase, aminopyrine demethylase, cytochromes b_5 and P 450, all previously classified into group *b*, still followed fairly well NADH

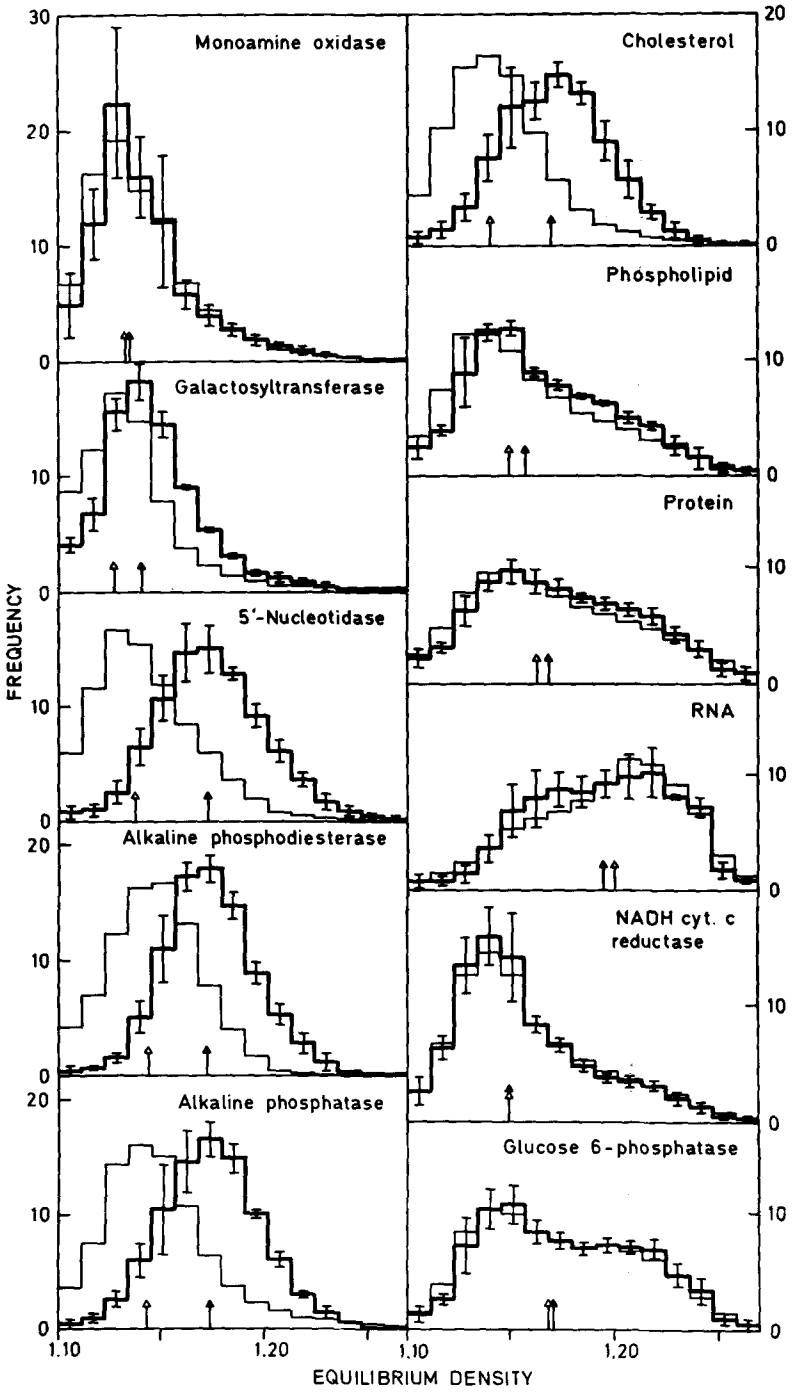

FIGURE 3 Influence of the treatment with digitonin on the density distribution of microsomal constituents in sucrose-H_2O gradient. Microsomes have been treated with digitonin, as described under Materials and Methods, and equilibrated afterwards in a linear gradient of sucrose. The frequency histograms obtained (thick lines) were averaged and normalized, as described under Presentation of Results. The represented portion extends from 1.10 to 1.27 in density. Vertical lines through histogram bars represent standard deviations. The density distributions obtained from untreated microsomes (thin lines) are reproduced from Fig. 2 of reference 7 or give the average results of 14 (monoamine oxidase), 2 (galactosyltransferase), 6 (alkaline phosphodiesterase I), and 5 (alkaline phosphatase) unpublished experiments. Arrows indicate the median densities of constituents in digitonin-treated (black arrow) or untreated (white arrow) microsomes.

cytochrome *c* reductase after digitonin treatment; likewise esterase, nucleoside diphosphatase, glucuronyltransferase and to a lesser extent β-glucuronidase, previously classified into group *c*, paralleled glucose 6-phosphatase.

Group *a* constituents exhibited a different picture. Whereas the distribution of monoamine oxidase was not significantly modified, those of 5'-nucleotidase, alkaline phosphodiesterase I, alkaline phosphatase, and cholesterol were markedly shifted towards higher densities. The median density of the four latter constituents showed an

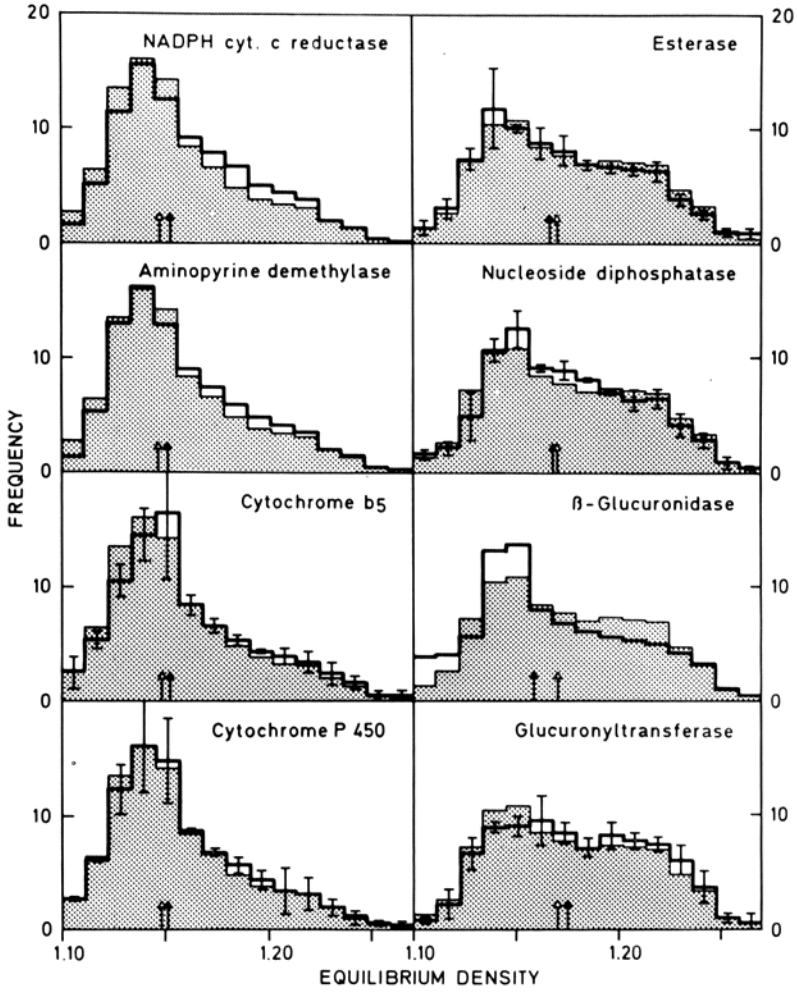

FIGURE 4 Density distribution of constituents of groups *b* (left-hand column) and *c* (right-hand column) after isopycnic equilibration of digitonin-treated microsomes in sucrose-H_2O gradient. Frequency histograms were averaged and normalized as explained under Presentation of Results. The represented portion of histograms extends from 1.10 to 1.27 in density. Vertical lines through histogram bars represent standard deviations. The distributions of constituents indicated in each compartment are represented by a thick line and the corresponding median densities, by a black arrow. To facilitate comparisons, the distribution of NADH cytochrome *c* reductase is represented by the thin line (shading) superimposed on the profiles of group *b* constituents (left-hand column), and the distribution of glucose 6-phosphatase is represented similarly on the profiles of group *c* constituents (right-hand column). The median density of these reference enzymes is marked by a white arrow. The profiles of glucuronyltransferase obtained with 4-methylumbelliferone (shown) and bilirubin (not shown) almost coincided.

TABLE III

Median Density and Recovery of Microsomal Constituents Equilibrated in Sucrose-H_2O Gradients after Treatment with Digitonin

Constituent	No. of exps.	Recovery*	Median density	
			Observed*	Excess over control‡ ($\times 10^4$)
		%		
Protein	6	105.5 ± 9.9	1.1692 ± .0009	55[b]
Phospholipid	3	97.5 ± 13.4	1.1580 ± .0016	84[c]
Galactosyltransferase	2	88.6 ± 11.4	1.1410 ± .0005	141[a]
Monoamine oxidase	5	88.8 ± 8.0	1.1355 ± .0058	23
5'-Nucleotidase	6	107.0 ± 13.2	1.1724 ± .0017	352[d]
Cholesterol	5	96.6 ± 14.8	1.1705 ± .0037	292[d]
Alkaline phosphodiesterase I	4	100.9 ± 11.6	1.1723 ± .0020	278[d]
Alkaline phosphatase	3	99.4 ± 14.7	1.1738 ± .0010	303[d]
NADH cytochrome *c* reductase	5	102.6 ± 20.6	1.1482 ± .0009	−6
Cytochrome b_5	3	112.4 ± 22.7	1.1519 ± .0025	23
Cytochrome P 450	2	86.5 ± 6.5	1.1508 ± .0041	−25
NADPH cytochrome *c* reductase	1	110.7	1.1537	1
Aminopyrine demethylase	1	131.3	1.1521	−6
Esterase	3	107.6 ± 13.8	1.1666 ± .0035	−1
Nucleoside diphosphatase	2	101.8 ± 7.6	1.1684 ± .0019	28
Glucose 6-phosphatase	5	100.6 ± 8.9	1.1703 ± .0050	25
Glucuronyltransferase	1	55.7	1.1836	48
β-Glucuronidase	1	108.2	1.1589	−36
Ribonucleic acid	3	72.9 ± 13.9	1.1951 ± .0054	−56

* Statistics refer to mean ± S.D.

