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LOCALIZATION OF ENZYMES IN SPECIALIZED REGIONS OF THE MICROSOMAL MEMBRANE

LARS WINQVIST and GUSTAV DALLNER

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm and Department of Pathology at Sabbatsberg Hospital, Karolinska Institutet, Stockholm (Sweden)

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SUMMARY

Microsomal vesicles were centrifuged through sucrose density gradients containing deoxycholate. With 0.15 % detergent electron transport enzymes and phosphatases could be separated. Increasing the deoxycholate concentration to 0.19 % resulted in separation of the microsomal material into five bands containing (in order from the top of the gradient) adenosine monophosphatase, inosine diphosphatase and some glucose-6-phosphatase (band 1); NADH-linked (band 2) and NADPH-linked (band 3) electron transport enzymes; and glucose-6-phosphatase (bands 4 and 5). It appears that enzymes are arranged in specialized patches in the microsomal membrane.

INTRODUCTION

The microsomal membrane contains a large number of enzymes, including several groups of enzymes catalyzing consecutive reactions. With subfractionation techniques it has not been possible to separate microsomal vesicles with single specialized functions. Enrichment of certain enzymes in different subfractions has, however, been achieved [1].

The endoplasmic reticulum does not only process metabolites for intracellular purposes but also produces a large number of macromolecules for secretion [2]. Among these macromolecules are albumin, various types of serum glycoproteins and different kinds of lipoproteins. These macromolecules are assembled in the lumen of the endoplasmic reticulum and transported as such or completed during the transport process [3]. It is conceivable that these synthetic reactions are mediated by enzymes which are not distributed randomly but localized in specific regions of the endoplasmic reticulum membrane system.

It has been established that the mitochondrial respiratory chain and energy-transducing system is composed of well-defined complexes which can be isolated by suitable techniques [4]. The presence of similar complexes in microsomal membranes is much less certain. Immunoprecipitates of detergent-solubilized microsomal membranes seem to contain several enzyme activities, as measured by histochemical

methods [5, 6]. In liver microsomes from adult rats there is a stoichiometric relationship between the amounts of the components of the electron transport chains [7, 8]. However, these relationships are in a state of change in the early postnatal period [9] or during the initial phase of phenobarbital induction [10]. Cytochrome b_5 and its reductase may or may not be organized in a complex; both cases have been argued in the literature [11, 12].

This study has been designed to investigate the possible existence of complexes in microsomal membranes. The experiments performed in detergent-containing sucrose density gradients indicate that functionally related enzymes are segregated onto specific patches of the membrane.

MATERIALS AND METHODS

Animals. Adult, male Sprague-Dawley rats weighing 180–200 g were used. All animals were starved for 20 h before sacrifice by decapitation.

Fractionation. Microsomes and microsomal subfractions were prepared as described earlier [13]. Except where otherwise stated, all fractions were subjected to a Tris-water-Tris washing procedure to remove adsorbed and secretory proteins [13]. In order to standardize the experiments, the protein content before gradient centrifugation was determined with the Biuret reaction [14]. The continuous sucrose gradients, with a total volume of 36 ml were prepared with a mixing chamber [15]. The linear density gradient in all experiments ranged from 1.05 to 1.23 g/cm^3. Where indicated, deoxycholate was added in equal amounts to both the light and heavy sucrose solutions in the mixing chamber. Thus, the detergent concentration was constant throughout the gradient. In all cases where deoxycholate was used the gradient also contained 50 mM KCl to increase the efficiency of the detergent [16]. 24 mg of microsomal protein in 3 ml 0.13 M sucrose was layered onto the gradient. Centrifugation was performed in an SW 27 rotor (Beckman-Spinco) at 80 000 $\times g$ for 48 h in a Beckman L2-65B centrifuge. The five bands were collected using a syringe fitted with a long needle.

Millipore filtration and incorporation. To estimate the size of the particles in the bands the fractions were filtered by a suction pump through Millipore filters of known pore size (Millipore Filter Co., Bedford, Mass.) [17]. 2 mg protein in 4 ml 0.6 M sucrose was passed through the filter, which was then rinsed with 2 ml 0.25 M sucrose. The absorption at 280 nm was measured before filtration and afterwards, and the percentage of ultraviolet-absorbing material that had passed through the filter was calculated.

