ENZYME-STRUCTURE RELATIONSHIPS IN THE ENDOPLASMIC RETICULUM OF RAT LIVER

A Morphological and Biochemical Study

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ABSTRACT

Subfractionation of preparations of rat liver microsomes with a suitable concentration of sodium deoxycholate has resulted in the isolation of a membrane fraction consisting of smooth surfaced vesicles virtually free of ribonucleoprotein particles. The membrane fraction is rich in phospholipids, and contains the microsomal NADH–cytochrome *c* reductase, NADH diaphorase, glucose-6-phosphatase, and ATPase in a concentrated form. The NADPH–cytochrome *c* reductase, a NADPH (or pyridine nucleotide unspecific) diaphorase, and cytochrome b_5 are recovered in the clear supernatant fraction. The ribonucleoprotein particles are devoid of, or relatively poor in, the enzyme activities mentioned. Those enzymes which are bound to the membranes vary in activity according to the structural state of the microsomes, whereas those which appear in the soluble fraction are stable. From these findings the conclusion is reached that certain enzymes of the endoplasmic reticulum are tightly bound to the membranes, whereas others either are loosely bound or are present in a soluble form within the lumina of the system. Some implications of these results as to the enzymic organization of the endoplasmic reticulum are discussed.

INTRODUCTION

It is well established that the microsomal fraction isolated by usual differential centrifugation from rat liver homogenates consists of vesicular and tubular fragments of the endoplasmic reticulum. Indeed, when examined in the electron microscope, hepatic microsomes appear as closed vesicles, bound by a single membrane which in most cases bears attached ribonucleoprotein (RNP) particles on its outer surface, an indication that most of these fragments are derived from the "rough surfaced" part of the endoplasmic reticulum (1). These findings are consistent with the view that, during the homogenization of the tissue, the endoplasmic network breaks down, by a generalized pinching off process, into a collection of separate vesicles, each containing a quantum of the soluble material present *in vivo* in the lumina of the reticular system. It follows that a typical hepatic microsome is comprised of a membrane, a content, and—for most of them at least—a set of attached RNP particles.

Whereas it is today fairly well established that the biochemical function of the RNP particles is concerned primarily with the incorporation of

"activated" amino acids into proteins (*cf.* review (2)), relatively little is settled about the biochemical function of the endoplasmic reticulum. Conversely—and paradoxically—however, only a few of those rather numerous enzymes hitherto detected in microsomes have proved to be associated with RNP particles (3–5). The majority probably occurs bound to, or included within, the endoplasmic membranes. Among these enzymes there are, in the case of the liver, a NADH[1]–cytochrome c reductase (1, 6–15), a NADPH–cytochrome c reductase (8, 12, 14, 16, 17), a pyridine nucleotide unspecific diaphorase (18, 19), cytochrome b_5 (1, 9, 11, 20–23), a glucose-6-phosphatase (24–27), an ATPase (26, 28–30), certain reductive enzymes involved in fatty acid and steroid synthesis (31, 32), and enzymes catalyzing the synthesis of ascorbic acid (33), as well as a number of enzymes carrying out various oxidative and conjugative detoxication reactions (34).

This disparate enzymic pattern suggests a multiplicity of functions, possibly associated with the local differentiations of the system. In attempting to elucidate these functions, it may be important to assess, in the first place, whether the various enzymes are merely enclosed within the membranes, as in a sac, or are bound to them by chemical forces. Moreover, dealing with "particulate" enzymes, the possibility must be considered that the activity of such enzymes may be influenced, quantitatively as well as qualitatively, by the state of the structure with which they are associated. Such modifications of enzyme activities are well known from studies on mitochondria (35, 36). The existence of similar modifying principles in microsomes, although not yet explored systematically, is suggested by several findings. For example, data indicating that the glucose-6-phosphatase of liver microsomes may be bound to the endoplasmic membranes (37–39) and that the bound state may influence the affinity of this enzyme for its substrate, have been recently reported (38).

With this end in view hepatic microsomes were treated with appropriate concentrations of deoxycholate and subsequently fractionated by differential centrifugation. Two subfractions of known cytological significance were obtained: attached ribosomes and smooth membranes, in addition to a supernatant. The latter may correspond to the original microsomal content enriched by whatever materials were solubilized by the detergent from the other microsomal components. The distribution of various microsomal enzymes among these subfractions was studied and the effect of structural changes upon their activities was assessed.

Certain parts of this work have already been reported briefly (40, 41)

[1] Abbreviations used are: NADH and NADPH, reduced nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate; DOC, deoxycholate; ATP and ATPase, adenosine triphosphate and adenosine triphosphatase; RNP, ribonucleoprotein; TCA, trichloroacetic acid.

EXPERIMENTAL

Preparation of Microsomes

Albino rats, 150 to 250 gm, were starved overnight and killed by decapitation. Each liver was quickly removed, washed with cold 0.25 M sucrose solution, blotted with filter paper, weighed, returned into the same solution, and finally cut into small pieces, which were rinsed with several portions of 0.25 M sucrose. All subsequent operations were carried out at 0–2°C. The minced tissue was homogenized in 0.25 M sucrose in a glass tube provided with a Teflon pestle. The final volume was made up with 0.25 M sucrose to contain about 100 mg fresh liver per ml. The homogenate was centrifuged in a Spinco model L centrifuge, rotor 40 (11 ml homogenate per tube), at 12,500 RPM (10,000 g) for 10 minutes and the ensuing supernatants were completely decanted and transferred to new tubes. In each tube the volume was adjusted to about 11 ml with sucrose. A second centrifugation at 40,000 RPM (105,000 g) for 60 minutes yielded microsomal supernatants and pellets. The supernatants were discarded, whereas the pellets were rinsed with several portions of sucrose and then resuspended in 0.25 M sucrose with the aid of a Teflon pestle made to fit the centrifuge tubes. The suspension was adjusted to contain microsomes from 0.2 gm liver (4 to 5 mg protein) per ml.

Subfractionation of Microsomes Treated with 0.26 Per Cent DOC

Stock solutions of deoxycholate, containing 2.6 gm deoxycholic acid (Wilson Co., Chicago) per 100 ml, were prepared by dissolving the acid in a minimum amount of NaOH and adjusting the pH with HCl to 7.7 to 7.8. The solution was filtered and kept at room temperature. Turbidity or precipitate in the solution interferes with the fractionation.

Treatment of microsomes with DOC in the cold was performed by adding 1 volume of DOC of desired concentration to 10 volumes of microsomal suspension. The tube was turned upside down 3 or 4 times, but not shaken. Subsequent centrifugation for 2 hours at 105,000 *g* of the microsomes so treated resulted in three fractions (Fig. 1): a tightly packed pellet, a loose reddish sediment on top of the pellet, and a clear supernatant. The top layer of the latter, however, was usually turbid with fat. The clear supernatant could be removed without disturbing the loose reddish sediment by using a fine tipped large volumetric pipette (15 to 20 ml) attached to a rubber aspirator; up to 10 to 10.5 ml out of a total volume of 11 ml could be removed in this way. The bottom layer containing the loose sediment ("M fraction," see under "Results") was afterward decanted as completely as possible, leaving behind the tightly packed pellet ("P fraction"). In some experiments, the clear supernatant was removed in 2 or 3 portions, again leaving behind the bottom layer, which was finally collected as a single fraction together with the loose sediment it contained. The volumes of all these fractions were measured carefully and expressed in terms of ml/gm liver. The tightly packed pellet was thoroughly rinsed with several portions of sucrose, and finally suspended in sucrose with the aid of a homogenizer.

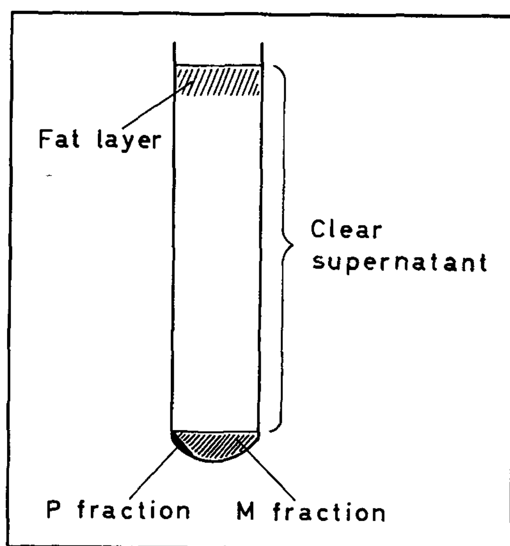


FIGURE 1

Schematic illustration of fractions obtained after treatment of microsomes with 0.26 per cent DOC and subsequent centrifugation. For explanation, see text.

Electron Microscopy

The pellets were fixed *in situ* by overlaying them with a 2 per cent OsO_4 solution in 0.25 M sucrose containing 0.26 per cent DOC. The fixation was carried out at 0–2°C for ~16 hours. The dehydration of the pellets was started *in situ* without previous washing. In 95 per cent ethanol the pellets were solid enough to withstand removal from the centrifuge tube and trimming into orientable strips without deformation or displacement of their layers. Dehydration of the strips was continued through 100 per cent ethanol, after which they were impregnated and finally embedded in a mixture of methyl and butyl methacrylate. Cradles of polymethacrylate were used to maintain the strip properly oriented during polymerization. Sections were cut perpendicular to the surface of the pellets, care being taken to include the whole depth of the sediment in the section. With such precautions, a systematic survey from top to bottom of the pellet was possible.

Some preparations were fixed in suspension and subsequently centrifuged to obtain a well packed pellet. The latter was then treated as above. All sections were stained with uranyl acetate or lead hydroxide, and examined with or without a carbon film blanket in an RCA electron microscope model EMU-2B, or a Siemens Elmiskop I.