‡ Value observed for digitonin-treated microsomes minus that observed for untreated microsomes (Table II of reference 7). a–d indicate that difference from control is significant at $P < 0.1$ (a), $P < 0.05$ (b), $P < 0.02$ (c) and $P < 0.001$ (d).

increase of 0.028–0.035 density unit, which is highly significant (Table III). Experiments in which microsomes were treated with digitonin at various concentrations have shown (Fig. 5) that the median equilibrium density of 5'-nucleotidase and alkaline phosphodiesterase I rises and levels off at a digitonin concentration of about 1.4 mg (1.14 μmol)/ml. Since digitonin preparations contain minor saponins which may not react with cholesterol, the concentration required to maximize the digitonin shift reduces to 1.08–0.97 mM. The corrected concentration coincides satisfactorily with that of microsomal cholesterol (found: 0.93 mM) in the reaction mixture. We do not know, however, what the partition of digitonin is between the water phase and the various microsomal membranes. A less pronounced shift was observed for galactosyltransferase, the median density of which showed an increase of 0.014 unit. The digitonin shift of galactosyltransferase was more pronounced in experiments not reported here. In particular, a relationship between density shift and digitonin concentration, similar to those presented in Fig. 5, has been found.

The distribution patterns of protein and phospholipid were only moderately modified, in a manner suggestive of the transfer of some microsomal components from the upper to the central part of the gradient. The corresponding increase of median densities appears significant, especially for phospholipids which were more markedly displaced. In contrast, the slight difference in the density distribution of RNA is uncertain. These findings are in agreement with the shift of some microsomal enzymes caused by digitonin treatment.

As shown in Fig. 6 and Table IV, the modifications of sedimentation properties noticed after treatment of microsomes with digitonin were small and fell within the range of experimental varia-

tions. These data, however, confirm that none of the microsomal entities has been extensively disaggregated by digitonin.

Modification of Microsomes by Treatment with PPi or EDTA

The morphometric study made by Wibo et al. (33) has shown that the ribosome load of microsomal vesicles is the major determinant of their equilibrium density in sucrose gradient. We have thus undertaken to analyze microsomes treated with reagents known to remove membrane-bound ribosomes, but which only minimally affect other membrane properties. EDTA (21, 22) and PPi (23) were selected.

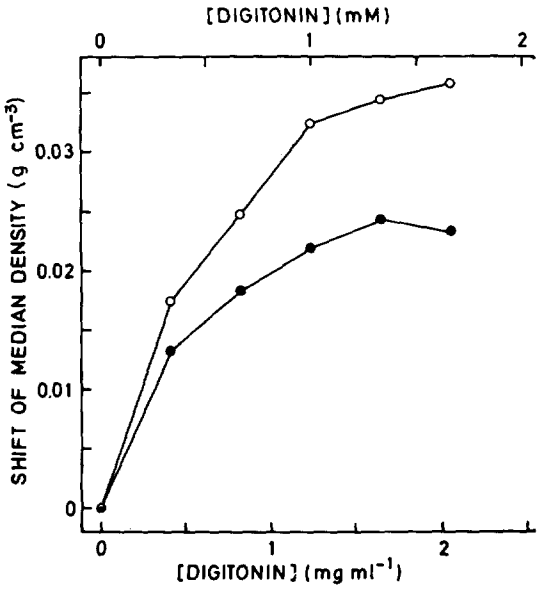

FIGURE 5 Influence of digitonin concentration on the density shift of 5′-nucleotidase (○) and alkaline phosphodiesterase I (●). Unwashed microsomes were divided into six samples and treated with digitonin as described under Materials and Methods, except that in the 0.25 M sucrose solution added to the microsome suspension the concentration of digitonin ranged from 0 to 0.28% (wt/vol). 0.5-ml portions of these preparations were layered on top of a linear gradient of sucrose extending from 1.10 to 1.22 in density over a total volume of approximately 5 ml. After 12-h centrifugation at 35,000 rpm ($W = 5.8 \times 10^{11}$ rad$^2 \cdot$s^{-1}) in the SW 50-1 rotor, the gradients were processed exactly as described by Beaufay et al. (8). The shift of median densities is plotted against the concentration of digitonin expressed in milligrams per milliliter (below), or in micromoles per milliliter (above). Microsomes contained 1.33 mg cholesterol/g liver from which they derived.

The influence of PPi concentration on the release of various constituents from microsomes is shown in Fig. 7. Over the whole range of concentration studied, glucose 6-phosphatase, esterase, and nucleoside diphosphatase remained essentially sedimentable. On the contrary, the amount of RNA sedimenting with the microsomes obtained from 1 g liver was lowered from 6.1 to 1.5 mg when the PPi concentration exceeded .10 mM. The protein content of these microsomes was simultaneously lowered from 44.2 to 35.5 mg. The detachment of ribosomes or ribosome subunits from membranes accounts thus for only a part (about 3.7 mg) of the protein released by PPi. Fumarase was completely released from microsomes by PPi, as were aldolase and glutamine synthetase (not shown). Data on the biochemical composition of PPi-treated microsomes are given in Table I, and their appearance under the electron microscope is illustrated by Fig. 8. Electron microscopy confirms the biochemical evidence for the removal of ribosomes. Both results agree with the earlier observations reported by Sachs (23), although the higher pH adopted may account for the more extensive release of RNA in our experiments.

Under the conditions used in this work, treatment of microsomes with EDTA exerted effects similar to those described for PPi; the content of microsomes in protein, fumarase, and aldolase was lowered to the same extent, but less RNA was removed (Table V). Fig. 9 shows the appearance of EDTA-treated microsomes under the electron microscope. A few residual particles can be seen on the outer surface of some vesicles, but they are much less numerous and smaller than in untreated microsomes. Our biochemical and morphological data are in full agreement with previous reports by Palade and Siekevitz (21) and Sabatini et al. (22).

A comparison of the density distributions of some typical constituents of untreated and of PPi- or EDTA-treated microsomes is presented in Figs. 10 and 11. The corresponding median densities are reported in Tables VI and VII. Treating microsomes with PPi or EDTA led to similar effects, apparently somewhat more pronounced in the second case. In contrast to the specific action of digitonin upon certain enzymes of group *a*, PPi and EDTA decreased the density of all the microsomal constituents studied. Nevertheless, the influence of these agents on the various groups is easily

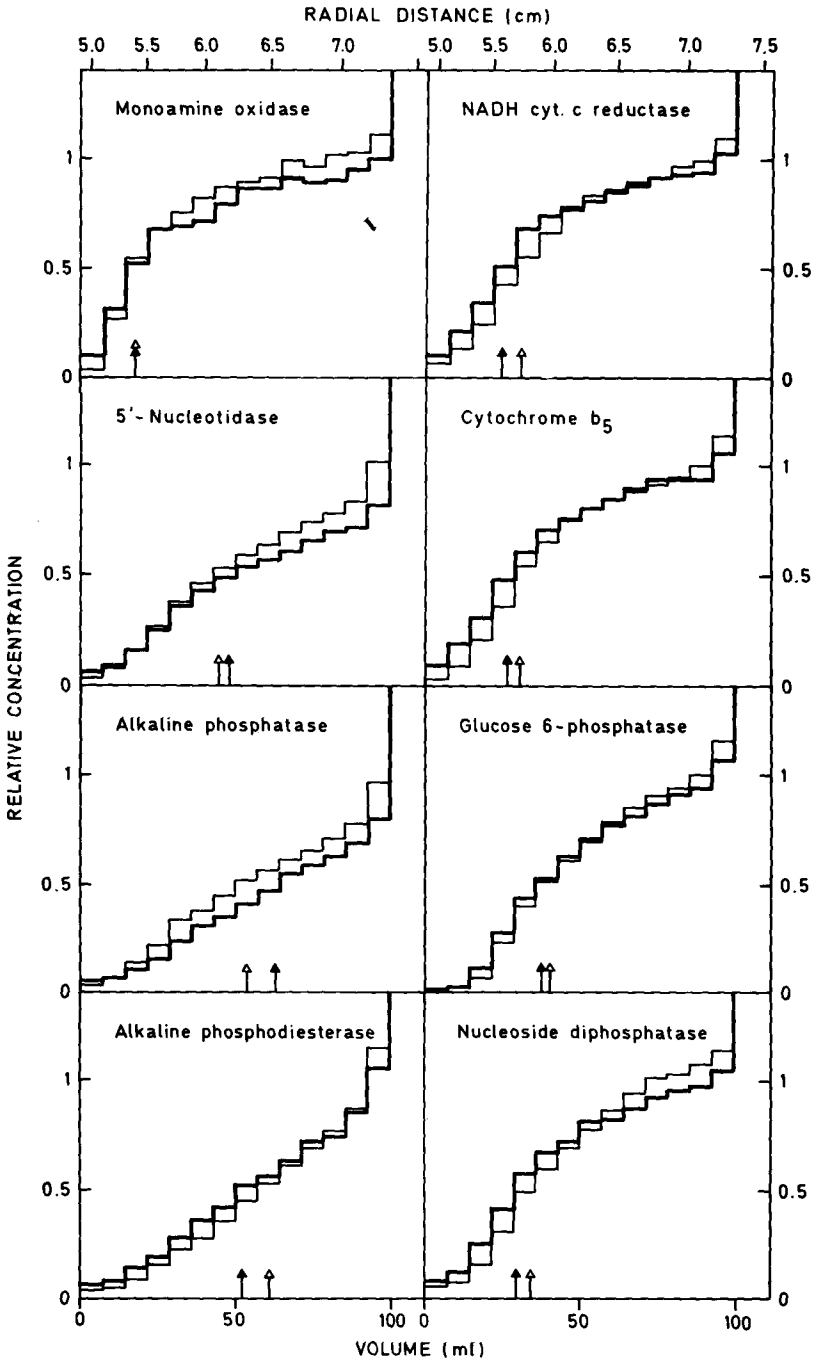

FIGURE 6 Influence of the treatment with digitonin on the sedimentation pattern of microsomal constituents after differential sedimentation in a density gradient. P′ fractions (two experiments) were treated with digitonin as described under Materials and Methods, and submitted afterwards to boundary analysis by differential sedimentation in a stabilizing gradient of sucrose ($W_{average} = 9.75 \times 10^9$ rad$^2 \cdot$s^{-1}). The sedimentation profiles obtained (thick lines) were averaged and normalized as explained under Presentation of Results. The sedimentation profiles obtained from untreated microsomes (thin lines) are reproduced from Fig. 9 of reference 7. The position in the gradient at which $C/C_i = 0.5$ is marked by a white (untreated microsomes) or a black arrow (digitonin-treated microsomes). 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. 6