50 μCi [^{14}C]leucine (50 Ci/mol) from the Radiochemical Centre, Amersham, U.K., in sterile Ringer's solution was injected into the portal vein of rats under Nembutal anaesthesia. After 60 min the rats were decapitated and the livers homogenized and fractionated. Radioactivity was determined in a Beckman liquid scintillation counter using Bray's solution [18].

Chemical and enzymatic analyses. Protein was measured according to Lowry et al. [19]. Phospholipid was analyzed as described previously [20]. The various enzyme activities and amounts were measured according to methods described earlier [21, 22]. In the case of deoxycholate-containing gradients glucose-6-phosphatase and inosine diphosphatase (IDPase) activities of the individual fractions were analyzed without the addition of more detergent to the assay medium.

RESULTS

Subfractionation of total microsomes

Total microsomes centrifuged to isopycnic equilibrium on a continuous sucrose gradient exhibit a broad distribution of protein between densities 1.10 and 1.25 g/cm^3 (Fig. 1). Various electron transport and phosphatase activities were measured and three of them are shown on the figure. Glucose-6-phosphatase, NADH- and NADPH-cytochrome *c* reductase activities are distributed in a similar fashion on a protein basis without significant enrichment in any fraction.

Appearance of bands in the presence of deoxycholate

The appearance of total microsomes centrifuged for 48 h in a continuous sucrose gradient ranging from 1.05 to 1.23 g/cm^3 and containing 0.15 % or 0.19 % deoxycholate is shown in Fig. 2. The relatively narrow band 1, lying in both cases at a density of about 1.085 g/cm^3, had a white flaky appearance. Bands 2 and 3 looked finely dispersed and were red in the 0.15 % deoxycholate gradient but the latter was white in the 0.19 % deoxycholate gradient. These two fractions were better separated in the presence of the higher detergent concentration, where they equilibrated at 1.11–1.12 and 1.14–1.15 g/cm^3, respectively. Fractions 4 and 5 were gray and grainy and displayed a somewhat higher density in the 0.19 % deoxycholate-containing gradient.

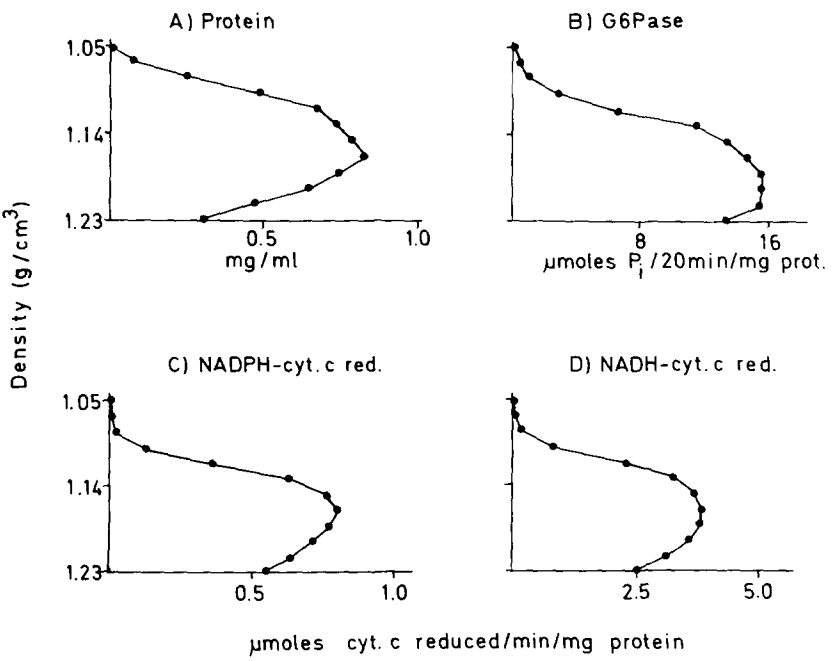

Fig. 1. Distribution of protein and some enzymes after isopycnic centrifugation of total microsomes. Non-washed microsomes were suspended in 0.13 M sucrose and subfractionated on a density gradient ranging from 1.05 to 1.23 g/cm^3. The density distribution is shown on the ordinate, and the protein amounts and enzyme activities on the abscissa. Each value represents the mean of 3 experiments.