Chemical Analyses

Cold TCA was added to all fractions to a final concentration of 5 per cent. The precipitated material was then washed twice with cold 5 per cent TCA. From this material, phospholipid, RNA, and RNA-free and fat-free protein were obtained by the method of Schneider (42). The Mejbaum orcinol method (43) was used to measure RNA, using a purified yeast RNA as a standard. Protein nitrogen was determined either by nesslerization after Kjeldahl digestion (44) or by the biuret method (45). The values were multiplied by 6.25 to obtain protein. Phospholipid phosphorus was determined by digestion of the pooled alcohol and alcohol-ether extracts and determining the phosphate of the digest by the Fiske and Subbarow method (46); phospholipid was obtained by multiplying these values by 25.

Enzyme Assays

For all enzyme assays, the samples (suspensions of microsomes or subfractions thereof) were kept in the undiluted state until testing. When necessary, they were diluted immediately before being added to the assay system. In the NADH–cytochrome *c* reductase assay of the loose sediment, for example, it was usually necessary to dilute this fraction 20- to 50-fold to get a suitable reading with 0.1 ml of the

diluted sample in the cuvette with a final volume of 3 ml. For this purpose the cuvette was prepared with all the reagents, the spectrophotometer was adjusted, and when all was ready, 0.1 ml of the loose sediment fraction was added to 1.9 or 4.9 ml of cold water (depending on the dilution needed), mixed, and 0.1 ml of this diluted sample *immediately* transferred to the cuvette. The same precaution was followed with all fractions, including the fresh microsomes.

When DOC was added to an assay system, it was introduced therein as the last ingredient before the addition of the enzyme.

The individual enzymes were assayed as follows:

CYTOCHROME *c* REDUCTASES: The assay system contained 0.05 M tris buffer, pH 7.5; 0.1 mM NADH or NADPH; 0.05 mM cytochrome *c;* and 0.33 mM KCN (for this addition, *cf.* (14)), in a final volume of 3 ml. The reduction of cytochrome *c* was followed at 550 mμ in a DU, or recording DK2, Beckman spectrophotometer; the time of recording was 3 to 5 minutes with the former, and 1 to 2 minutes with the latter instrument. The rates were linear. Glass cuvettes of 1 cm light path were used. The temperature was about 28°C.

DIAPHORASE: The assay system was the same as above except that cytochrome *c* was replaced by 0.04 mM 2,6-dichlorophenolindophenol. The reduction of the dyestuff was recorded at 600 mμ. The addition of KCN was not essential.

CYTOCHROME b_5: This was estimated in the clear supernatant and loose sediment obtained by subfractionating the microsomes with 0.26 per cent DOC. The difference spectrum (reduced minus oxidized) was measured by using two cuvettes containing the same fraction, diluted with 0.02 M phosphate buffer, pH 7.5, and 0.4 per cent DOC (final concentrations) in a volume of 3 ml. In one of the cuvettes, reduction was performed by the addition of 10 mM $Na_2S_2O_4$ (final concentration). The difference spectrum so obtained was closely similar to that reported by Garfinkel (10). The difference between the 427 mμ maximum and the 410 mμ minimum was taken as an arbitrary estimate of cytochrome b_5 (see Fig. 7).

GLUCOSE-6-PHOSPHATASE: The assay used was that of Swanson (47) with an incubation of 20 minutes at 30°C. Activity was proportional to tissue concentration.

ATPASE: The assay system contained 0.16 M sucrose; 0.05 M tris buffer, pH 7.5; 4 mM $MgCl_2$; and 5 mM disodium-ATP, in a final volume of 2 ml. Incubation lasted 20 minutes at 30°C.

Inorganic orthophosphate, produced as a result of ATPase and glucose-6-phosphatase activities, was determined according to the modified Martin and Doty method (48).

RESULTS

NADH- and NADPH-Cytochrome c Reductase Activities of Freshly Prepared Microsomes

The specific activity of microsomal NADH-cytochrome *c* reductase varied as much as 6-fold from one preparation to another, whereas the specific activity of NADPH-cytochrome *c* reductase changed only slightly under the same conditions. The possible reasons for this variation and difference will be discussed below. In accord with the results of others (8, 14, 16), the NADH-cytochrome *c* reductase activity of liver microsomes was from 10 to 50 times higher than the NADPH-cytochrome *c* reductase activity.

Effects of Aging, Dilution, and DOC Treatment on Microsomal Cytochrome c Reductase Activities

Data in Table I demonstrate the lability of the NADH enzyme and the stability of the NADPH enzyme, as a function of microsome storage in sucrose or in DOC, or as a function of microsome dilution with sucrose. It can be noted that the activity of the NADH enzyme can be either increased or decreased as a result of these treatments, as if in some cases the isolated microsomes already had a fully activated enzyme, and treatment inactivated it, whereas in other cases the microsomal enzyme could be further activated by storage in sucrose or in DOC. Dilution in sucrose markedly inactivated the NADH-cytochrome *c* activity, but dilution in the assay system neither inactivated nor activated the enzyme, for its activity remained unchanged at least during the 10 minutes needed for the assay. It follows that some, or all, of the components of the assay system could stabilize the enzyme. Added separately to the assay system, NADH or cytochrome *c* did not stabilize the enzymic activity, but introduced together they protected it. No stabilization was observed upon addition of albumin, Versene, or Mg^{++}.

Effect of DOC on Cytochrome c Reductases When Added to Assay

In the previous section, DOC was added in the cold before storage and subsequently diluted in the assay. In Table II, the effect of DOC added directly to the assay system is shown. NADH-

TABLE I

Effects of Storage, Dilution, and DOC Treatment on Microsomal Cytochrome c-Reductase Activities

The "undiluted" suspensions contained microsomes from 200 mg liver per ml 0.25 M sucrose. All dilutions were made with 0.25 M sucrose, and all agings were carried out at 0–2°C. Immediately (within 15 seconds) before assay, all samples were diluted to contain microsomes from 4 mg liver per ml, *i.e.*, 50-fold with regard to the "undiluted" suspensions. Assay conditions are given in the text.

Exp. no.	Procedure	NADH–cyt. *c* red. (ΔE_{550}/min./gm liver)	NADPH–cyt. *c* red. (ΔE_{550}/min./gm liver)
1	Microsomes in sucrose:		
	Fresh	292	
	After 6 hr.	550	
	Microsomes in 0.026 % DOC:		
	After 6 hr.	711	
	Microsomes in 0.26 % DOC:		
	After 0.5 hr.	780	
	After 3.3 hr.	588	
	After 3.3 hr., dil. 50 ×:		
	Assayed after 7 min.	350	
	″ ″ 14 ″	198	
	″ ″ 21 ″	156	
	″ ″ 28 ″	144	
	″ ″ 90 ″	144	
	After 6 hr.	453	
	Dil. 2.5 ×, assayed after 6 hr.	270	
	″ 5 ×, ″ ″ ″ ″	157	
	″ 10 ×, ″ ″ ″ ″	136	
2	Microsomes in sucrose:		
	Dil. 50 ×:		
	Assayed immediately	684	
	″ after 6 min.	533	
	″ ″ 12 ″	298	
	″ ″ 18 ″	254	
	″ ″ 24 ″	232	
	″ ″ 30 ″	207	
	Undiluted:		
	Assayed after 40 min.	473	
	Microsomes in 0.26 % DOC:		
	Dil. 50 ×:		
	Assayed immediately	473	
	″ after 6 min.	279	
	″ ″ 12 ″	270	
	″ ″ 18 ″	132	
	″ ″ 24 ″	113	
	″ ″ 33 ″	113	
	Undiluted:		
	Assayed after 50 min.	430	
3	Microsomes in sucrose:		
	Fresh	558	8.0
	After 8 hr.	266	9.2
	Microsomes in 0.26 % DOC:		
	Dil. 5 ×, tested after 6 hr.	148	8.6

cytochrome *c* reductase was almost completely inhibited above 0.05 per cent DOC, whereas NADPH–cytochrome *c* reductase was unaffected at a DOC concentration as high as 0.35 per cent. The sensitivity of the NADH–cytochrome *c* reductase proved somewhat dependent on the amount of microsomal protein present in the cuvette. Since NADH–cytochrome *c* reductase is usually tested with 10 to 20 times less microsomes than NADPH–cytochrome *c* reductase, the great difference in sensitivity to DOC found in Experiment 1 of Table II might be due to this fact. However, in Experiment 2 the two activities are compared using equal amounts of microsomes (except for the "no DOC" sample). A true difference in sensitivity is clearly shown. Moreover, it is seen that NADPH–cytochrome *c* reductase activity can, under these circumstances, considerably exceed NADH–cytochrome *c* reductase activity.

TABLE II

Effect of DOC on NADH– and NADPH–Cytochrome c Reductase Activities of Liver Microsomes

The amounts of microsomes used in the assay were, in terms of mg tissue equivalent:
Experiment 1: NADH, 1.6; NADPH, 16.
Experiment 2: NADH (no DOC), 2; NADH (DOC) or NADPH, 40.

		Cyt. *c* reductase (ΔE_{550}/min./gm liver)	
Exp. no.	% DOC in assay	NADH	NADPH
1	0	60.0	3.0
	0.01	60.0	2.8
	0.02	62.4	3.0
	0.05	49.8	3.5
	0.075	4.8	3.5
	0.1	2.4	3.5
2	0	97.0	5.0
	0.17	2.3	5.0
	0.26	0.9	4.6
	0.35	0.5	5.2

Reversibility of NADH–Cytochrome c Reductase Inhibition by DOC

As Fig. 2 shows, the inhibition caused by DOC is reversible. In an assay system completely inhibited by 0.13 or 0.26 per cent DOC, activity is fully restored upon a 10-fold dilution with sucrose.