Constituent	Recovery	Amount recovered beyond 7.34 cm from axis*		Radial distance traveled at $C/C_i = 0.5$§	
		Observed	Excess over control‡	Observed	Excess over control‡
	%				
Monoamine oxidase	102.3 ± 5.2	35.3 ± 5.0	2.1	5.3 ± 0.6	−0.1
5'-Nucleotidase	105.8 ± 3.0	58.9 ± 6.9	3.2	13.9 ± 3.5	1.2
Alkaline phosphodiesterase I	65.7 ± 2.2	58.1 ± 8.5	−2.9	14.3 ± 3.5	−1.9
Alkaline phosphatase	96.7 ± 1.6	65.5 ± 4.7	5.3	17.4 ± 2.6	2.8
NADH cytochrome *c* reductase	99.5 ± 4.1	38.3 ± 4.4	−3.7	7.3 ± 0.9	−1.4
Cytochrome b_5	93.3 ± 2.3	38.9 ± 5.3	−4.3	7.7 ± 1.4	−1.2
Glucose 6-phosphatase	105.1 ± 0.4	49.1 ± 4.5	−1.6	10.6 ± 1.4	−0.7
Nucleoside diphosphatase	99.3	41.4	−2.9	8.4	−1.4

Results of two experiments, except for nucleoside diphosphatase. Statistics refer to mean ± SEM.

* Amount of constituent recovered in the last fraction of Fig. 6 and in the cushion, expressed in percent of the total amount recovered.

‡ Value observed for digitonin-treated microsomes minus that observed for untreated microsomes (Table IV of reference 7). Except for 5'-nucleotidase ($P < 0.05$) these differences are not significant.

§ Expressed in mm from the meniscus and computed by linear interpolation, assuming that the average values of C/C_i are attained in the middle of the fraction.

distinguishable in two respects: (*a*) the lowering of the equilibrium density of cholesterol and of enzymes classified into group *a* was weak and variable, being greater in the case of NADH cytochrome *c* reductase and still stronger in that of glucose 6-phosphatase. (*b*) The distribution profiles of constituents of group *a* were merely shifted towards slightly lower densities, whereas those of NADH cytochrome *c* reductase and glucose 6-phosphatase became sharper and more symmetrical than in untreated microsomes. The density of microsomes which normally equilibrate in the densest part of the gradient was clearly lowered to a much greater extent than that of lighter elements, which resulted in a greater physical homogeneity. This behavior was displayed by all enzymes of groups *b* and *c*. As illustrated by Fig. 12, NADPH cytochrome *c* reductase and aminopyrine demethylase on the one hand, and esterase, nucleoside diphosphatase and β-glucuronidase on the other hand, still followed fairly well NADH cytochrome *c* reductase and glucose 6-phosphatase, respectively, after PPi treatment. Furthermore, the distributions of phospholipid and protein suffered modifications very similar to those of enzymes of groups *b* and *c* (Figs. 10 and 11). Finally, the density distribution of RNA after treatment with EDTA was shifted more extensively than any other one and, unexpectedly, the ratio of RNA to phospholipid or protein in microsomal subfractions did not increase anymore with density. In fact, all microsomal constituents equilibrated within about the same relatively narrow density range after treatment with PPi or EDTA. Agglutination of microsomes was obviously a possible explanation for these results. This possibility was tested by submitting EDTA-treated microsomes to differential sedimentation in a density gradient. The data presented in Fig. 13 and Table VIII clearly show that no agglutination occurred after the treatment. On the contrary, the sedimentation rate of all constituents was decreased, probably as a consequence of the lowering in density produced by EDTA.

Modification of Microsomes by Subsequent Treatments with EDTA and Digitonin

The result of the combined actions of digitonin and EDTA on microsomes was roughly equivalent to the sum of the effects of the agents used separately, with respect to the biochemical composition of microsomes, to the density of constituents in sucrose gradient (Fig. 14 and Table IX), and to

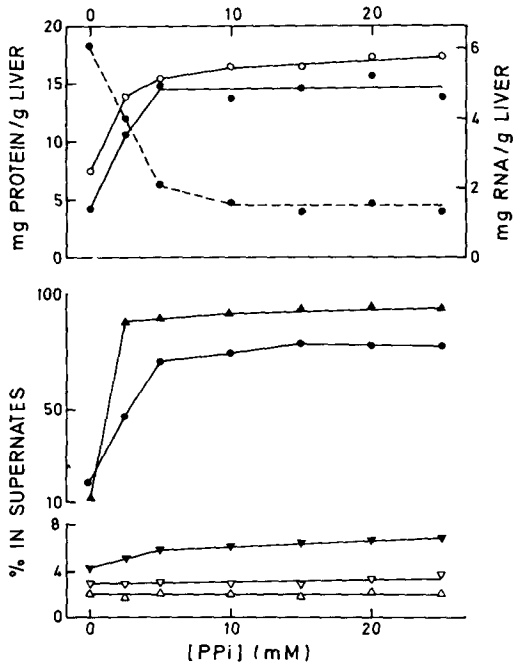

FIGURE 7 Release of constituents from microsomes treated with PPi. Microsomes (68.5 mg protein obtained from 1.38 g liver) were incubated 15 min at 0°C and pH 8.2 in 11 ml 0.25 M sucrose containing Na PPi at various concentrations (abscissa), centrifuged at $W = 3 \times 10^{10}$ rad$^2 \cdot$s^{-1}, washed, and resuspended in 0.25 M sucrose. The supernates and the sedimented material were assayed for the following constituents: protein (○), RNA (●), glucose 6-phosphatase (▽), nucleoside diphosphatase (△), esterase (▼) and fumarase (▲). Continuous lines give the amount of constituent present in the supernates. The dashed line gives the amount of RNA present in the sediments. Absolute values (upper graph) refer to 1 g fresh weight of liver; percentage values (lower graph) refer to the recovered amount of constituent. Recoveries ranged between 87 and 109%, except for RNA (76–101%).

morphological alterations (Fig. 15 *a*). Similar additivity relationships resulted also from subsequent treatments of microsomes with PPi and digitonin (not shown). The combined use of EDTA and digitonin is of great analytical significance. Indeed, microsomal constituents responded to this treatment in three distinct ways: (*a*) the density distributions of 5′-nucleotidase, alkaline phosphodiesterase I, and cholesterol shifted towards higher densities. (*b*) The distribution pattern of monoamine oxidase did not change noticeably. (*c*) NADH cytochrome *c* reductase and glucose 6-phosphatase, accompanied by protein, shifted towards lower densities and gained in symmetry. Most dramatic was the shift of RNA which apparently became the lightest constituent. Very likely, part of this RNA represents ribosome fragments detached from membranes and remote from their equilibrium position. The occurrence of ribosome fragments is explainable by the higher integrated force used for sedimentation of particles after the treatments with EDTA and digitonin (see Materials and Methods).

In practice, the distribution patterns were modified to such an extent that 5′-nucleotidase, alkaline phosphodiesterase I, and cholesterol equilibrated at a higher density than all other enzymes studied and were the only ones recovered in measurable amount beyond 1.20 in density. Fig. 15 *b* shows the appearance of a dense subfraction obtained from microsomes treated with EDTA and digitonin. The broken-looking elements detected as a minor component in digitonin-treated microsomes (Figs. 2 and 15 *a*) predominate so largely that other components, such as vesicles bearing remnants of ribosomes, appear as rare contaminants within the subfraction.

DISCUSSION

Action of Digitonin on Microsomal Components

Digitonin may act upon subcellular membranes by its capacity to form a complex with cholesterol, by its detergent properties or both. In this work, digitonin was consistently added in very slight molar excess over microsomal cholesterol to study the physical and morphological alterations induced. Under these standard conditions it acted in a strikingly specific manner upon some microsomal components.

Vesicles derived from ER did not suffer any detectable alteration. This is supported by the following facts. (*a*) The chemical (Table I) and enzymic composition of digitonin-treated microsomes does not differ significantly from that of untreated microsomes. (*b*) The binding of nucleoside diphosphatase to microsomes and the structure-linked latency of this releasable intracisternal enzyme are maintained (Fig. 1). We are inclined to attribute the release occurring at higher concentrations to the detergent properties of digitonin. (*c*)