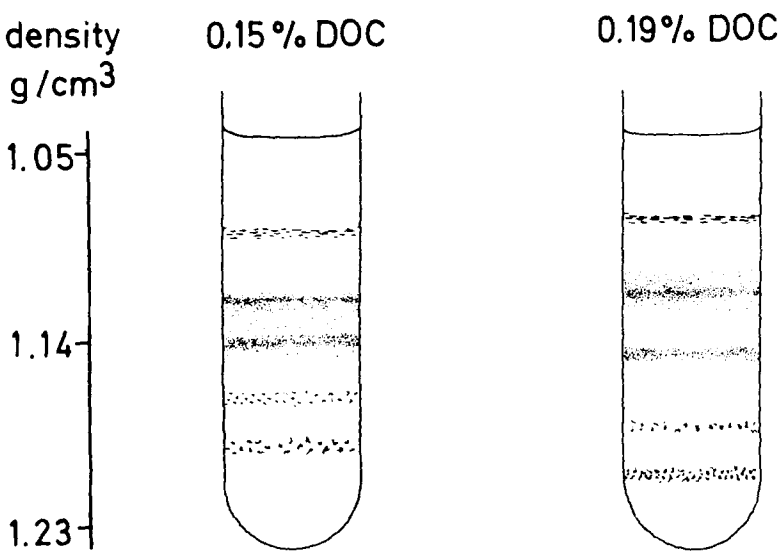

Fig. 2. Appearance of bands after equilibrium centrifugation of total Tris-water-Tris washed microsomes in sucrose gradients containing 0.15 % deoxycholate (DOC) (A) and 0.19 % deoxycholate (B).

Millipore filtration

After centrifugation in a sucrose gradient containing detergent, five bands were seen. In order to determine the size of the vesicles in these bands, they were subjected to Millipore filtration. This procedure was previously used to study rough microsomes after sonication, and good correlation was found between the sizes determined by electron microscopy and by filtration [17].

Microsomes were Millipore filtered in the presence of various concentrations of deoxycholate. In the absence of detergent only a small amount of material passed through the 0.22-μm pores, two-thirds passed through 0.45-μm pores, and almost all the microsomal material appeared in the filtrate when the pores were 0.8 μm in diameter (Fig. 3A). Increasing the deoxycholate concentration permits more material to go through all of the filters used. The greatest change occurred between 0.1 and 0.2 % deoxycholate and at the latter concentration two thirds of the microsomal material passed through 0.22-μm pores. Thus, this range of deoxycholate concentration seemed to be most interesting to investigate.

Fig. 3B shows the Millipore filtration pattern for the individual bands obtained by centrifugation in the presence of 0.19 % deoxycholate. Since these bands were centrifuged to equilibrium in a density gradient, particle size should have no influence on their final positions. Filtration demonstrated that the particles in bands 2 and 3 are relatively small, all of them passing through 0.22-μm pores. Particles from bands 4 and 5 do not pass trough this filter, so they are relatively large; while the material in band 1 is intermediate in size.

Protein and phospholipid distribution

The five bands obtained after density gradient centrifugation were removed and analyzed for protein and phospholipid content (Table I). The individual bands were removed completely but the clear regions between the bands were discarded.