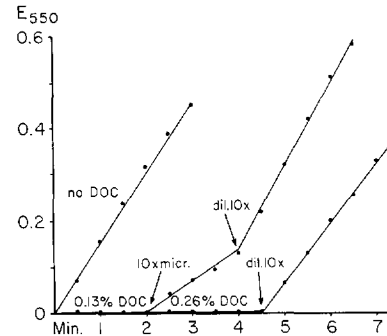


FIGURE 2

Reversibility of DOC inhibition of NADH–cytochrome *c* reductase. Microsomes were assayed in the absence and in the presence of 0.13 per cent and 0.26 per cent DOC. 2 minutes after the start of the assay, 10 times as much microsomes was added to the assay cuvettes. At 4 minutes and at 4½ minutes a 1/10 aliquot of the reaction mixture was mixed with 10 times its volume of the normal, non-DOC-containing assay mixture and was then immediately assayed.

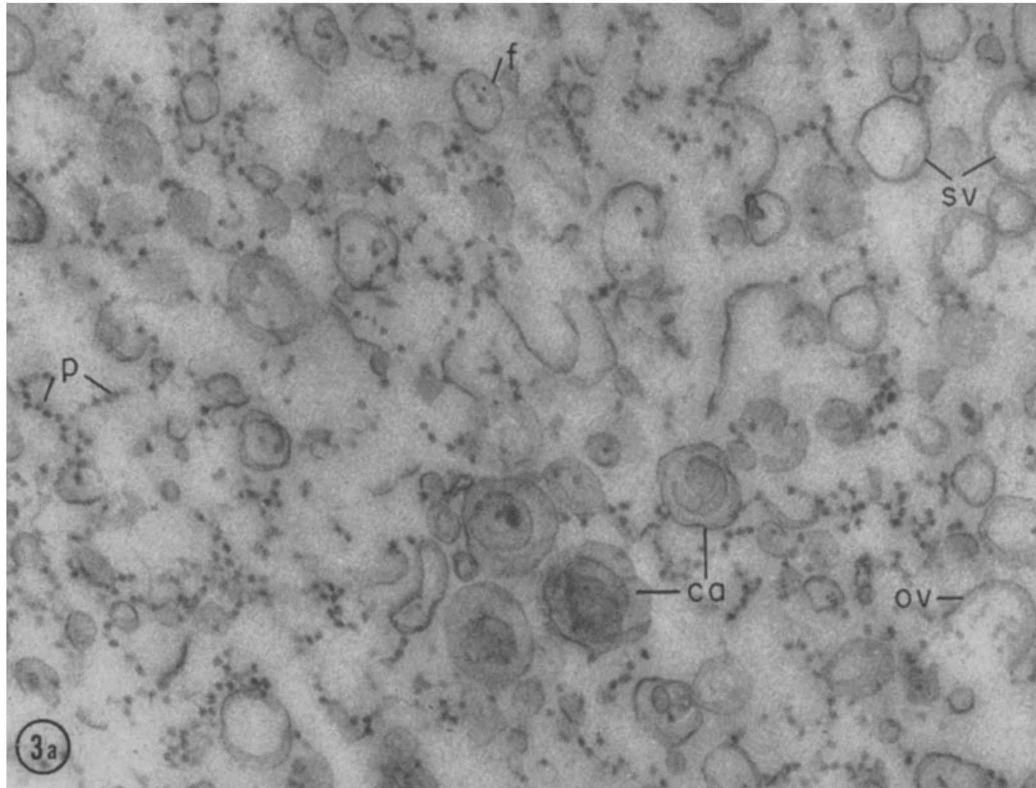
Changing the ratio DOC:microsomes, by adding more microsomes to the assay, releases the inhibition only partially at 0.13 and not at all at 0.26 per cent DOC, whereas subsequent dilution with sucrose again fully restores the activity of the enzyme at both DOC concentrations.

"Solubilization" of Cytochrome c Reductase by DOC

Suspensions containing microsomes from 200 mg fresh liver per ml were treated with varying concentrations of DOC in the cold, and subsequently

FIGURE 3

Pellet of the P fraction. *a*, top of the pellet; *b*, bottom of the pellet. The main component through most of the depth of the pellet is RNP particles (*p*) in chains or clusters. Smooth surfaced vesicles (*sv*) are rare at the bottom and rather frequent at the top of the pellet. Ferritin (*f*) can be recognized throughout the preparation. Note that, presumably as a result of the DOC treatment, concentric aggregates (*ca*) of membranes and broken open vesicles (*ov*) appear in the pellet. × 80,000.


f
sv
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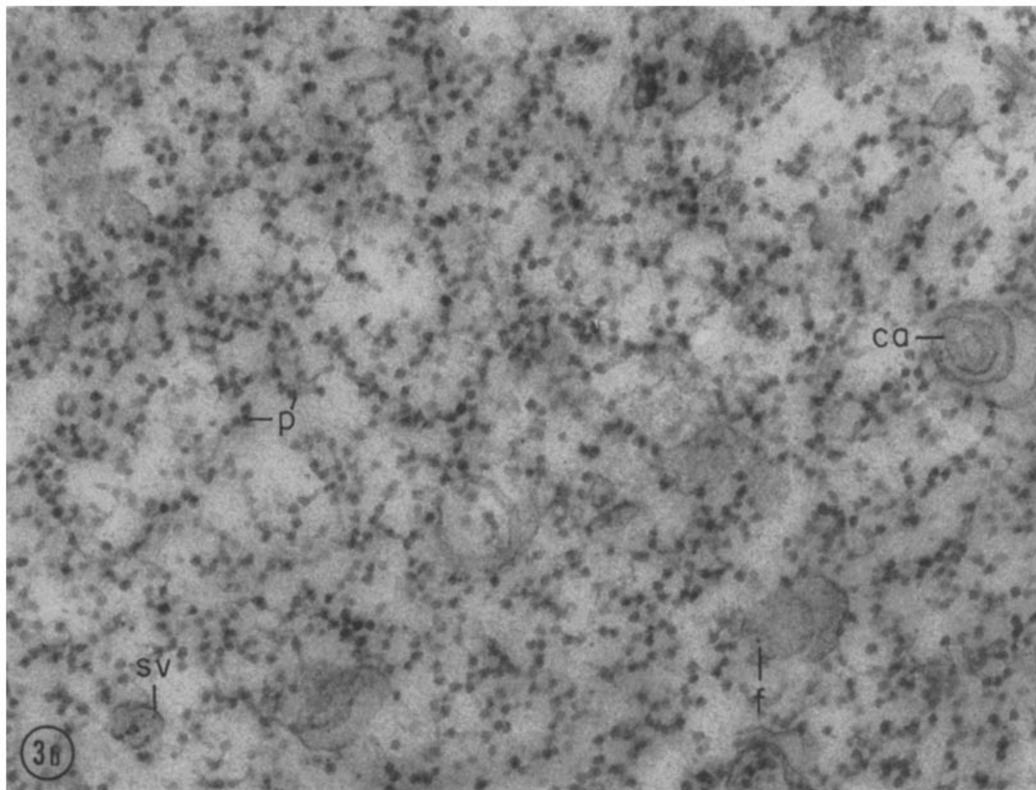
ca
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centrifuged for 2 hours at 105,000 *g*. Protein content and NADH– and NADPH–cytochrome *c* reductase activity were determined in the *clear* supernatants and expressed as percentages of the values found in the fresh microsomes. At 0.13 per cent, DOC "solubilized" 37 per cent of the protein, 38 per cent of the NADPH–cytochrome *c* reductase, and 10 per cent of the NADH–cytochrome *c* reductase. At 0.26 per cent, DOC "solubilized" 67.5 per cent of the protein, 90 per cent of the NADPH–cytochrome *c* reductase, and 17 per cent of the original NADH–cytochrome *c* reductase activity.

Fractionation of Microsomes with 0.26 Per Cent DOC

Microsomes treated with 0.065 per cent and 0.13 per cent DOC yielded, upon subsequent centrifugation for 2 hours at 105,000 *g*, tightly packed, "homogeneous" pellets and clear supernatants. When the DOC concentration was increased to 0.26 per cent a loose reddish sediment appeared on top of the tightly packed bottom pellet. The three recognizable subfractions, *i.e.* the clear supernatant, the loose sediment, and the tight pellet, were easily separated from one another by pipetting off the first, and decanting the second subfraction; the third was packed tightly enough to remain, apparently undisturbed, at the bottom of the tube. The isolated subfractions were used for the morphological, chemical, and enzymological studies reported below.

A. *Electron Microscopy*

The systematic examination of the main microsomal subfractions revealed the following:

1. *The tight pellets* fixed *in situ* were composed of a mixture of small dense particles ~150 A in diameter, organized in chains or small clusters (Fig. 3 *b*). The particles were similar in general morphology to the RNP particles previously (1) prepared from hepatic microsomes by treatment with higher (up to 0.5 per cent) concentrations of DOC. Vesicles appeared occasionally in the lower (Fig. 3 *b*), and relatively frequently in the upper strata of the pellet (Fig. 3 *a*). Ferritin molecules occurred as a noticeable contaminant especially in the deep layers. The tight pellet can be described therefore as predominantly consisting of RNP particles and will be referred to as the "P fraction" in the rest of the text.

2. *The loose sediment* was fixed by mixing the bottom layer (see Fig. 1) with 2 per cent OsO_4 in 0.25 M sucrose with 0.26 per cent DOC. Pellets obtained by centrifuging this mixture at 105,000 *g* for 60 minutes proved to consist, almost exclusively, of closed, smooth surfaced vesicles (Fig. 4 *b* and *c*) more tightly packed toward the bottom of the pellet (Fig. 4 *c*). Their diameter varied noticeably, being smaller for the vesicles of the upper layers and larger for those of the middle and lower layers, although within the latter this detail was partially obscured by excessive packing. Recognizable RNP particles and ferritin molecules were infrequently encountered. A thin top layer, representing about one-tenth of the depth of the whole pellet, appeared to consist of "granular" elements. At higher magnification most of these elements proved to be extremely small vesicles (15 to 30 mμ in diameter) frequently disposed in branched chains (Fig. 4 *a*).