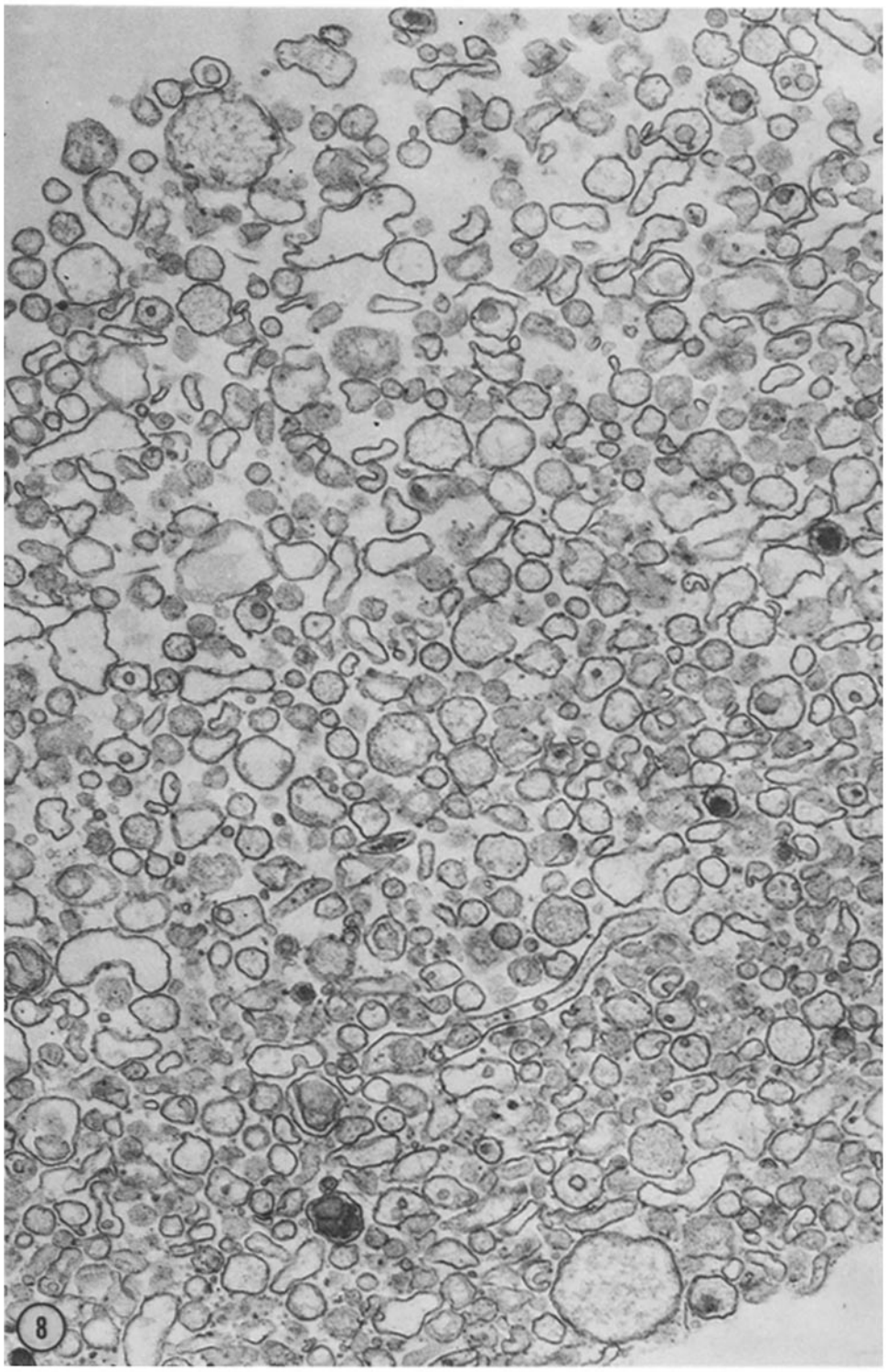

FIGURE 8 Morphology of PPi-treated microsomes. Unwashed P fraction treated with PPi as described under Materials and Methods. Ribosomal profiles are less frequent than in untreated (compare with Fig. 2 *a* of reference 4) or in digitonin-treated microsomes (compare with Fig. 2). Most ribosomal remnants appear membrane-bound and many of them are smaller than normal ribosomes. Otherwise the microsomal vesicles display the usual morphology. × 38,000.

TABLE V

*Release of Constituents by Treatment of Microsomes with EDTA**

Constituent	Unwashed microsomes	EDTA		Control	
		Microsome preparation	Supernate	Microsome preparation	Supernate
Protein	56.8	38.6	21.6	47.2	11.1
Phospholipid	17.9	20.2	1.4	17.7	2.0
Ribonucleic acid	7.3	3.5	4.4	6.6	1.0
Esterase	165.0	159.0	6.0	177.0	8.0
Nucleoside diphosphatase	49.6	39.6	1.2	38.3	0.7
Glucose 6-phosphatase	13.5	13.3	0.1	12.6	0.5
Fumarase	8.1	0.5	10.1	7.0	0.5
Aldolase	3.6	0.2	3.6	3.3	0.8

* Pellets obtained from a postmitochondrial supernate by centrifugation at 40,000 rpm ($W = 3 \times 10^{10}$ rad^2s^{-1}) were resuspended in 0.25 m sucrose (unwashed microsomes) or washed twice with 0.25 M sucrose, supplemented (EDTA) or not (Control) with 50 mM EDTA, as described under Materials and Methods. Enzymes and constituents were determined in the microsome prepatations and in the supernatant fluids. Results are given in milligrams (protein, RNA), in micromoles of organic phsophorus (phospholipid), or in units of enzyme activity, and refer to 1 g fresh weight of liver.

Electron microscopy fails to reveal any structural modification of many smooth microsomal vesicles and of all rough vesicles which obviously derive from ER (Fig. 2). (*d*) RNA and enzymes of groups *b* and *c*, attributed to ER (7), equilibrate in sucrose-H_2O gradient in a manner indistinguishable from that exhibited by untreated microsomes (Figs. 3 and 4). (*e*) No pronounced and significant difference in the sedimentation patterns of these constituents results from the treatment with digitonin (Fig. 6).

The microsomal particles bearing monoamine oxidase also appear to be unaffected by digitonin at the concentration used in this study.

In contrast, the microsomal component bearing the enzymes of group *a2* suffered physical alterations evidenced by a marked increase in equilibrium density (Fig. 3). Furthermore, its appearance under the electron microscope was transformed into broken-looking profiles of membranes and vesicles (Fig. 2). Indeed, the infrequent elements which exhibit that appearance in digitonin-treated microsomes may be unambiguously identified with the microsomes bearing the group *a2*. The subfraction of density 1.204–1.224 obtained from microsomes treated with EDTA and digitonin (Fig. 14) brings out a clear-cut correlation between the frequency of broken-looking membranes (Fig. 15 *b*) and the selective concentration of 5'-nucleotidase and alkaline phosphodiesterase I. At first glance, electron microscopy suggests the disaggregation of the membranes into small fragments. Nevertheless, this opinion is untenable. A careful examination of Figs. 2, 15 *a* and 15 *b* reveals that the visible elements of the membranes are not randomly distributed in small fragments, but arrange themselves into easily recognizable large vesicles and leaflets. Furthermore, the rate of sedimentation of group *a2* remains unchanged (or increases) and is still higher than that of other constituents after treatment of microsomes with digitonin (Fig. 6). The vesicles and the membranes related with group *a2* are thus not decreased in size by digitonin. Another relevant observation is that none of the enzymes of group *a2* is detached from microsomes.

It is undoubtedly significant that the particles physically and morphologically altered by digitonin represent the minor microsomal component which carries most of the cholesterol of the microsome fraction (7). Further, the extent of the shift increases with the amount of digitonin added. A plateau is attained when the molar concentration of digitonin is close to that of microsomal cholesterol (Fig. 5). Accordingly, it appears justified to attribute the alterations observed to the formation of a digitonin-cholesterol complex which remains associated with the membranes. We know, indeed, that cholesterol is not removed from microsomes by digitonin (Table I), and that its original localization is largely preserved, since its density distribution in sucrose gradient still follows

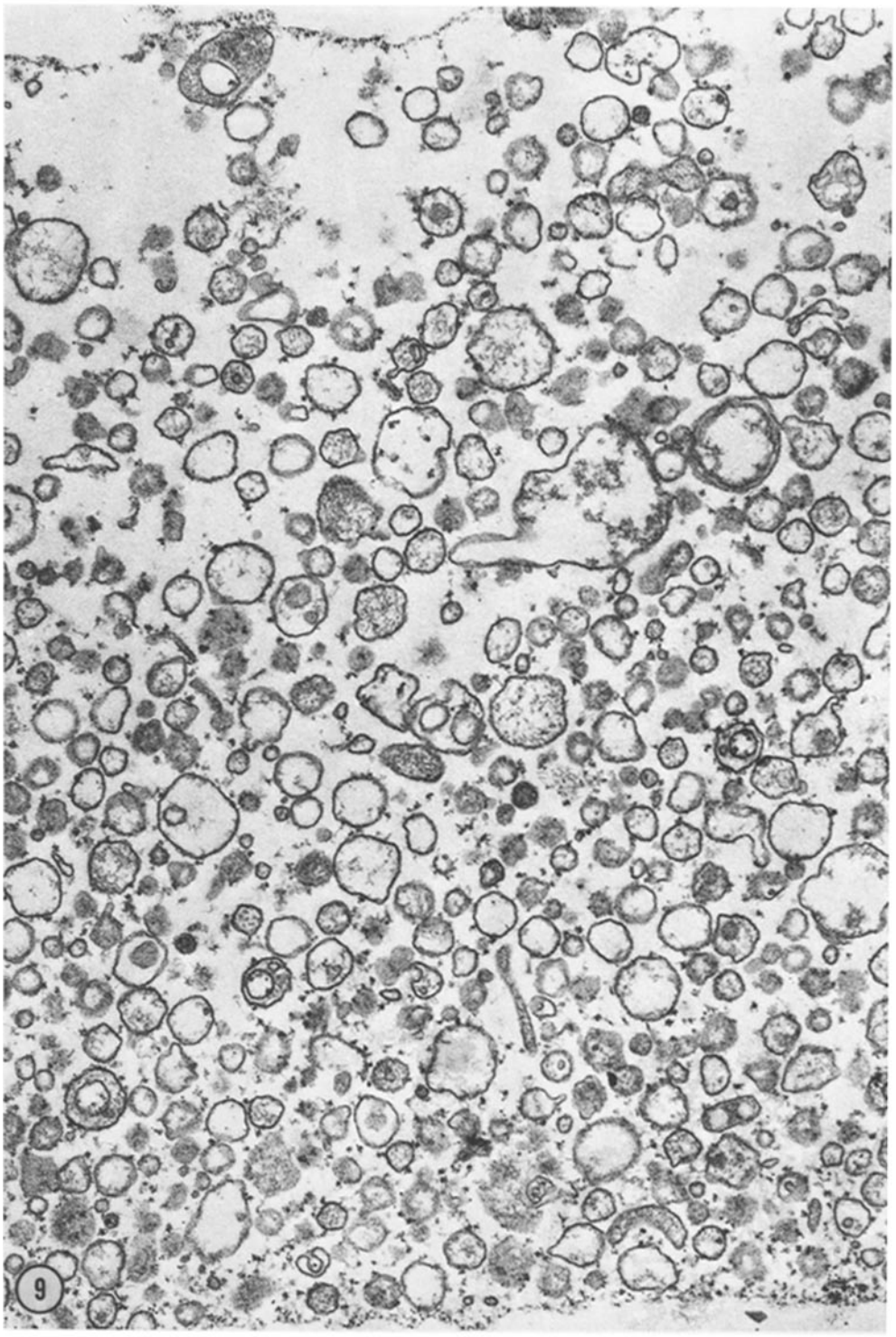

FIGURE 9 Morphology of EDTA-treated microsomes. P′ fraction treated as described under Materials and Methods. Few ribosomal profiles are visible and most of them are smaller than usual. Ferritin particles are present at the lower limit of the pellicle. × 39,000.