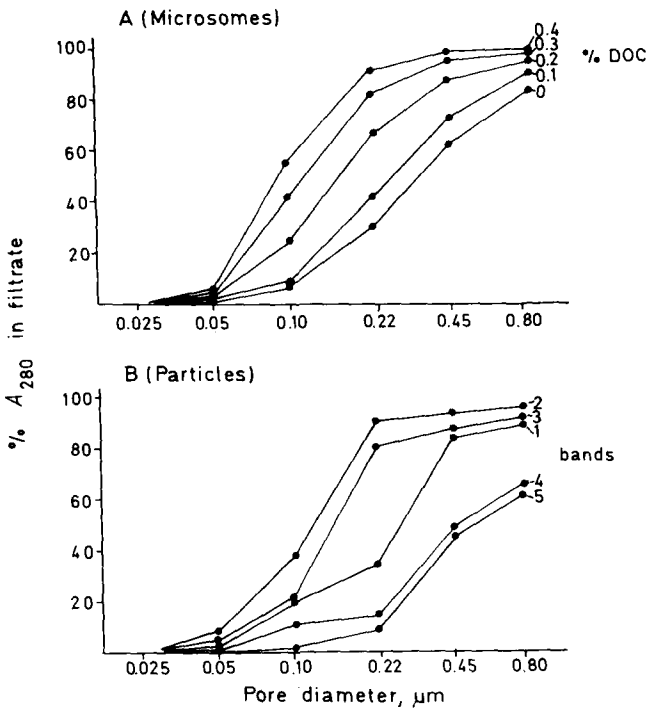

Fig. 3. Millipore filtration of Tris-water-Tris washed microsomes and isolated particles. (A) The microsomal pellet was suspended in 0.15 M Tris · HCl, pH 8.0 to give a concentration of 40 mg protein per 10 ml and sedimented by centrifugation. For the water thermal treatment the microsomal pellet was suspended in cold distilled water (1 mg protein/ml), incubated at 30 °C for 15 min, cooled rapidly in an ice-water bath and sedimented by centrifugation, whereupon the Tris washing was repeated, and the pellet was suspended in 0.6 M sucrose at a concentration of 0.6 mg protein/ml. This corresponds to the protein concentration one would get if the subfractionated microsomal protein was uniformly distributed throughout the gradient. Deoxycholate (DOC) was added to separate aliquots to give final concentrations of 0.1, 0.2, 0.3, and 0.4 %. Millipore filtration was performed as described in Materials and Methods. The amount of protein was estimated by measurement of the A_{280nm} in the filtrate. (B) The five bands obtained after isopycnic centrifugation of microsomes in the presence of 0.19 % deoxycholate were Millipore filtered as in A. The values present the means of 3 experiments.

This clear medium proved to contain only 30 % of the total protein and such low enzyme activities, that they were considered less interesting; the assay results were also uncertain. At all detergent concentrations most of the protein was found in bands 2 and 3. At higher deoxycholate concentrations there is a redistribution of material leading to an increase of protein in bands 4 and 5. Generally the phospholipid to protein ratio decreases as the density increases, this trend being more pronounced at higher detergent concentrations.

Enzyme distribution

The enzyme activities and amounts on the various figures are given as relative specific activities. Unfractionated microsomes with the same protein and deoxycholate concentration as in the gradient were stored during a time period corresponding to

TABLE I

DISTRIBUTION OF PROTEIN AND PHOSPHOLIPID/PROTEIN RATIO IN MICROSOME-DERIVED PARTICLES

The deoxycholate concentrations in the first column refers to the concentration in the gradient during centrifugation. The bands are numbered from the top of the gradient, i.e. band 1 is the uppermost. The protein is given in relative concentrations, i.e. the protein concentration of the fractions divided by the protein concentration of the original material, homogenously distributed throughout the tube and stored during the centrifugation. The relative phospholipid/protein ratio was calculated in an analogous manner. The values are the means ±S.E.M. of 6 experiments.

	Deoxycholate concentration (%)	Bands				
		1	2	3	4	5
Protein (relative concentration)	0.11	1.11±0.09	2.06±0.11	2.29±0.36	0.78±0.06	0.66±0.12
	0.13	1.10±0.10	2.01±0.16	2.22±0.21	1.25±0.49	0.58±0.10
	0.15	1.30±0.21	2.54±0.50	2.35±0.16	0.81±0.15	0.56±0.19
	0.17	1.19±0.18	2.66±0.47	2.11±0.15	1.41±0.17	0.60±0.14
	0.19	1.34±0.21	2.55±0.16	2.14±0.13	1.31±0.31	1.15±0.20
	0.21	1.32±0.09	2.29±0.14	2.04±0.29	1.11±0.21	1.32±0.35
Phospholipid/ protein ratio (relative ratio)	0.11	1.49±0.15	1.22±0.12	1.05±0.10	0.62±0.12	0.36±0.14
	0.13	1.61±0.10	1.19±0.19	1.00±0.09	0.56±0.14	0.21±0.10
	0.15	1.91±0.25	1.21±0.20	0.90±0.16	0.61±0.09	0.40±0.09
	0.17	2.04±0.11	1.08±0.19	0.86±0.10	0.34±0.08	0.23±0.17
	0.19	2.51±0.21	1.37±0.16	1.01±0.08	0.38±0.11	0.11±0.09
	0.21	2.98±0.26	1.34±0.10	0.86±0.08	0.25±0.06	0.16±0.09

the time of centrifugation at 4 °C (Table II). As expected, deoxycholate effects the enzyme activities to some extent.