When the loose sediment was pelleted by recentrifugation before fixation, its composition was found to be similar except for the upper layer of fine vesicles, which was missing, and for the tighter packing of the entire preparation. The loose sediment appears, therefore, to consist primarily of membranes and as such will be referred to in the rest of the text as the "M fraction."

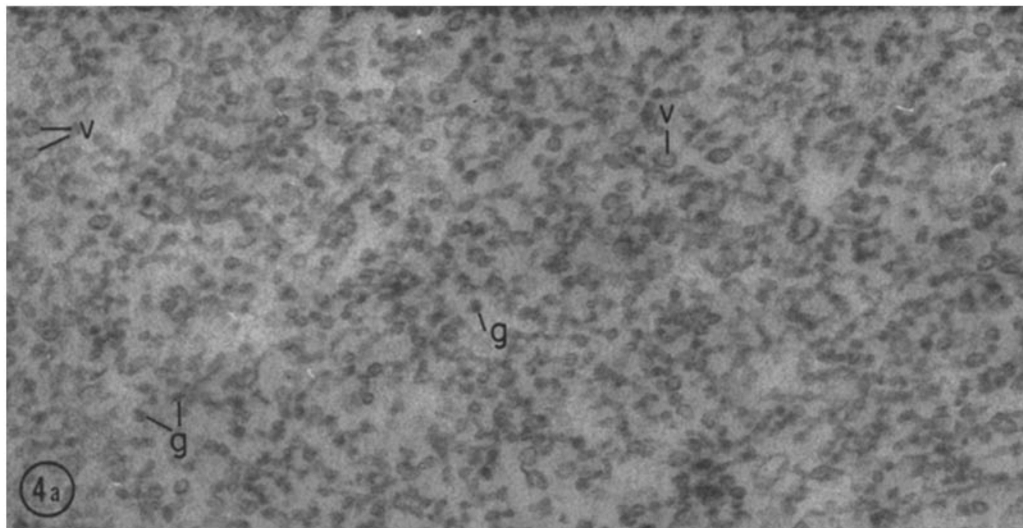
FIGURE 4

Pellet of the M fraction. × 80,000.

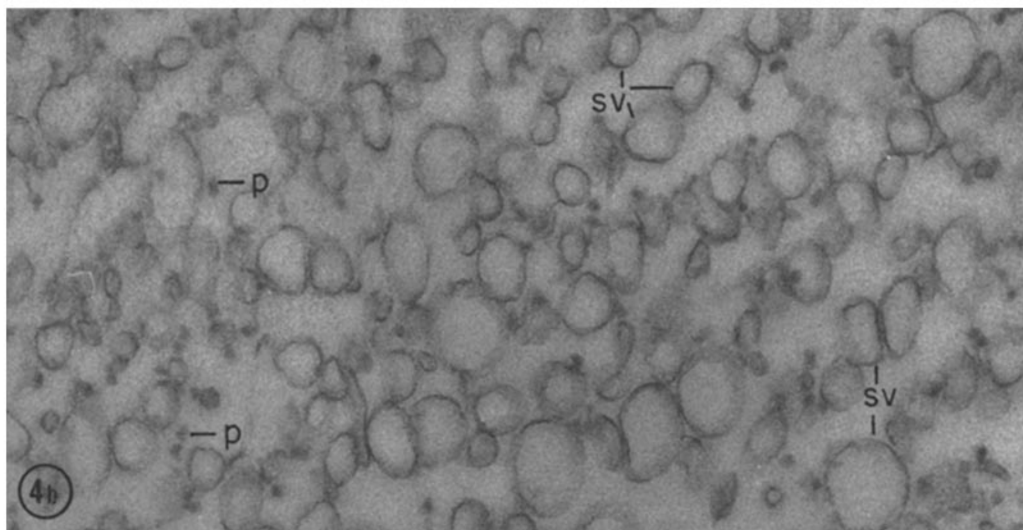
a. Top layer. Most of the elements at this level appear to be small vesicles (*v*) with a distinct lumen. Elements in which a lumen is not apparent (*g*) may represent small vesicles entirely included in the thickness of the section.

b. The middle layer consists of relatively large, smooth surfaced vesicles (*sv*) with an admixture of particles (*p*). Most of the latter are probably RNP particles, but some could be small vesicles of the type seen in Fig. 4 *a* (*v*).

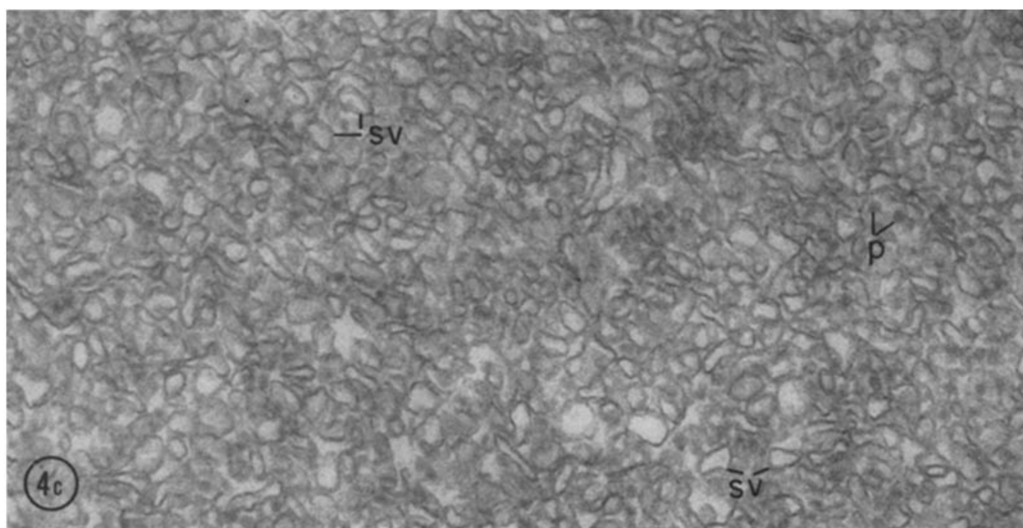
c. The bottom layer consists of tightly packed smooth surfaced vesicles (*sv*) with a few dense particles (*p*), presumably RNP particles, scattered among them.



v
v
g
g
4a



sv
p
p
sv
4b



sv
p
sv
4c

3. *The clear supernatant* mixed with an equal volume of 2 per cent OsO_4 in 0.25 M sucrose containing 0.26 per cent DOC yielded, upon centrifugation for 2 hours at 105,000 *g*, a small pellet which consisted mainly of extremely fine vesicles frequently organized in chains (Fig. 5 *a*) and similar in size to those observed in the thin top layer of the M fraction. In the deeper layers of the pellet there was an increasing admixture of large vesicles (Fig. 5 *b*). When a DOC solution was treated with OsO_4, a pellet was formed but it dissolved during subsequent dehydration.

A more substantial pellet was obtained when the clear DOC supernatant was diluted with 5 to 10 volumes of sucrose (or water) and centrifuged at 105,000 *g* for 2 to 16 hours (*cf.* "Recentrifugation Experiments," below, and Table VII). After fixation *in situ* with OsO_4 this pellet (Figs. 6 *a* and *b*) proved to consist of vesicles better outlined and generally of larger size than those found in the presence of undiluted DOC. Here again the size of the vesicles increased from the upper (Fig. 6 *a*) to the lower (Fig. 6 *b*) strata of the pellet.

B. *Gross Chemistry of Microsomal Subfractions*

The relevant data are summarized in Table III. The salient findings are:

1. A large amount of RNA (~70 per cent) is recovered in the P fraction. The concentration of RNA is somewhat lower than previously reported (1) for DOC-insoluble subfractions, presumably on account of the contamination of the pellet by some membranous material.

2. A small but definite amount of RNA is present in the M fraction; the clear supernatant contains practically none.

3. A more than 2-fold concentration of phospholipid is found in the M fraction in comparison with the original microsomes and the other subfractions.

C. *Enzymic Activities*

Cytochrome *c* Reductases

DISTRIBUTION AND RECOVERY: Enzymic assays showed that the M fraction is much richer in NADH–cytochrome *c* reductase than the clear supernatant (Table IV). The enzyme appeared to be associated with the membranous material, for, in a series of experiments in which the clear supernatant was removed in two or three successive samples, the specific activity did not increase from the upper to the lower layer. The activity increased sharply in amount and concentration only in the M fraction. In contrast to this situation, the specific NADPH–cytochrome *c* reductase activity was roughly equal in the M fraction and the clear supernatant. The enzymic activity of the P fraction was negligible for both reductases.[2]

The recovery of NADPH–cytochrome *c* reductase activity was almost quantitative (98 and 118 per cent in the two experiments), whereas that of NADH-cytochrome *c* reductase varied greatly (106 and 265 per cent in the same experiments). Overrecovery was frequent but underrecovery was also encountered (*cf.* Table VI). Also in line with these observations was the finding that the NADH–cytochrome *c* reductase activity of the M fraction was highly sensitive to dilution. In the experiment shown in Fig. 7, a part of this fraction