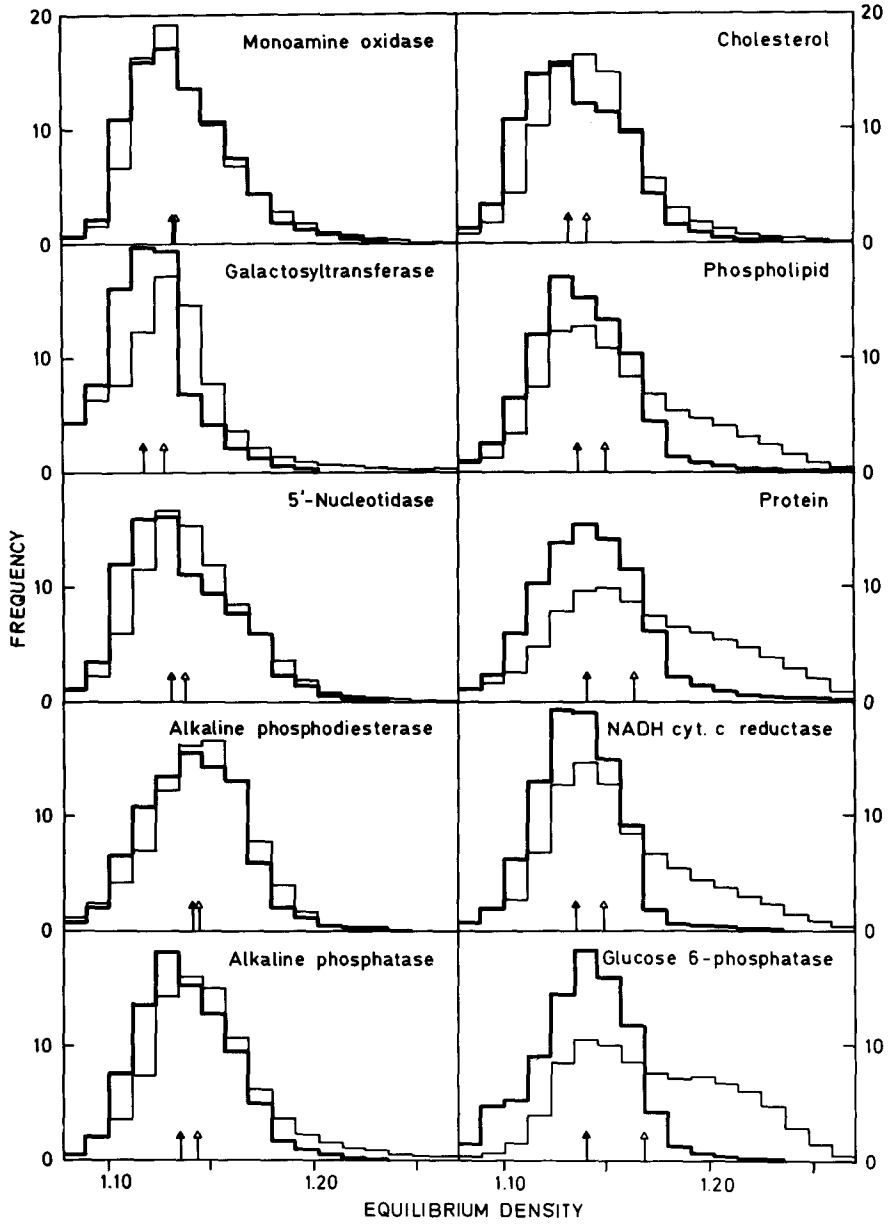

FIGURE 10 Influence of the treatment with PPi on the density distribution of microsomal constituents in sucrose-H_2O gradient. Microsomes were treated with PPi, as described under Materials and Methods and equilibrated afterwards in a linear gradient of sucrose. The frequency histograms obtained are given by the thick lines in a normalized form (see Presentation of Results). The represented portion extends from 1.0773 to 1.27 in density. The density distributions for untreated microsomes (thin lines) were constructed from experiments reported in Fig. 3. Arrows indicate the median densities of constituents in PPi-treated (black arrow) or untreated (white arrow) microsomes.

that of group *a2* enzymes (Fig. 3). Although we have no direct evidence supporting the association of digitonin with microsomal membranes related to group *a2*, their physical and morphological modifications can be interpreted on that basis. Indeed, the theoretical digitonin-binding capacity of these membranes is extremely high. If we attribute to them 67% of cholesterol (which is a

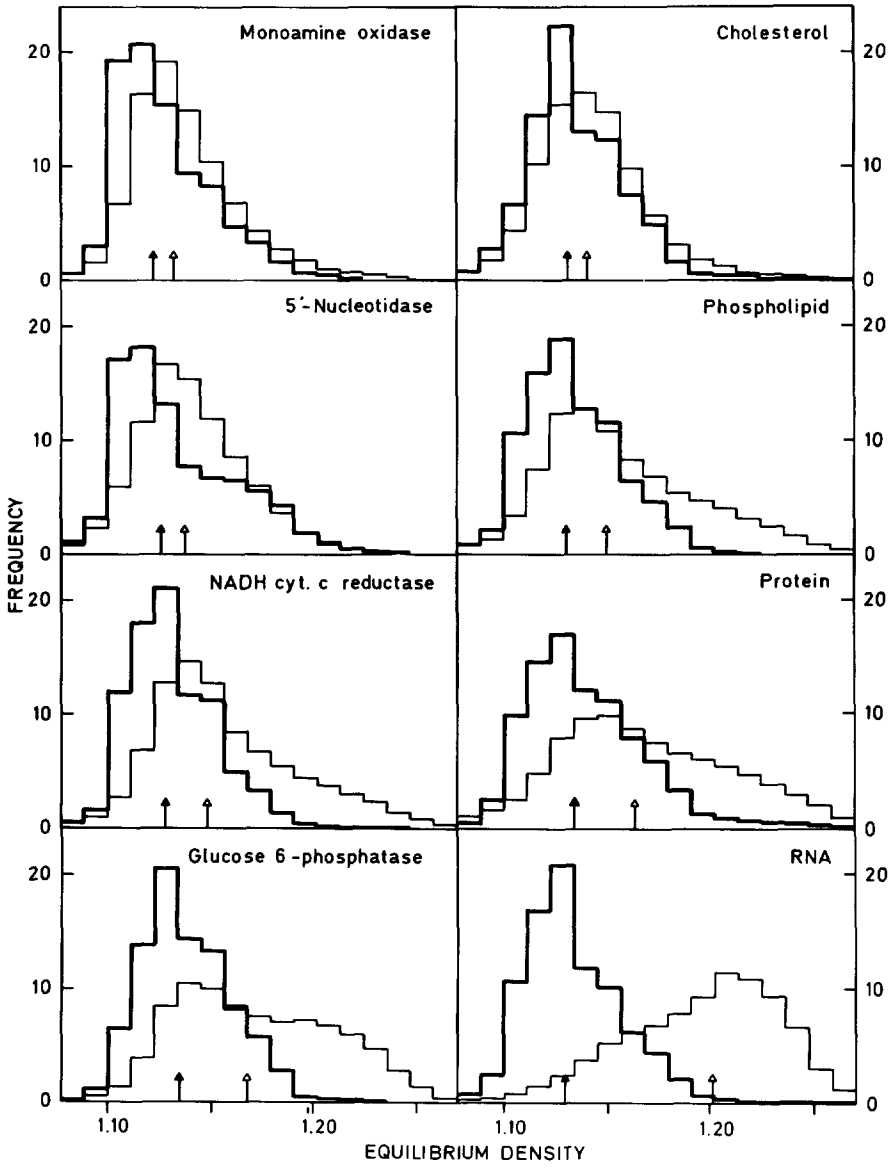

FIGURE 11 Influence of the treatment with EDTA on the density distribution of microsomal constituents in sucrose-H_2O gradient. Microsomes were treated with EDTA, as described under Materials and Methods and equilibrated afterwards in a linear gradient of sucrose. The frequency histograms obtained are given by the thick lines in a normalized form (see Presentation of Results). The represented portion extends from 1.0773 to 1.27. The density distributions for untreated microsomes (thin lines) were constructed from experiments reported in Fig. 3. Arrows indicate the median densities of constituents in EDTA-treated (black arrow) or untreated (white arrow) microsomes.

minimum) and 7.5% of protein of the microsome fraction (7), the *a2* component contains 0.35 mg total cholesterol/mg protein. This value, which agrees with determinations made by Touster et al. (30) on rat liver plasma membranes, permits the binding of 1.1 mg digitonin to form 1.45 mg digitonin-cholesterol complex per milligram protein, since it is generally admitted that cholesterol

TABLE VI

Median Density and Recovery of Microsomal Constituents Equilibrated in Sucrose-H_2O Gradient after Treatment with PPi

Constituent	Recovery	Median density: Observed	Median density: Excess over control* ($\times 10^4$)
	%		
Protein	94.1	1.1410	−227
Phospholipid	94.0	1.1366	−130
Galactosyltransferase	90.2	1.1171	−98
Monoamine oxidase	94.0	1.1317	−15
5'-Nucleotidase	128.8	1.1302	−70
Cholesterol	99.6	1.1316	−97
Alkaline phosphodiesterase I	101.0	1.1411	−34
Alkaline phosphatase	105.2	1.1353	−82
NADH cytochrome *c* reductase	93.4	1.1353	−135
NADPH cytochrome *c* reductase	102.5	1.1384	−152
Aminopyrine demethylase	107.9	1.1394	−133
Esterase	97.9	1.1426	−241
Nucleoside diphosphatase	126.1	1.1436	−220
Glucose 6-phosphatase	96.7	1.1393	−285
β-Glucuronidase	134.6	1.1440	−185

* Value observed for PPi-treated microsomes minus that observed for untreated microsomes (Table II of reference 7).