The distribution of various phosphatase activities after subfractionation in a sucrose density gradient containing detergent is shown in Table III. Adenosine monophosphatase (AMPase) is highly enriched in band 1, much less is present in bands

TABLE II

ACTIVITY OF MICROSOMAL ENZYMES STORED IN VARIOUS CONCENTRATIONS OF DEOXYCHOLATE

The microsomal suspension in 1.10 M sucrose (0.6 mg protein/ml) was supplemented with deoxycholate and stored for 48 h at 4 °C before estimation of enzyme activities. No extra deoxycholate was added during the assays. The results are the means of 6 experiments. G6Pase = glucose-6-phosphatase; cyt. = cytochrome; red. = reductase.

Activities	Deoxycholate (%)					
	0.11	0.13	0.15	0.17	0.19	0.21
AMPase*	3.7	3.5	3.6	3.7	3.9	3.6
IDPase*	26	27	30	29	36	33
G6Pase*	3.6	3.8	4.1	4.2	5.4	5.4
NADPH-cyt. *c* red.**	0.46	0.45	0.44	0.46	0.50	0.48
NADH-cyt. *c* red.**	1.3	1.5	1.6	1.6	1.6	1.7

* μmol P_i/20 min/mg protein.

** Cyt. *c* reduced/min/mg protein.

TABLE III

DISTRIBUTION OF PHOSPHATASE ACTIVITIES IN MICROSOME-DERIVED PARTICLES

The relative specific activities were calculated as in Table I. The values are the means ±S.E.M. of 6 experiments

Relative specific activity	Deoxycholate concentration (%)	Bands				
		1	2	3	4	5
Adenosine monophosphatase	0.11	2.48±0.22	0.78±0.08	0.95±0.14	0.30±0.11	0.14±0.05
	0.13	3.69±0.36	0.75±0.17	0.74±0.10	0.16±0.06	0.06±0.04
	0.15	5.08±0.48	0.79±0.08	0.63±0.14	0.06±0.03	0.03±0.02
	0.17	4.38±0.56	0.53±0.11	0.32±0.05	0.05±0.04	0.04±0.02
	0.19	3.67±0.31	0.50±0.10	0.51±0.16	0.11±0.05	0.07±0.03
	0.21	4.03±0.34	0.61±0.09	0.82±0.13	0.18±0.07	0.11±0.04
Inosine diphosphatase	0.11	1.16±0.12	1.05±0.09	0.11±0.07	0.04±0.02	0.05±0.03
	0.13	3.48±0.45	0.86±0.11	0.23±0.07	0.04±0.03	0.02±0.01
	0.15	2.42±0.39	0.87±0.13	0.09±0.06	0.10±0.06	0.05±0.03
	0.17	2.29±0.15	0.38±0.12	0.03±0.02	0.04±0.02	0.04±0.02
	0.19	1.12±0.13	0.86±0.10	0.10±0.05	0.05±0.02	0.03±0.01
	0.21	1.03±0.17	1.18±0.09	0.07±0.04	0.08±0.05	0.05±0.03
Glucose-6-phosphatase	0.11	1.71±0.17	1.02±0.09	1.75±0.14	0.09±0.05	0.06±0.03
	0.13	1.62±0.24	0.66±0.11	0.97±0.10	0.25±0.09	0.23±0.08
	0.15	1.93±0.13	0.36±0.06	1.11±0.12	1.10±0.10	0.76±0.21
	0.17	1.44±0.26	0.34±0.08	0.72±0.07	2.66±0.27	1.63±0.39
	0.19	1.27±0.16	0.10±0.06	0.52±0.11	1.42±0.21	3.64±0.37
	0.21	1.13±0.12	0.08±0.05	0.22±0.07	2.04±0.23	4.89±0.57

2 and 3, while bands 4 and 5 are almost devoid of AMPase activity. In general, the distribution of IDPase is similar, with highest activity in band 1 and next highest in band 2. With 0.21 % deoxycholate, however, the activity in band 2 is slightly higher than in band 1. There is almost no IDPase activity in the three lower bands. Interestingly, IDPase activity is not much affected by storage of microsomes in the presence of deoxycholate. However, when fractions are isolated by a long centrifugation the enzyme is inactivated to a large degree.