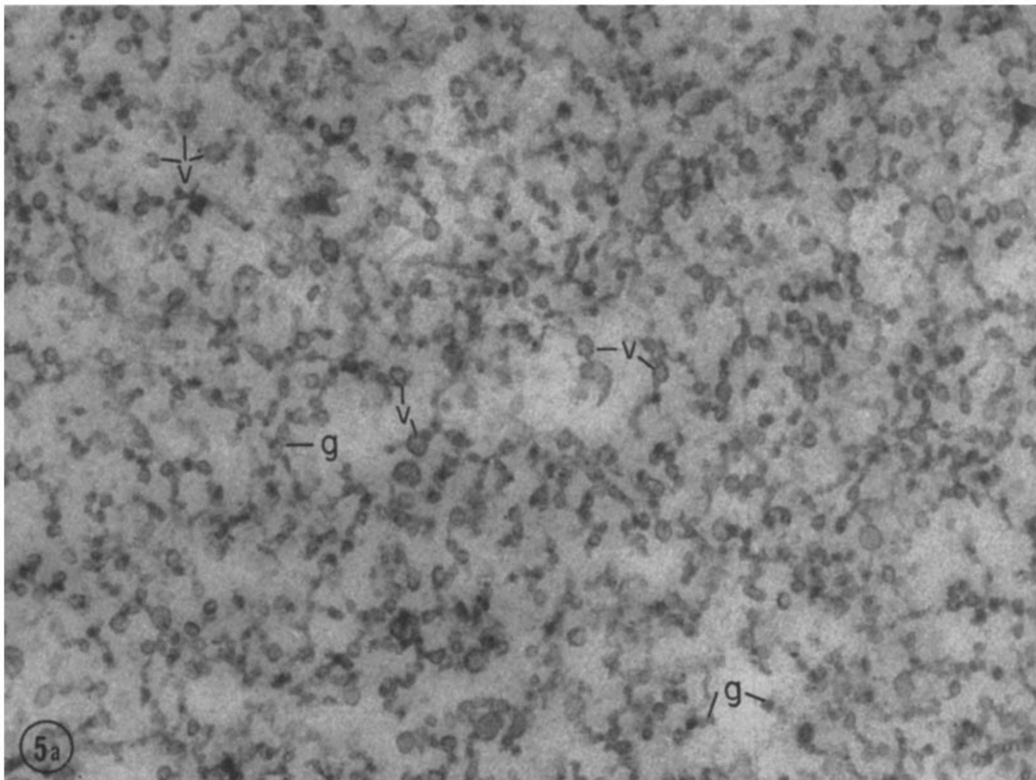
[2] In a recent note, Packer (49) reports on the occurrence of a considerable accumulation, about 50 per cent of the total, of the liver microsomal NADPH–cytochrome *c* reductase activity in the RNP particles. No evidence, however, is given to exclude the admixture of membranous elements in the detached RNP particle fraction.

FIGURE 5

Pellet obtained by centrifuging (2 hours at 105,000 *g*) the clear supernatant after mixing it with an equal amount of 2 per cent OsO_4 in 0.25 M sucrose containing 0.26 per cent DOC. × 80,000.

a. The upper layer consists mostly of small vesicles (*v*) frequently disposed in chains. Elements without an apparent lumen (*g*) are probably small vesicles entirely included in the thickness of the section. More than half of the pellet has the appearance shown in this field.

b. The lower layer contains smooth surfaced vesicles (*sv*) of various shapes and sizes, and particles (*p*, *g*). Many of the latter probably represent small vesicles similar to those found in the upper layers.


v
v
v
g
g
5a

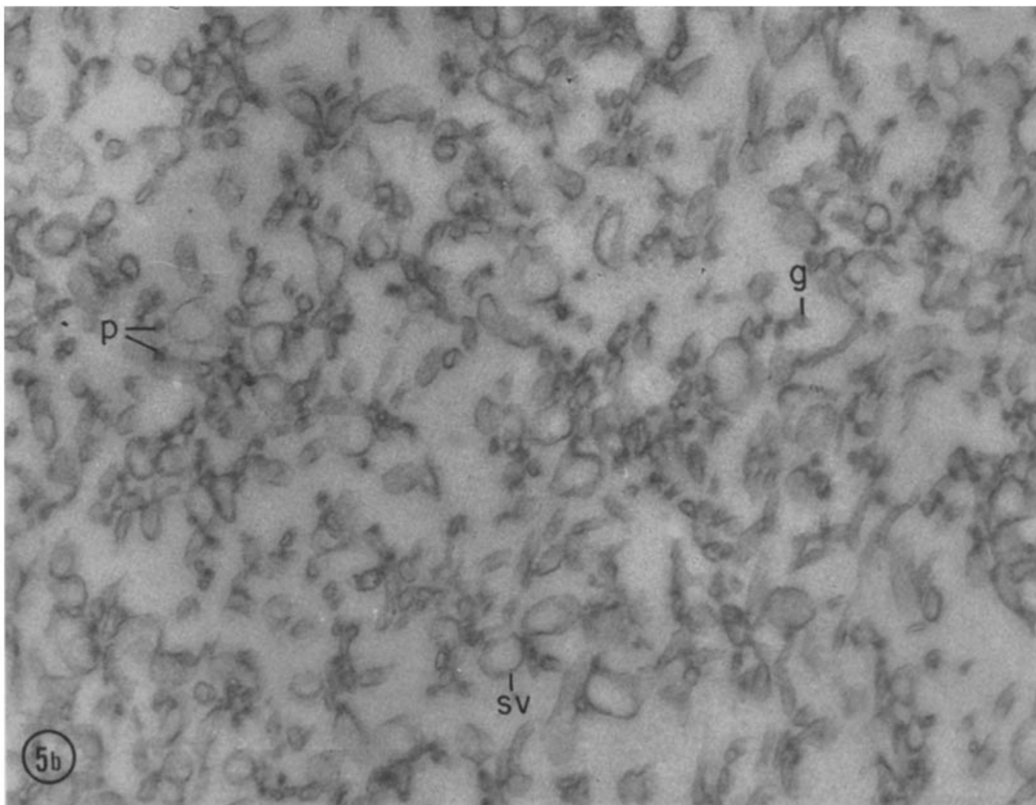

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TABLE III

Distribution of Protein, RNA, and Phospholipid in Microsomal Subfractions Obtained by DOC Treatment

Exp. no	Fraction	Protein (mg/gm liver)	RNA (mg/gm liver)	Phospholipid (mg/gm liver)	RNA (mg) / Protein (mg)	Phospholipid (mg) / Protein (mg)
1	Microsomes	20.6	4.23	12.5	0.20	0.61
	Subfractions after DOC treatment:					
	Clear supernatant (4.3 ml/gm liver)	7.1	0.12	3.6	0.02	0.51
	M fraction (0.7 ml/gm liver)	5.1	0.69	6.6	0.13	1.30
	P fraction	6.2	3.36	2.9	0.54	0.47
2	Microsomes	32.8	2.23		0.07	
	Subfractions after DOC treatment:					
	M fraction (0.7 ml/gm liver)	5.1	0.40		0.07	
	P fraction	6.1	1.78		0.29	
3	Microsomes	26.7	2.80	16.0	0.10	0.60
	M fraction (0.7 ml/gm liver)	4.6	0.39	6.8	0.08	1.47
4	Microsomes	28.2		10.8		0.37
	M fraction (0.7 ml/gm liver)	6.3		5.1		0.81

was diluted 50-fold with cold water and allowed to stand in an ice bath for varying lengths of time. It is seen that after only 5 minutes, the activity dropped to about one-fourth of the initial value. The undiluted sample maintained maximal activity even after 20 minutes. The inactivation following dilution did not drop below a certain level (*cf.* also Table I). Furthermore, once the assay was started, no further inactivation occurred. No protection was provided by 1 per cent albumin or by 0.26 per cent DOC. All these findings agree with those made on unfractionated microsomes (cf. Table I). The NADH–cytochrome *c* reductase as well as the NADPH–cytochrome *c* reductase activity of the clear supernatant was generally not affected by dilution.

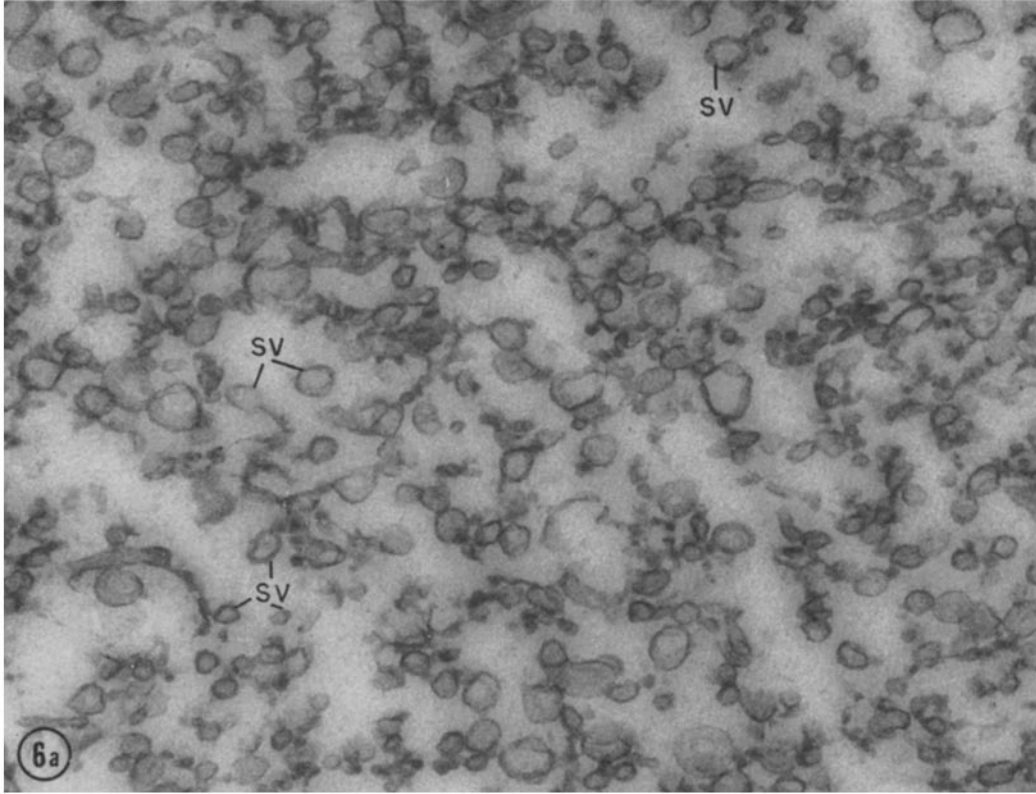
RECENTRIFUGATION EXPERIMENTS: Two types of recentrifugation experiments were carried out. In one type, an attempt was made to recover the membrane fraction as a tightly packed sediment free from supernatant. This was done, *either* by swirling up the entire DOC supernatant (including the loose pellet) and transferring it into a new centrifuge tube (leaving behind the P fraction), then centrifuging it from 30 to 120 minutes at 105,000 *g*; *or* by pipetting off the clear supernatant as completely as possible, transferring the bottom layer with the membrane fraction into a new centrifuge tube, filling up the latter with sucrose, and centrifuging it at 105,000 *g* for 2 hours. In both ways tightly packed pellets were obtained, still rich in NADH–cytochrome *c* reductase, but less active on the protein basis than the original membrane subfractions, the missing activity being only partly recovered in the supernatant. Thus, a further solubilization as well as a partial inactivation had occurred. This type of preparation was nevertheless suited for electron microscopic observations. As before, the