TABLE VII

Median Density and Recovery of Microsomal Constituents Equilibrated in Sucrose-H_2O Gradient after Treatment with EDTA

Constituent	Recovery	Median density: Observed	Median density: Excess over control* ($\times 10^4$)
	%		
Protein	111.5	1.1344	−293
Phospholipid	94.0	1.1306	−190
Monoamine oxidase	94.9	1.1227	−105
5'-Nucleotidase	141.7	1.1262	−110
Cholesterol	68.0	1.1318	−95
NADH cytochrome *c* reductase	96.2	1.1288	−200
Glucose 6-phosphatase	105.2	1.1348	−330
Ribonucleic acid	97.5	1.1296	−711

* Value observed for EDTA-treated microsomes minus that observed for untreated microsomes (Table II of reference 7).

of plasma membranes exists in the unesterified form (for a review see 27). The density of digitonin is 1.35 according to pycnometric determinations. A density increase exceeding 0.05 unit may thus be expected for group *a2* at total cholesterol saturation. The average shift of 0.03 density unit (Table III) can thus be accounted for solely by digitonin loading, and may indicate that complete saturation of cholesterol has not been achieved. Another possible explanation of the digitonin shift, which, however, appears superfluous, could be that digitonin increases the membrane permeability to sucrose. In consideration of the amount of digitonin which may be associated with the membranes, ultrastructural modifications must be expected. Apparently the affinity of some parts of the membrane for the heavy atoms used to stain positively ultrathin sections has been noticeably weakened. Less frequently, the broken appearance was replaced by a stiff three-layer aspect, similar to that reported by Colbeau et al. (10). It is tempting to assume that the unstained patches correspond to aggregates of the digitonin-cholesterol complex. Such an interpretation of the electron microscope picture remains, however, tentative, and requires further biochemical and ultrastructural studies.

The microsomal component bearing galactosyltransferase also acquires a higher equilibrium density in sucrose gradient, but the shift is less pronounced than that of group *a2*. Interestingly, the shift occurs at digitonin concentrations insufficient to unmask the latent activity of galactosyltransferase (Fig. 1), which indicates again that the extent of membrane alteration remains limited under the standard conditions used for the treatment. The action of digitonin upon Golgi elements will be considered extensively in a following paper of this series.

Action of EDTA and PPi on Microsomal Components

The purpose of this part of our work was not to study the mechanisms by which ribosomes or ribosome subunits may be detached from ER

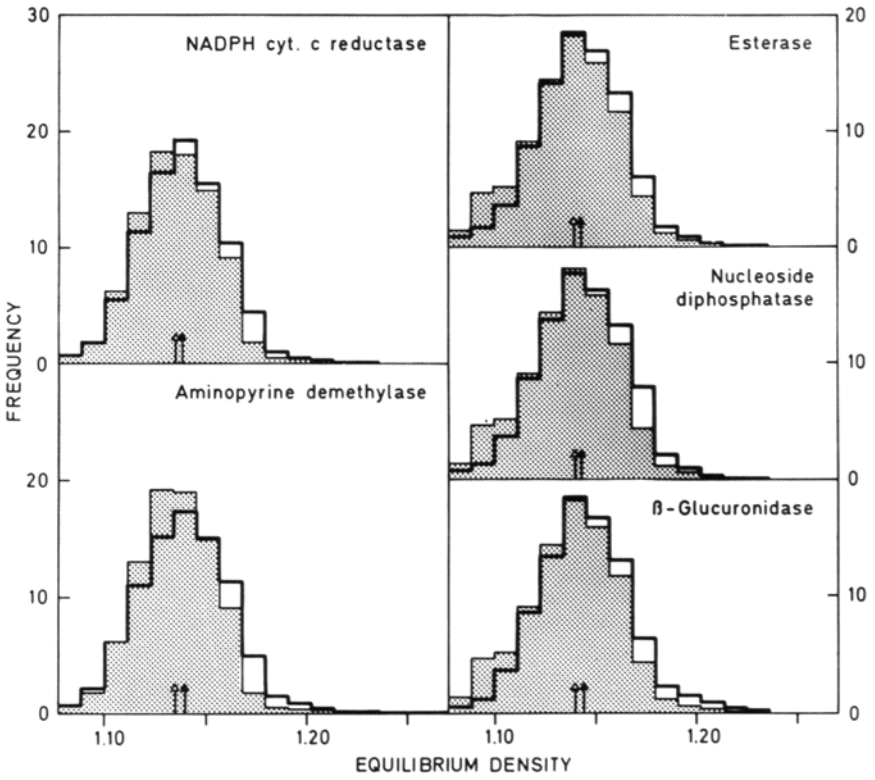

FIGURE 12 Density distribution of constituents of groups *b* (left-hand column) and *c* (right-hand column) after isopycnic equilibration of PPi-treated microsomes in sucrose-H_2O gradient. The represented portion of frequency histograms extends from 1.0773 to 1.27 in density. The distributions of constituents indicated in each compartment are represented by a thick line and the corresponding median densities by a black arrow. To facilitate comparisons the distribution of NADH cytochrome *c* reductase in PPi-treated microsomes, represented by the thin line (shading), is superimposed upon the profiles of group *b* constituents (left-hand column), and similarly the distribution of glucose 6-phosphatase on the profiles of group *c* constituents (right-hand column). The median density of these reference enzymes is marked by a white arrow, whereas the median density of other constituents is marked by a black arrow.

membranes in vitro. This matter is already well documented for EDTA (22) and, to a lesser extent, for PPi (23). Our biochemical and morphological data agree satisfactorily with these reports. It is worth noticing, however, that under our conditions PPi appears more effective than EDTA in detaching ribosomes.

Judging from our biochemical (Tables I and V, Fig. 7) and morphological results (Figs. 8 and 9), membranes *stricto sensu* do not suffer noticeable alteration from our treatment with PPi or EDTA. Referred to 1 g fresh weight of liver, the phospholipid and cholesterol contents are very close to those reported for untreated P fractions (4). Except for 5'-nucleotidase (see Results), enzyme activities of groups *a*, *b*, and *c* in the treated microsomes are normal. Particularly important is the fact that enzymes such as nucleoside diphosphatase or esterase are not released.

Effects of EDTA and PPi are, however, not restricted to the detachment of ribosomes or ribosomal subunits from membranes. We have stressed above that the amount of protein lost by microsomes exceeds markedly that which can be accounted for by the loss of RNA, on the basis of a protein to RNA ratio of 0.8 in ribosomes (16, 26). Furthermore, the release of protein does not parallel that of RNA (Fig. 7). The unaccounted for proteins which were detached most likely represent soluble proteins adsorbed on microsomal

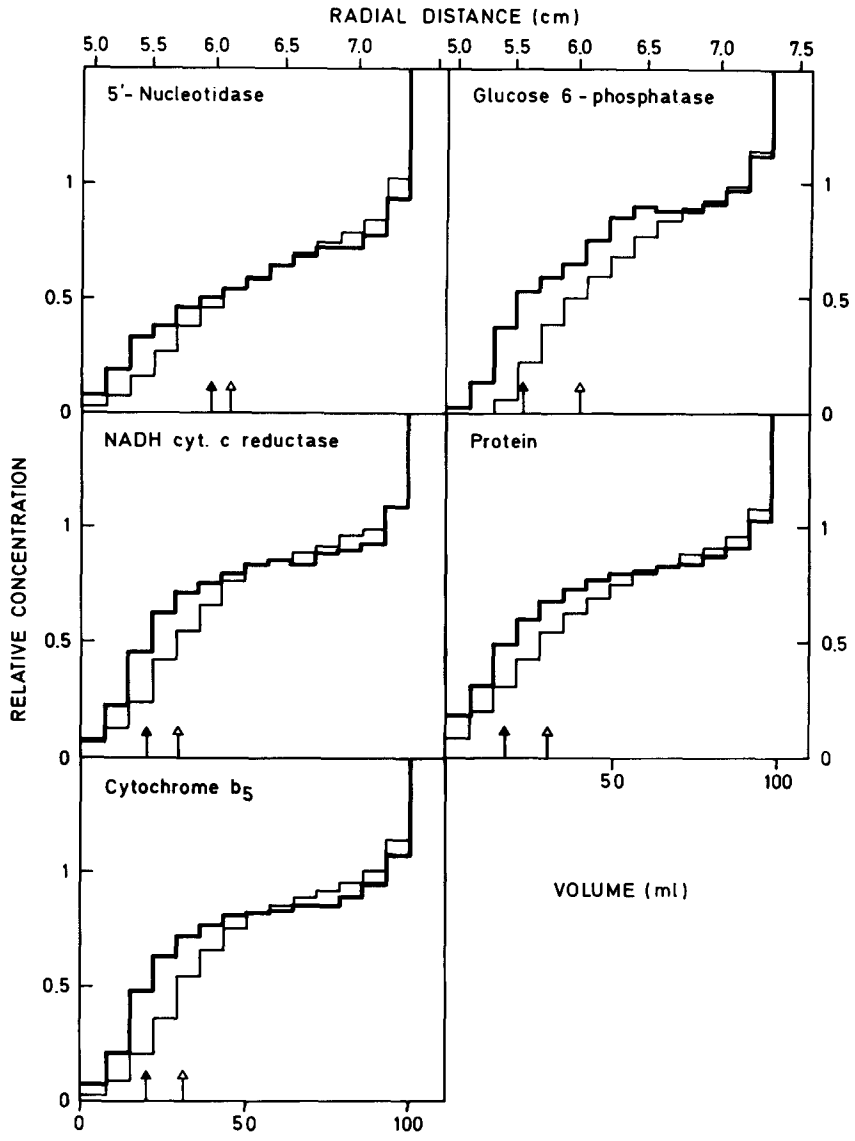

FIGURE 13 Influence of the treatment with EDTA on the sedimentation pattern of microsomal constituents after differential sedimentation in a density gradient. The P' fraction was treated with EDTA as described under Materials and Methods, and submitted afterwards to boundary analysis by differential sedimentation in a stabilizing gradient of sucrose ($W = 9.19 \times 10^9$ rad$^2 \cdot$s^{-1}). The sedimentation profiles obtained (thick lines) were averaged and normalized as explained under Presentation of Results. The sedimentation profiles obtained from untreated microsomes (thin lines) are reproduced from Fig. 9 of reference 7. The position in the gradient at which $C/C_1 = 0.5$ is marked by a white (untreated microsomes) or a black arrow (EDTA-treated microsomes). The upper abscissa gives the radial distance as a function of volume measured from the meniscus. Complementary data are given in Table VIII.

elements. In this respect, it is significant that fumarase and other adsorbed group *d* enzymes (4, 7) are almost completely solubilized by PPi or EDTA. Of course, some proteins detached by these agents may be structural constituents of microsomal membranes, but their loose bonding argues against that possibility.