The situation with glucose-6-phosphatase is different. At low detergent concentrations the enzyme appears to be concentrated in the upper band. At 0.17 % and higher concentrations of deoxycholate, a redistribution takes place and most of this hydrolytic activity is present in band 4 and particularly in band 5. Significant activity also remains in fraction 1.

NADPH-cytochrome *c* reductase activity can be detected in almost all the bands, even at higher deoxycholate concentrations (Table IV). A great enrichment of the activity is, however, obtained in band 3. The localization of NADH-cytochrome *c* reductase activity is different from the NADPH-dependent activity. At deoxycholate concentrations of 0.15 % and more, all of the NADH-cytochrome *c* reductase activity is recovered in bands 1 and 2, the activity in 2 being higher than that in 1.

In order to investigate the distribution of other enzymes participating in the two microsomal electron transport chains, other NADPH- and NADH-dependent reactions were analyzed on the 0.15 % and 0.19 % deoxycholate-containing gradient (Table V). NADPH-neotetrazolium reductase follows its cytochrome *c* reductase

TABLE IV

DISTRIBUTION OF NADPH- AND NADH-CYTOCHROME *c* REDUCTASE ACTIVITIES IN MICROSOME-DERIVED PARTICLES

The relative specific activities are calculated as in Table I. The values are the means ±S.E.M. of 6 experiments.

Relative specific activity	Deoxycholate concentration (%)	Bands				
		1	2	3	4	5
NADPH-cytochrome *c*-reductase	0.11	0.07±0.04	1.02±0.12	1.28±0.19	1.03±0.13	0.35±0.16
	0.13	0.21±0.06	1.03±0.18	1.42±0.18	0.49±0.15	0.44±0.09
	0.15	0.11±0.05	0.98±0.12	1.54±0.26	0.32±0.07	0.16±0.05
	0.17	0.10±0.07	0.99±0.15	1.57±0.12	0.21±0.09	0.18±0.04
	0.19	0.07±0.03	0.90±0.10	1.67±0.17	0.35±0.11	0.08±0.05
	0.21	0.11±0.04	0.51±0.10	1.84±0.19	0.24±0.07	0.09±0.05
NADH-cytochrome *c*-reductase	0.11	0.37±0.06	1.51±0.26	1.78±0.28	0.46±0.12	0.10±0.04
	0.13	0.41±0.09	1.64±0.21	1.46±0.11	0.16±0.05	0.18±0.07
	0.15	0.97±0.22	1.49±0.16	1.04±0.10	0.27±0.11	0.04±0.02
	0.17	1.16±0.14	1.86±0.14	0.71±0.11	0.10±0.04	0.05±0.03
	0.19	1.52±0.12	1.93±0.10	0.35±0.11	0.07±0.04	0.05±0.02
	0.21	1.18±0.16	2.03±0.13	0.32±0.07	0.04±0.03	0.04±0.02

counterpart; it is mainly enriched in band 3. DT-diaphorase, an enzyme reacting with both pyridine nucleotides, has its highest specific activity in band 3 in the presence of 0.15 % deoxycholate and in band 2 in the presence of 0.19 % detergent.