FIGURE 6

Pellet obtained by recentrifuging (2 hours at 105,000 *g*) the clear DOC supernate after diluting the DOC 10 times with 0.25 M sucrose. The pellet was fixed *in situ* in 2 per cent OsO_4 in 0.25 M sucrose. $\times$ 80,000.

a. The upper layer consists of smooth surfaced vesicles (*sv*) better defined and larger than those found at the same level in the presence of undiluted DOC.

b. The lower layer is comprised of smooth surfaced vesicles (*sv*) of larger size.


sv
sv
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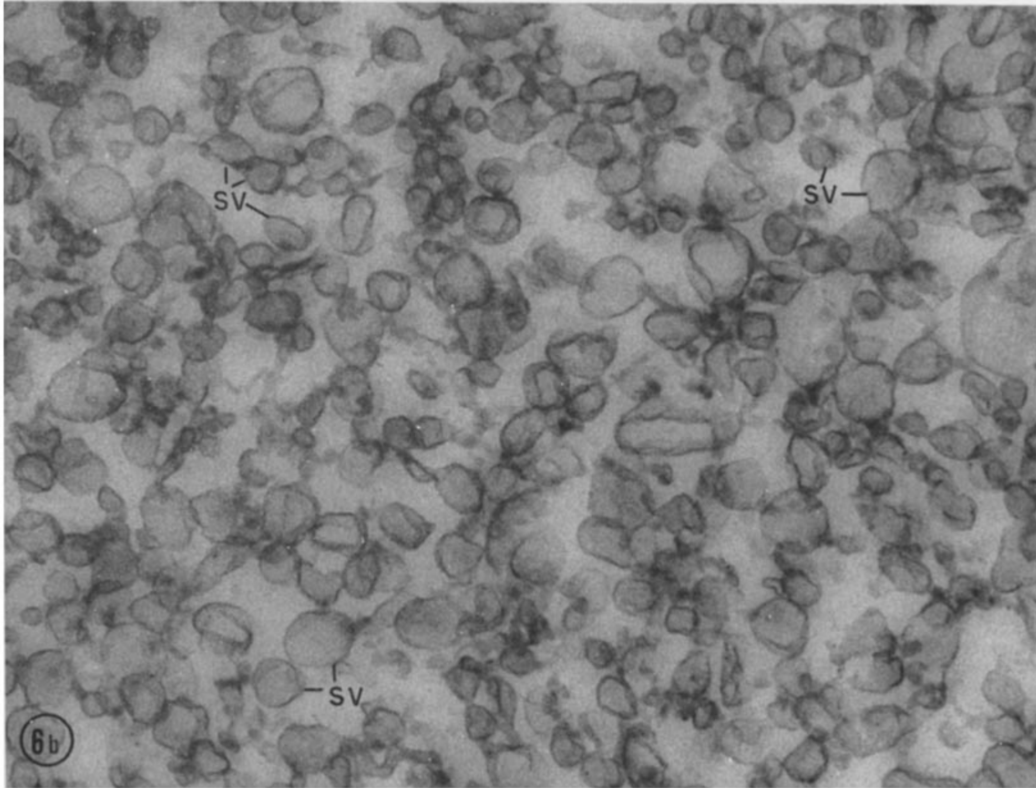
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TABLE IV

Distribution of NADH- and NADPH-Cytochrome c Reductase Activities among Subfractions of Rat Liver Microsomes Obtained by DOC Treatment

The treatment, centrifugation, and description of the subfractions are given in the text.

		Protein (mg)		Cytochrome *c* reductase (ΔE_{550}/min.)					
				NADH			NADPH		
Exp. no.	Fraction	Per ml	Per gm liver	Per ml	Per gm liver	Per mg protein	Per ml	Per gm liver	Per mg protein
1	Microsomes		23.6		140	6.2		11.6	0.51
	Subfractions after DOC treatment:								
	Clear supernatant (4.32 ml/gm liver):								
	Upper 0.45 ml	2.31	1.2	6.0	3	2.6	1.28	0.6	0.55
	Lower 3.87 ml	3.65	14.1	8.0	31	2.2	2.04	7.9	0.56
	M fraction (0.68 ml/gm liver)	8.85	6.0	168	114	19.0	4.20	2.9	0.47
	P fraction		2.0		1	0.5		0.0	0.00
	Subfractions, total		23.3		149			11.4	
2	Microsomes		31.7		121	3.8		9.0	0.28
	Subfractions after DOC treatment:								
	Clear supernatant (6.65 ml/gm liver):								
	Upper 1.85 ml	3.4	6.2	3.7	7	1.1	1.15	2.1	0.34
	Middle 3.25 ml	3.8	12.4	4.0	13	1.1	1.21	3.9	0.32
	Lower 1.55 ml	4.9	7.6	6.9	11	1.4	1.43	2.2	0.29
	M fraction (0.35 ml/gm liver)	16.4	5.8	828	290	50.2	6.90	2.4	0.42
	P fraction		5.9		1	0.2		0.0	0.00
	Subfractions, total		37.9		322			10.6	

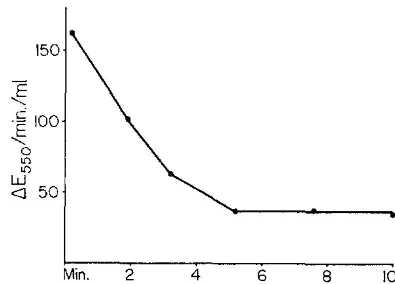

FIGURE 7

Effect of dilution on the NADH-cytochrome *c* reductase activity of the M fraction. The M fraction (0.25 ml/gm liver) was diluted 50-fold with cold water and kept in an ice bath until assayed. The NADH-cytochrome *c* reductase activity (E_{550}/min/mg protein) was 4.7 in the original microsomes, 1.28 in the clear DOC supernatant, and 14.2 in the M fraction, the latter being measured 12 seconds after dilution.

distribution pattern of NADPH-cytochrome *c* activity was different, no concentration being found in any new subfraction.

Another type of recentrifugation experiments was done with the *clear* DOC supernatant. It was observed (Table V) that when the clear DOC supernatant was diluted with sucrose (or with water), so as to lower the concentration of DOC from 2- to 10-fold, a pellet was obtained upon recentrifuging the diluted sample at 105,000 *g* for 2 to 16 hours. No pellet was obtained with the undiluted sample, or when the dilution was made with 0.26 per cent DOC. The pellet collected upon DOC dilution had a peculiar, silky consistency after homogenization in sucrose or water (but not in DOC) and sedimented rapidly on standing. It contained NADH-cytochrome *c* reductase in higher proportion than NADPH-cytochrome *c* reductase, especially after 2 to 6

hours' centrifugation; after 16 hours of centrifugation, however, both enzymes were largely found in the pellet. This pellet was about twice as rich in phospholipid (per protein) as the supernatant (*cf.* footnote to Table V).

Other Enzymes

DIAPHORASES: The distribution of these enzymes among the microsomal subfractions obtained by treatment with 0.26 per cent DOC is similar to that of the cytochrome *c* reductases (Table VI). The NADH diaphorase activity was concentrated in the M fraction, while there was no discernible concentration of the NADPH diaphorase. The P fraction was virtually free of diaphorase activity. If the distributions of the two NAD-dependent enzymes are compared, a difference in pattern becomes evident: the solubilization of the NADH diaphorase is more extensive than that of the corresponding reductase. No such difference appears between the distribution patterns of the two NADP-dependent enzymes (*cf.* last two columns in Table VI). This finding may be due to the fact that part of the microsomal diaphorase is DT diaphorase (18, 19), which reacts equally with NADH and NADPH,

TABLE V

Recentrifugation of Clear 0.26 Per Cent DOC Supernatants after Dilution with Sucrose

The clear supernatants were obtained by treating microsomes with 0.26 per cent DOC and centrifuging at 105,000 *g* for 2 hours. The *clear* supernatant was removed and treated as indicated. All dilutions were carried out with 0.25 M sucrose.