In practice, if nonribosomal proteins are de-

TABLE VIII

Complementary Data on Experiment of Fig. 13

Constituent	Recovery	Amount recovered beyond 7.34 cm from axis*		Radial distance traveled at $C/C_i = 0.5$§	
		Observed	Excess over control‡	Observed	Excess over control‡
	%				
Protein	115.8	37.2	−6.6	5.6	−3.2
5′-Nucleotidase	103.1	52.1	−3.6	11.2	−1.5
NADH cytochrome *c* reductase	97.1	36.2	−5.8	6.0	−2.7
Cytochrome b_5	101.1	36.7	−6.5	5.8	−3.1
Glucose 6-phosphatase	109.3	39.3	−11.4	7.1	−4.2

* Amount of constituent recovered in the last fraction of Fig. 13 and in the cushion, expressed in percent of the total amount recovered.

‡ Value observed for digitonin-treated microsomes minus that observed for untreated microsomes (Table IV of reference 7).

§ 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.

tached from all the microsomes by PPi and EDTA, the selectivity of action expected towards elements derived from rough ER is reduced accordingly. Indeed, the logical consequence of lowering the protein to lipid ratio is to decrease the density of microsomal particles. This is probably the sole reason why constituents of group *a* acquire a lower equilibrium density, and we consider that the slight decrease in density observed in no way demonstrates that they are related to ribosome-loaded microsomes. As stressed in the Results section, we are simply dealing with a shift of the distribution profiles, without increase in physical homogeneity. Thus, if it was nevertheless concluded that the shift of group *a* results from the removal of ribosome subunits, it should be admitted that each constituent of group *a* belongs to one particular class of rough microsomes almost homogeneous with respect to their ribosome load per unit mass. This appears unlikely in view of the wide variations in ribosome coating of rat liver microsomes (33).

PPi and EDTA affect groups *b* and *c* differently than group *a*. As stressed in the Results section, the lightening is more pronounced than for group *a* and produces a marked decrease in the spreading of the density distributions. These properties are most clearly exhibited by group *c*, and also are reflected in the phospholipid and protein profiles. The shift of the densest microsomes, which normally equilibrate at the density 1.25–1.26, is thus much more pronounced than that of the less dense vesicles. It amounts at least to 0.06–0.07 density unit, which is high, but not unreasonable in view of the normal chemical composition of these vesicles, and if the shift is attributed largely to the uncoating of vesicles heavily loaded with ribosomes.

CONCLUSION

The results of this study confirm the classification of microsomal constituents proposed earlier (3, 7) and strengthen the individuality of the groups by attributing new properties to each. They also enlighten us on their cytological import. These aspects are summarized in Table X and briefly examined below.

Microsomal monoamine oxidase is the only enzyme so far associated with group *a1*. It belongs to a component which acquires a slightly lower density by treatment with PPi or EDTA and retains its physical properties after treatment with digitonin. This latter observation supports a possible identification with outer mitochondrial membranes which have been found unaltered by the treatment with digitonin (unpublished observation), but it does not clear up the difficulties of this interpretation alluded to previously (7). In any case, the microsomal component bearing monoamine oxidase is a possible contaminant of low density microsomal subfractions, in particular of Golgi preparations, and its presence cannot be excluded by the lack of cytochrome oxidase activ-

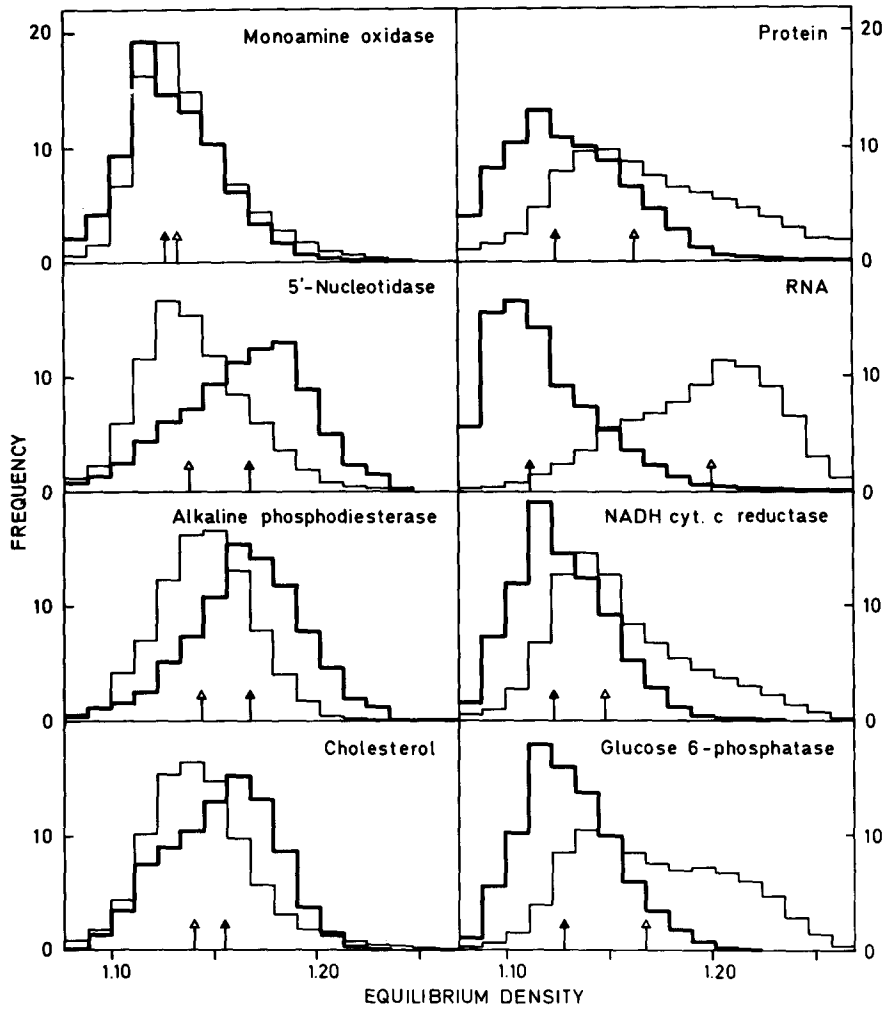

FIGURE 14 Influence of subsequent treatments with EDTA and digitonin on the density distribution of microsomal constituents in sucrose-H_2O gradient. Microsomes were treated with EDTA and digitonin as described under Materials and Methods and equilibrated afterwards in a linear gradient of sucrose. The frequency histograms obtained are given by the thick lines in a normalized form (see Presentation of Results). The represented portion extends from 1.0773 to 1.27 in density. The density distributions for untreated microsomes (thin lines) were constructed from experiments reported in Fig. 3. Arrows indicate the median densities of constituents in EDTA-digitonin-treated (black arrow) or untreated (white arrow) microsomes.

ity. To the extent that we are dealing with outer mitochondrial membranes which contain cytochrome b_5 (25), it may account for a part of this cytochrome detected in Golgi preparations (9).

The enzymes 5'-nucleotidase, alkaline phosphodiesterase I, and alkaline phosphatase, classified into group *a2*, belong to a microsomal component which displays a marked digitonin shift and a moderate sensitivity to PPi or EDTA. After digitonin treatment the membranes related to this group undergo a very typical modification of their electron microscope appearance. An obvious relationship with isolated plasma membranes is thus established, since the latter suffer the same modifications of their density distribution (1, 12) and of their morphology (12, 34) on treatment with

TABLE IX

Median Density and Recovery of Microsomal Constituents Equilibrated in Sucrose-H_2O Gradient after Treatment with EDTA and Digitonin

Constituent	Recovery	Median density	
		Observed	Excess over control* ($\times 10^4$)
	%		
Protein	100.9	1.1265	−372
Monoamine oxidase	97.4	1.1282	−50
5′-Nucleotidase	144.1	1.1687	315
Alkaline phosphodiesterase I	112.8	1.1678	233
Cholesterol	91.6	1.1561	148
NADH cytochrome *c* reductase	102.8	1.1248	−240
Glucose 6-phosphatase	97.1	1.1287	−391
Ribonucleic acid	161.8	1.1129	−878

* Value observed for EDTA and digitonin-treated microsomes minus that observed for untreated microsomes (Table II of reference 7).

digitonin. The type of relationship involved (fragments of the pericellular membranes or intracellular precursors or derivatives) remains to be cleared up. Besides, the association of the largest part of the microsomal cholesterol with the group *a2* component is now convincingly established, as argued above. Phospholipid distribution shifts more markedly than that of protein, and so it seems that the phospholipid to protein ratio is higher for group *a2* component than for total microsomes. Use of digitonin also confirms the existence of some heterogeneity within group *a2* (7), since the shift of 5′-nucleotidase is consistently more pronounced than that of alkaline phosphodiesterase I. Finally, our conclusions are at variance with those drawn from biochemical and cytochemical data by Widnell (35) who attributes an important part (at least 20%) of microsomal 5′-nucleotidase to rough vesicles. The dissociation between this enzyme and marker enzymes of ER has been extensively documented in this paper as well as in the preceding one (7).