The terminal enzyme of the NADPH-driven chain is cytochrome *P*-450. It is well documented that this cytochrome is easily denatured by detergents [23] and it was completely denatured under the conditions used here. If the centrifugation time was decreased to 15 h, equilibrium was not attained and therefore the distribution of the remaining *P*-450 (about 50 %) was only indicative; but this distribution closely resembled that of the NADPH-cytochrome *c* reductase activity. In additional experiments, centrifugation was performed with 10 % glycerol in the gradient, using correspondingly less sucrose to keep the density profile unchanged. 48 h centrifugation gave an enzyme distribution similar to that obtained without glycerol, but again about 50 % of the cytochrome *P*-450 was denatured. Therefore, the distribution pattern for this protein is not fully reliable. However, cytochrome *P*-450 could be found in high specific amounts in band 3 and also in band 2. In addition, after sodium dodecyl sulfate polyacrylamide gel electrophoresis the characteristic large cytochrome *P*-450 peak was found in bands 3 and 2.

The two components of the NADH chain were measured separately. Both ferricyanide reductase, which reflects the activity of the flavoprotein alone, and the amount of cytochrome b_5 show the same distribution pattern as NADH-cytochrome *c* reductase activity which involves both the flavoprotein and cytochrome b_5. At lower deoxycholate concentrations these activities are found in the upper three bands, while at higher deoxycholate concentrations they are restricted to bands 1 and 2. This is particularly true for cytochrome b_5 reduced enzymatically with NADH. The cytochrome was also reduced chemically to measure its amount independently of the flavoprotein. Both ferricyanide reductase and dithionite reduceable cytochrome b_5

TABLE V

DISTRIBUTION OF SOME NADPH- AND NADH-LINKED ENZYMES IN MICROSOME-DERIVED PARTICLES IN DENSITY GRADIENTS CONTAINING 0.15 % OR 0.19 % DEOXYCHOLATE

Cytochrome b_5 was reduced by NADH or dithionite. The values are the means ±S.E.M. of 6–9 experiments. NT = neotetrazolium; red. = reductase; FeCN = ferricyanide; cyt. = cytochrome; dith. = dithionite.

	Deoxycholate concentration (%)	Bands				
		1	2	3	4	5
NADPH-NT red.*	0.15	0.24±0.06	0.58±0.08	1.54±0.20	0.52±0.19	0.26±0.09
DT-diaphorase*	0.15	0.52±0.17	0.99±0.29	2.10±0.16	0.28±0.07	0.42±0.17
NADH-FeCN red.*	0.15	1.05±0.21	1.51±0.14	0.98±0.08	0.33±0.12	0.32±0.08
Cyt. b_5 (NADH)**	0.15	1.13±0.12	1.84±0.59	1.48±0.23	0.06±0.03	0.05±0.03
Cyt. b_5 (dith.)**	0.15	1.07±0.22	1.80±0.31	1.49±0.25	0.08±0.05	0.06±0.04
NADPH-NT red.*	0.19	0.31±0.05	1.82±0.12	1.50±0.08	0.51±0.15	0.27±0.06
DT-diaphorase*	0.19	0.42±0.11	1.83±0.22	0.57±0.13	0.25±0.11	0.01±0.01
NADH-FeCN red.*	0.19	1.44±0.09	1.48±0.18	0.27±0.08	0.38±0.13	0.45±0.19
Cyt. b_5 (NADH)**	0.19	1.60±0.20	1.79±0.21	0.53±0.11	0.06±0.03	0.04±0.02
Cyt. b_5 (dith.)**	0.19	1.21±0.29	1.95±0.21	1.15±0.09	0.62±0.17	0.20±0.04

* Relative specific activities as in Table I.

** Relative specific amounts as in Table I.

could be found in the bottom bands with no accompanying NADH-cytochrome *c* reductase activity, indicating some dissociation of the chain.

A number of other microsomal enzymes were also tested, such as ATPase, UDP-glucuronic acid transferase, naphthalene hydroxylation and various glycosyl transferase activities but the presence of deoxycholate influenced the measurements to such an extent that the results were unreliable.

Rough and smooth microsomes

Subfractionation studies show that microsomes exhibit a certain heterogeneity [1] and the possibility arose that the enrichment of individual enzymes seen on our gradients is due to separation of subfractions having some degree of specialization. For this reason, rough and smooth microsomes were separated, washed to remove adsorbed and secretory proteins, and centrifuged on separate gradients containing 0.19 % deoxycholate (Table VI). As can be seen, the distributions of both protein and NADH-cytochrome *c* reductase from both rough and smooth microsomes are the same as those found with total microsomes. Nor did investigation of other enzymes show any significant differences between rough, smooth and total microsomes (not shown in Table).