Exp. no.	Treatment of clear DOC supernatant	NADH–cyt. *c* red. (ΔE_{550}/min./gm liver)	NADPH–cyt. *c* red. (ΔE_{550}/min./gm liver)
1*	Diluted 5 ×, centrifuged at 105,000 *g* for 6 hr.:		
	Pellet‖	82	1.8
	Supernatant‖	25	5.4
2‡	Undiluted, centrifuged at 105,000 *g* for 15 hr., pellet	13	
	Diluted 2.5 ×:		
	Centrifuged at 105,000 *g* for 1 hr., pellet	2	
	" " " " " 3 " "	12	
	" " " " " 15 " "	59	
	Diluted 5 ×:		
	Centrifuged at 105,000 *g* for 1 hr., pellet	2	
	" " " " " 3 " "	15	
	" " " " " 15 " "	62	
	Diluted 10 ×:		
	Centrifuged at 105,000 *g* for 1 hr., pellet	5	
	" " " " " 3 " "	22	
	" " " " " 15 " "	64	
3§	Diluted 10 ×, centrifuged at 105,000 *g* for 2 hr.:		
	Pellet	18.2	1.9
	Supernatant	13.6	4.1
	Diluted 10 ×, centrifuged at 105,000 *g* for 16 hr.:		
	Pellet	34.5	3.3
	Supernatant	1.6	1.6

* The initial activity of the microsomes was: NADH–cytochrome *c* reductase, 558; NADPH–cytochrome *c* reductase, 8.0.

‡ The initial activity of the microsomes was: NADH–cytochrome *c* reductase, 397.

§ The initial activity of the microsomes was: NADH–cytochrome *c* reductase, 82; NADPH–cytochrome *c* reductase, 9.1.

‖ The pellet contained 7.8 mg protein and 4 mg phospholipid, and the supernatant 4.2 mg protein and 0.11 mg phospholipid per gm liver. Thus, the phospholipid/protein ratio was about 0.5 in the pellet and 0.02 in the supernatant.

TABLE VI

A Comparison of the Distribution of NADH and NADPH Diaphorase and Cytochrome c Reductase Activities in Microsomal Subfractions Obtained by DOC Treatment

Fraction	Protein (mg)	Cyt. *c* red. (ΔE_{550}/min.)				Diaphorase (ΔE_{600}/min.)				Cyt. *c* red./diaph.	
		NADH		NADPH		NADH		NADPH			
	Per gm liver	Per gm liver	Per mg protein	Per gm liver	Per mg protein	Per gm liver	Per mg protein	Per gm liver	Per mg protein	NADH	NADPH
Microsomes	17.8	241	13.5	5.3	0.30	37.5	2.10	4.1	0.23	6.4	1.3
Subfractions after DOC treatment:											
Clear supernatant (4.6 ml/gm liver):											
Upper 1.0 ml	2.4	5	1.9	0.9	0.37	1.3	0.52	0.7	0.28	3.7	1.3
Middle 2.2 ml	6.0	9	1.6	2.0	0.33	2.6	0.43	1.5	0.24	3.8	1.4
Lower 1.4 ml	4.6	9	2.0	1.3	0.28	2.3	0.49	1.1	0.23	4.0	1.2
M fraction (0.2 ml/gm liver)	2.7	134	49.8	0.9	0.33	19.3	7.18	0.6	0.24	6.9	1.4
Subfractions, total	15.7	157		5.1		28.5		3.9			

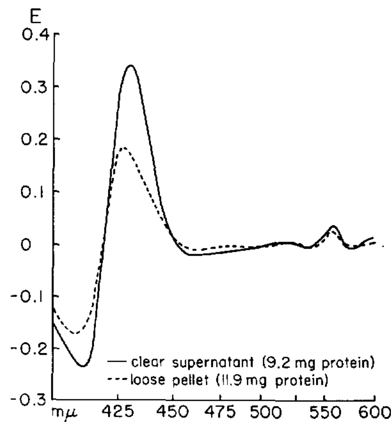

FIGURE 8

Difference spectra (reduced-oxidized) of loose sediment (M fraction), and clear DOC supernatant. Solid line, clear supernatant, 9.2 mg protein; dashed line, loose pellet (M fraction), 11.9 mg protein.

exhibits no cytochrome *c* reductase activity, and is easily "solubilized" from the microsomes. It has also been found earlier (12) that NADH diaphorase is only partially inhibited by concentrations of DOC, which completely suppress NADH–cytochrome *c* reductase activity.

CYTOCHROME b_5: Typical difference spectra obtained with the membrane fraction and the clear supernatant are shown in Fig. 8. The spectra were similar to those reported by Garfinkel (10, 21) with the possible exception of a slight shift of the 427 mμ peak to the left in the case of the membrane subfraction. This shift was consistent from one experiment to another and gives the impression of being due to a deflection of the base line. Reduction of the cytochrome b_5 by NADH or NADPH did not occur at the DOC concentration used, but reduction by NADH does occur if the DOC content is lowered.[3] DOC was added to the cuvette in the present case in order to eliminate turbidity when examining the membrane fraction. Taking the extinction difference between the absorption maximum at 427 mμ and the minimum at 410 mμ as an arbitrary estimate of cytochrome b_5 content, it is seen that the M fraction was poorer in cytochrome b_5 than the clear supernatant, since the measurements in Fig. 8 were made with roughly equal amounts of protein. It may be pointed out that cytochrome b_5 was the only enzyme among those investigated in this work, including NADPH–cytochrome *c* reductase and NADPH diaphorase, whose concentration was found to be definitely lower in the M fraction than in the clear supernatant.

This point is further illustrated by the comparison of the distribution of cytochrome b_5 with that of NADH–cytochrome *c* reductase, as shown in Table VII. In the two experiments tabulated, only about 10 per cent of the microsomal cytochrome b_5 was recovered in the mem-

[3] Personal communication from M. Klingenberg, from unpublished observations made by him in the laboratory of B. Chance.

brane fraction without an increase in specific activity. As a result, there was about 24 times more NADH–cytochrome *c* reductase per cytochrome b_5 in the membrane fraction than in the clear supernatant.

ATPASE: The typical distribution pattern of this enzyme (Table VIII) is similar to that of NADH–cytochrome *c* reductase, except that its recovery is always below 100 per cent (usually between 50 and 70 per cent), irrespective of the NADH–cytochrome *c* reductase recovery, which may vary between 30 and 300 per cent. The ATPase is thus concentrated in the M fraction, only a minor part being found in the clear supernatant. However, some activity was always present in the P fraction, at variance with the other enzymes investigated in this paper. More about this ATPase, which resembles in several respects the Mg^{++}-activated ATPase of damaged mitochondria, except that it is present in the *fresh* microsomes, has been briefly reported elsewhere (50) and is published in another paper in this issue (51). It has been found (50) that rat liver microsomes contain also a diphosphatase acting on GDP, UDP, and IDP, but not on ADP and CDP, which, in contrast to the ATPase, is recovered to a major part in the clear DOC supernatant. By histochemical procedures, the same enzyme was localized in the Golgi complex of a variety of cells (52).

GLUCOSE-6-PHOSPHATASE: It is already known (38, 53) that detergents such as DOC can "solubilize" the glucose-6-phosphatase activity of liver microsomes, and moreover that after "solubilization" a loose pellet which contains a concentrate of enzymic activity (38) can be obtained by high speed centrifugation. This procedure and these results resemble our own, for, as Table VIII shows, glucose-6-phosphatase appears concentrated in the same membrane fraction as do the NADH–cytochrome *c* reductase and the ATPase, with an increase in specific activity 5- to 6-fold over the original microsomes. The distribution and recovery pattern of the glucose-6-phosphatase differs, however, from those of the two other enzymes, in that the recovery is consistently above 100 per cent (usually 160 to 170 per cent) and the "excess" is recovered in the clear supernatant. A similar pattern is found, as is shown elsewhere (51), in the case of the aforementioned nucleoside diphosphatase. Only an insignificant fraction of the recovered glucose-6-phosphatase activity (~3 per cent) is found in the P fraction.

TABLE VII

A Comparison of the Distribution of NADH–Cytochrome c Reductase and Cytochrome b_5 in Microsomal Subfractions Obtained by DOC Treatment

Exp. no.	Fraction	Protein (mg) Per gm liver	NADH–cyt. *c* red. (ΔE_{550}/min.)		Cyt. b_5 ($\Delta E_{427-410}$(red-ox))		NADH–cyt. *c* red./b_5
			Per gm liver	Per mg protein	Per gm liver	Per mg protein	
1	Clear supernatant (5.33 ml/gm liver)	24.5	13.3	0.54	1.55	0.063	8.6
	M fraction (0.20 ml/gm liver)	4.7	30.0	6.38	0.14	0.030	212
	M fraction/clear supernatant	0.19	2.3	11.8	0.09	0.48	24.6
2	Clear supernatant (5.32 ml/gm liver)	21.3	19.7	0.93	1.12	0.053	17.6
	M fraction (0.23 ml/gm liver)	6.7	61.0	9.14	0.15	0.022	408
	M fraction/clear supernatant	0.31	3.1	9.8	0.13	0.42	23.2

In further experiments it was found that low concentrations of Mg^{++} had no effect on the M fraction enzyme, as is already known for microsomal glucose-6-phosphatase (54). Previous workers (53, 55) have found that low concentrations of DOC increase the glucose-6-phosphatase activity of the microsomes, whereas higher concentrations are inhibitory. The same effect, though less pronounced, has been found here. In the case of the M fraction enzyme, which is assayed by necessity in a low DOC concentration (0.01 per cent), an activating effect could not be demonstrated.