Group *a3*, represented here by the enzyme galactosyltransferase, acquires a somewhat lower density by treatment with PPi. It distinguishes itself markedly from monoamine oxidase (group *a1*) by a digitonin shift which, however, appears less pronounced than that of group *a2*. More recent experiments have clearly shown that sialyltransferase (acceptor: sialidase-treated α_1-acid glycoprotein) and *N*-acetylglucosaminyltransferase (acceptor: ovalbumin) activities are recovered in our microsome fraction to the same extent as galactosyltransferase, and display, in density gradient centrifugation, the characteristic behavior presented here for the latter enzyme. The identification of the *a3* microsomal entity with components of the Golgi apparatus is thus given a stronger basis (20, 24, 31, 32). Tentatively, the digitonin shift of group *a3* is attributed to formation of a complex between digitonin and cholesterol. This latter constituent has indeed been detected in Golgi preparations (7, 17). Qualitatively similar effects of digitonin on groups *a2* and *a3* are interesting, in view of the functional relationships which exist between the Golgi complex and the pericellular membrane. An enzymic analogy has been proposed by Bergeron et al. (9), who attribute to Golgi membranes the 5′-nucleotidase activity detected biochemically (9) and cytochemically (14) in Golgi-rich fractions. This interpretation is not ruled out by our present results, although the dissociation achieved by differential sedimentation in density gradient shows that the bulk of microsomal 5′-nucleotidase activity is not exclusively associated with the component which bears galactosyltransferase activity.

The distinction between groups *b* and *c* is confirmed by the results presented here, but it remains more quantitative than qualitative, the typical properties of these groups being more pronounced for *c* than for *b*. Groups *b* and *c* never dissociate noticeably from protein, even after treatment with digitonin, PPi, or EDTA. They both acquire a much lower density and a greater physical homogeneity in sucrose gradient after treatment with PPi or EDTA, and in this respect these agents can be regarded as acting specifically on groups *b* and *c*. Their effect is more pronounced on group *c* than on group *b*, and consequently they noticeably weaken the dissociation between the constituents of these groups. Since, as stated above, the effects of PPi and EDTA must mainly be attributed to the uncoating of rough microsomes, two conclusions drawn previously from the biochemical and morphological analysis of rat liver microsomes made in this laboratory are strengthened. (*a*) The ribosome load per unit weight is the main factor responsible for the broad

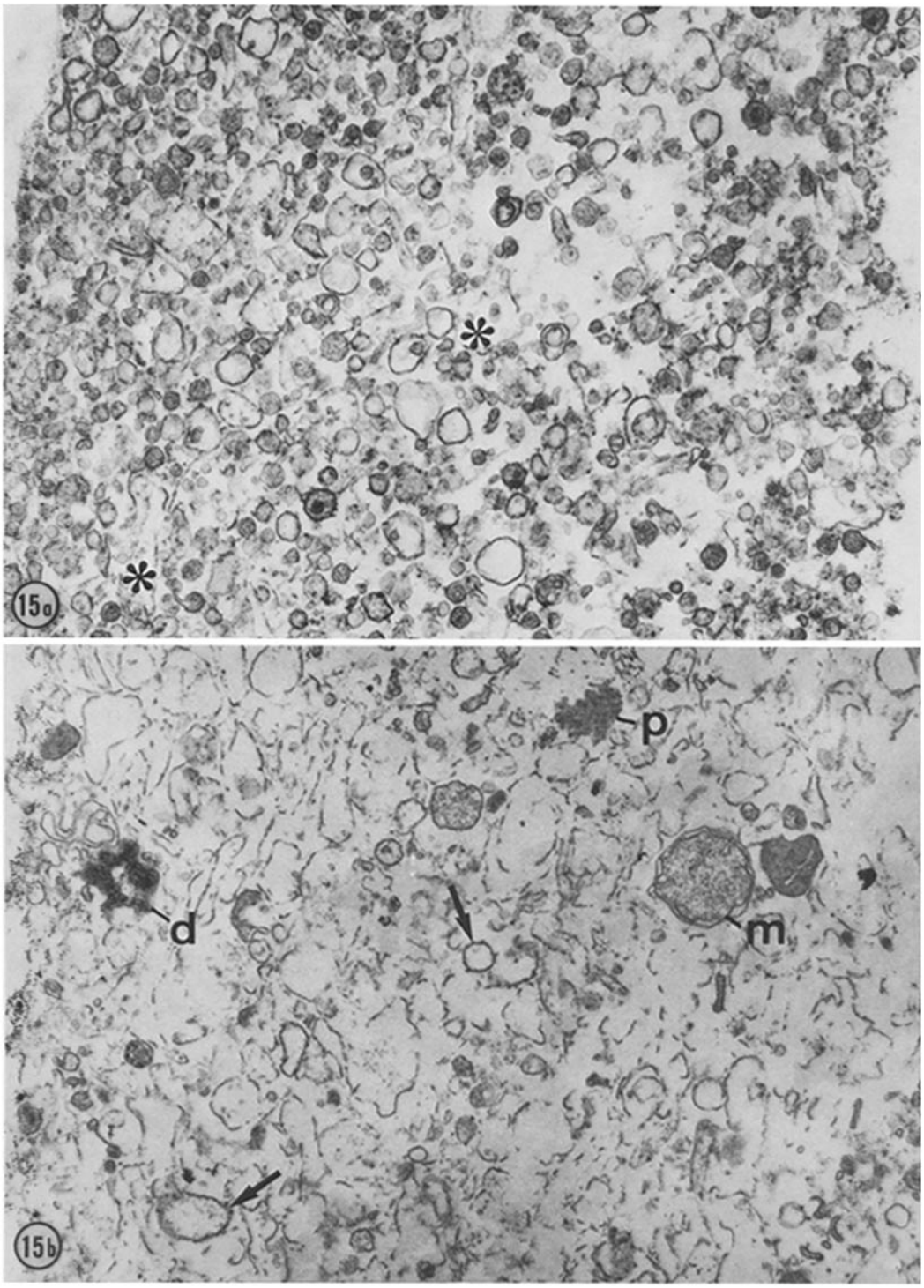

FIGURE 15 (*a*) Morphology of microsomes treated with digitonin and EDTA as described under Materials and Methods. Ribosomal profiles are infrequent. A number of rather large vesicles show a broken appearance (asterisks). × 29,000. (*b*) Morphology of a dense subfraction (density ≈ 1.204–1.224) obtained from the preparation shown in Fig. 15 *a* after density equilibration in a sucrose gradient. Many vesicular profiles appear broken. Note a mitochondrion with apparently normal membranes (*m*), a peroxisomal core (*p*), an altered dense body (*d*), and a few vesicles derived from rough ER, recognizable by the ribosomal remnants seen on their outer surface (arrows). × 31,000.

TABLE X

Classification and Main Properties of Microsomal Constituents*

Group	Constituent	Properties in untreated microsomes: Density distribution in sucrose gradient‡	Properties in untreated microsomes: Rate of sedimentation in 0.25–0.50 M sucrose gradient, relative to that of protein	Modification of the density distribution on treatment of microsomes with: Digitonin	Modification of the density distribution on treatment of microsomes with: PPi or EDTA	Modification of the density distribution on treatment of microsomes with: PPi or EDTA + Digitonin	Cellular localization§
a1	Monoamine oxidase	1.10–1.20 sharp	slower	insignificant	slight −shift	insignificant	Outer membrane of mitochondria (and other?)
a2	5′-Nucleotidase Alkaline phosphodiesterase I Alkaline phosphatase Cholesterol	1.10–1.20 almost symmetrical	faster	marked +shift¶	slight −shift	marked +shift	Plasma membranes (and related intracellular component?)
a3	Galactosyltransferase *N*-Acetylglucosaminyl transferase‖ Sialyltransferase‖	1.08–1.18	slower	+shift less marked than for *a2*	slight −shift	—	Golgi complex
b	NADH cytochrome *c* reductase NADPH cytochrome *c* reductase Aminopyrine demethylase Cytochrome b_5 Cytochrome P 450 Phospholipid	1.10–1.27 skewed main peak at 1.14	close or slightly slower	insignificant	marked − shift and gain in symmetry		Smooth and rough parts of ER

c	Glucose 6-phosphatase Esterase Nucleoside diphosphatase Glucuronyltransferase β-Glucuronidase Protein	1.10–1.27 skewer than *b* main peak at 1.14	close or slightly faster	insignificant	marked −shift and gain in symmetry	smooth and rough parts of ER
d	RNA Ribosomes ----------	1.10–1.27 skewed main peak at 1.22		insignificant	marked −shift and gain in symmetry	Rough portion of ER ----------
	Glutamine synthetase Aldolase Fumarase			insignificant		Cell sap

* The properties described summarize the results presented in this paper and in reference 7. They were established for one, several, or all constituents of the groups.

‡ Density limits within which the constituents are recovered to the extent of 90% or more.

§ This assignment of microsomal constituents to a cell component does not imply that small amounts are not associated with other components. This restriction is valid for about 20% of microsomal phospholipid and protein (7). We consider however that the association of a minor part of the constituents to other cell components should be convincingly established, as it is for the occurrence of cytochrome b_5 and NADH cytochrome *c* reductase in outer membranes of mitochondria (25).

‖ Unpublished results of M. Wibo, A. Amar-Costesec, D. Godelaine, and H. Beaufay. Acceptors: ovalbumin and sialidase-treated α_1-acid glycoprotein.

¶ + and − indicate that the shift occurred towards higher or lower densities, respectively.

density frequency distributions of constituents associated with microsomes derived from ER, and a continuous spectrum of microsomal entities ranging from entirely ribosome-free vesicles to vesicles heavily coated with ribosomes exists (3, 33). (*b*) Enzymes classified in group *b* or *c* are all present in rough and in smooth microsomes derived from ER, and the ratio of group *c* to group *b* constituents increases with the coating of vesicles with ribosomes (3, 7).

In contrast to group *a2* and *a3* enzymes, those of group *b* or *c* appear entirely insensitive to digitonin, even upon careful examination of the density distributions in the low density range (Figs. 3 and 4). Thus the smooth microsomes derived from ER do not contain appreciable amounts of cholesterol per unit mass, at least in a form accessible to digitonin. Since we have previously shown that rough microsomes are free of cholesterol (7), we consider, despite repeated allegations to the contrary in the literature (11, 15), that in rat liver the ER membranes contain little or no cholesterol, the bulk of this substance being associated with other cell components.

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