The glucose-6-phosphatase activity of microsomes kept in sucrose or in 0.026 per cent DOC is rather stable, whereas in 0.26 per cent DOC about three-quarters of the activity is lost within 20 hours. The enzyme "solubilized" by DOC, as well as that present in the "reconstituted" membranes isolated from a diluted DOC suspension, lost activity upon standing. A similar loss of activity in DOC was noticed by Beaufay and de Duve (53), who also showed that it can be prevented by including substrate in the storage medium. These storage- and DOC-induced changes in the activity of glucose-6-phosphatase are strikingly reminiscent of those already described for NADH–cytochrome *c* reductase activity (see Table I).

suggested by the fact that only 25 per cent of the original microsomal phospholipid fails to sediment after 2 hours at 105,000 *g*. Furthermore, even if we can safely assume that the membrane subfraction studied is mainly derived from the endoplasmic reticulum, we can neither ascertain to what extent the various parts of this system are represented in the subfraction, nor rule out the presence therein of membranous material from other sources (cell membrane, for instance).

Other attempts to fractionate liver microsomes were carried out by Chauveau *et al.* (56) and by Rothschild (57). The first authors succeeded in separating smooth membranes from particles starting with a "light" microsomal fraction. The membranous fraction so obtained apparently represents the smooth surfaced part of the endoplasmic reticulum of the liver cell. From their data it follows that these membranes possess approximately the same levels of NADH–cytochrome *c* reductase and glucose-6-phosphatase activities and phospholipid contents per unit protein as do the predominantly rough surfaced microsomes employed in the present study. In agreement with earlier (1) and present data, these authors also find that the RNP particles are practically devoid of the above enzyme activities and are relatively poor in phospholipids. Rothschild (57) reported the separation of smooth from rough surfaced microsomes by a combination of differential and isopycnic centrifugation. Employing this procedure, Fouts (58) found that smooth surfaced rat liver microsomes exhibit much higher NADPH oxidase and NADPH-dependent detoxicating activities than do their rough sur-

TABLE VIII

Distribution of ATPase, Glucose-6-Phosphatase, and NADH–Cytochrome c Reductase Activity in Microsomal Subfractions Obtained by DOC Treatment

Fraction	Protein (mg) Per gm liver	ATPase (μmoles P_i/20 min.)		Glucose-6-phosphatase (μmoles P_i/20 min.)		NADH–cyt. *c* red. (ΔE_{550}/min.)	
		Per gm liver	Per mg protein	Per gm liver	Per mg protein	Per gm liver	Per mg protein
Microsomes	20.2	38.7	1.9	86.3	4.3	91.0	4.5
Subfractions after DOC treatment:							
Clear supernatant (5.2 ml/gm liver)	12.2	7.5	0.6	59.7	4.9	23.8	2.0
M fraction (0.3 ml/gm liver)	3.3	17.9	5.2	83.3	24.1	61.2	17.7
P fraction	2.9	1.7	0.6	3.0	1.0	0.1	0.04
Subfractions, total	18.4	27.1		146.0		85.1	

DISCUSSION

We have reported in this paper the isolation of a membrane subfraction from liver microsomes and its partial chemical and enzymic characterization. This fraction consists of closed, smooth surfaced vesicles comparable in dimensions to the original microsomes. As a result of these findings we assume that, at the concentration used (0.26 per cent DOC), the detergent causes an extensive detachment of RNP particles, not necessarily accompanied by "solubilization" of the membranes to which they have been attached. In view of their abundance, it is unlikely that these vesicles represent only the smooth surfaced elements of the original microsomal fraction; actually many of them appear to be former rough surfaced elements which have lost their attached RNP particles. On the other hand, "solubilization" of membranous material is not entirely avoided, although it seems to occur on a limited scale as

faced counterparts. Provided that these activities and the NADPH–cytochrome *c* reductase activity studied in this paper are manifestations of the same enzyme, this may mean that DOC treatment may preferentially solubilize smooth surfaced microsomes. However, liver microsomes have been shown (19) to contain several types of NADPH-oxidizing enzymes, and hence, a settlement of this question requires further studies.

Our data indicate that these membranes consist of phospholipid and protein, in a ratio higher than that of the original microsome fraction, and that they contain a certain amount of RNA ($\sim$25 per cent of the original microsomal RNA), which seems to be more than could be accounted for by contamination with RNP particles. Our experiments, finally, demonstrate that certain microsomal enzymes, namely NADH–cytochrome *c* reductase, NADH diaphorase, Mg^{++}-activated ATPase, and glucose-6-phosphatase, either are part of these membranes or are tightly bound to them. Other enzymes, *e.g.* NADPH–cytochrome *c* reductase, DT diaphorase, and cytochrome b_5, do not seem to be part of the membranes or appear to be loosely attached thereon.

An intriguing but still poorly understood finding concerns the appearance of sedimentable vesicles in the clear supernatant upon diluting its DOC concentration. These vesicles, which consist of phospholipid and protein and exhibit NADH–cytochrome *c* reductase and glucose-6-phosphatase activities, may result from the coalescence of smaller vesicular units known to exist in the supernatant, or may arise *de novo* from the aggregation of even smaller micelles and individual molecules. Solubilization by DOC seems to be a rather complex process which, in addition to extensive detachment of RNP particles, causes a fine dispersion of the membranous material into progressively smaller vesicular units. To what extent true solubilization occurs remains unknown. The fact that membrane-bound enzymic activity NADH–cytochrome *c* reductase and glucose-6-phosphatase) reacts to storage, dilution, and DOC treatment otherwise than a "soluble" enzyme (NADPH–cytochrome *c* reductase) may have important implications. It suggests that the activity of an enzyme could be influenced and controlled by agents acting not upon the enzyme itself but upon its position within the cellular structure to which it belongs.

A set of enzymes comparable to that associated with liver microsomes, and including NADH and NADPH reductases and diaphorases, cytochrome b_5, and a Mg^{++}-activated ATPase, is known to occur in liver mitochondria. There are some differences between these two enzyme sets, but the similarities are preponderant. In both cases, for instance, the NADPH–cytochrome *c* reductase (14) and the DT-diaphorase (19, 59) are more easily extractable than the NADH–cytochrome *c* reductase. The DOC effects upon mitochondrial and microsomal reductases and diaphorases are comparable, and the DOC inactivation of mitochondrial (NADH–cytochrome *c* reductase can be reversed by dilution as in the case of the microsomes.[4] The Mg^{++}-dependent ATPase activity is in both cases tightly bound to a membranous component (60), and the cytochrome b_5 extracted from mitochondria is similar to that of microsomal origin (61). A possible explanation of this parallelism is an extensive contamination of each of these two fractions by whole units or subunits that properly belong to the other. This possibility is ruled out or rendered negligible, however, by the fact that certain activities are not shared by the two fractions: glucose-6-phosphatase is restricted to the microsomes, and succinoxidase activity is found only in mitochondria, more precisely in mitochondrial membranes (62). Mitochondrial subunits poor in succinoxidase but rich in NADH oxidase activity have been isolated (63, 64) by a rather drastic and elaborate procedure, but it is hardly conceivable that such subunits could form at the expense of mitochondria during tissue homogenization (to be available for the contamination of the microsomes). Moreover, even among the enzymes shared in common there are small but significant differences. The mitochondrial NADPH–cytochrome *c* reductase can be readily extracted with water (14), whereas the extraction of the microsomal counterpart requires a more drastic treatment(14, and this paper). The mitochondrial and microsomal suspensions react differently to DOC treatment: though both are clarified by the detergent, the treated mitochondria yield upon high speed centrifugation a tight pellet which contains succinoxidase activity but neither NADH–cytochrome *c* reductase (62) nor NADH diaphorase activity (60). Under similar conditions only a loose sediment which contains the latter enzymes is obtained from microsomes. Finally, since the

[4] Unpublished experiments of L. Ernster.

cytochrome *c* reductases are distributed between mitochondria and microsomes in ratios varying from 3:7 to 5:5 (6–8, 14, 16), it follows that the explanation envisaged requires the contamination of one fraction by 30 to 50 per cent of the subcellular units of the other. This is clearly an improbable assumption (*cf.* (35)).

What could be the physiological meaning of this seemingly uniform organization of certain electron- and energy-transferring enzymes in the endoplasmic membranes and in mitochondria? May it be that we are here dealing with a common functional principle, which is carrying out a basically similar process, though with major or minor variations in the end result (65)? In the two cases under consideration, this enzyme system might provide the energy required for active transport across the membrane from one cell compartment to another. In the case of the endoplasmic reticulum the two compartments may be the cytoplasmic matrix, which is the truly intracellular medium, and the content of the canalicular system, which probably is related to the extracellular medium (40). In the case of the mitochondria the first compartment again could be the cytoplasmic matrix, whereas the second could be one of the two intramitochondrial spaces. The NADH–cytochrome *c* reductase system could be located only in the external mitochondrial membrane, whose basic relations to the cytoplasmic matrix are, at least in part, similar to those of the endoplasmic membranes. In some instances continuity between these two membranes has been reported (66).

There is evidence for active transport between mitochondria and the surrounding medium (67–73) but none so far between microsomes and their surrounding phase. However, ATPase activities have already been associated with membranous components in both mammalian (74–77) and bacterial cells (78) and have been implicated in sodium and potassium transport across erythrocyte (79) and nerve cell (80, 81) membranes. Another type of membrane-linked energy transfer, revealed by a participation of the sarcotubular ATPase in muscle fiber relaxation, has recently been demonstrated by Muscatello *et al.* (82). In the case of the mitochondria, it might be further speculated that the enzyme system of the mitochondrial membranes may have evolved into an energy-producing system as it exists today in the oxidative-phosphorylation complex. A similar view has recently been expressed in the "chemiosmotic theory" of Mitchell (83). The idea that electron transport–coupled generation of ATP and ATP-dependent energy and ion transport may be specialized manifestations of a common enzymic function, inherent in cellular membrane structures, is a fascinating one and stimulates further experimentation (51, 84).

This work was initiated in the spring of 1958, during a stay of Dr. Ernster at The Rockefeller Institute, and was pursued as a joint project between The Rockefeller Institute and The Wenner-Gren Institute.

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