Incorporation of [^{14}C]leucine.

The rate of incorporation of radioactivity after in vivo injection of [^{14}C]leucine 1 h before decapitation was studied. This time period is necessary to avoid possible interference from labelled secretory proteins, whose transport out of the cell takes about half an hour. The individual bands on the gradient containing 0.19 % deoxycholate exhibit different labeling (Table VII). The highest labeling is found in the top band and the specific activity displays a gradual decrease from band 1 to band 5. Clearly the different bands are not only heterogenous as regard enzyme activities, but they also have different protein turnover rates.

TABLE VI

DISTRIBUTION OF PROTEIN AND NADH-CYTOCHROME *c* REDUCTASE ACTIVITY IN PARTICLES DERIVED FROM ROUGH AND SMOOTH MICROSOMES

The values represent the means of 3 experiments.

	Deoxycholate concentration (%)	Bands				
		1	2	3	4	5
Protein*						
Rough	0.19	1.25±0.11	2.61±0.24	2.18±0.17	1.26±0.10	1.10±0.08
Smooth	0.19	1.49±0.15	2.59±0.34	2.29±0.19	1.14±0.11	1.03±0.06
NADH-cytochrome *c*-reductase**						
Rough	0.19	1.01±0.08	2.21±0.16	0.18±0.11	0.05±0.03	0.04±0.02
Smooth	0.19	0.94±0.21	2.00±0.17	0.31±0.13	0.04±0.02	0.05±0.02

* Relative concentration as in Table I.

** Relative specific activity as in Table I.

TABLE VII

DISTRIBUTION OF INCORPORATED [^{14}C]-LEUCINE IN PARTICLES DERIVED FROM MICROSOMES AFTER IN VIVO LABELING

The labeling procedure is given in Materials and Methods. The table shows the relative specific radioactivity (cpm/protein in the fractions divided by cpm/protein in total). The values are the means ±S.E.M. of 5 experiments.

	Deoxycholate concentration (%)	Bands				
		1	2	3	4	5
Relative specific radioactivity	0.19	1.47±0.15	1.15±0.11	0.83±0.08	0.74±0.07	0.24±0.04

DISCUSSION

The experiments performed in this study indicate that enzymes and enzyme systems are not distributed randomly in the endoplasmic reticulum membrane, but that specialized patches can be isolated. A significant separation of two main enzyme groups occurs at relatively low deoxycholate concentrations, where glucose-6-phosphatase and other phosphatases are found mainly in the upper bands of the gradient, while the various electron transport enzymes are recovered in bands with higher density. At a concentration of about 0.15 % deoxycholate certain changes occur and with 0.19 % deoxycholate bands with characteristic enzyme patterns appear. Band 1 contains particles which are larger than those in bands 2 and 3; but because of their higher lipid content they equilibrate at a lower density. The bands are characterized by enrichments of functionally related enzymes. This does not mean that complete purification was obtained; there is still sizeable cross-contamination between neighboring fractions. Considering the main enzymes in each band, band 1 contains AMPase, most of the IDPase and also some glucose-6-phosphatase activity. Some of the NADH-oxidizing system is, however, also found here, probably as a contaminant. Band 2 contains mainly enzymes involved in the oxidation of NADH, i.e. the majority of the NADH-cytochrome *c* reductase, NADH-ferricyanide reductase and most of the cytochrome b_5 and DT-diaphorase. Band 3 contains enzymes specifically involved in the oxidation of NADPH. NADPH-cytochrome *c* reductase, neotetrazolium reductase and probably a large amount of cytochrome *P*-450 are recovered here. Bands 4 and 5 are very similar to one another. Particles in both these bands are large, have high equilibrium densities, contain relatively little phospholipid, and have glucose-6-phosphatase as their main enzyme component. In addition, they exhibit a very similar protein pattern upon sodium dodecyl sulphate-disc gel electrophoresis. These particles are probably the same as those isolated by Cori et al. [24].

Our basic assumption is that the bands isolated in the gradient correspond to specialized regions of the membrane. Low deoxycholate concentrations dissociate regions catalyzing electron transport and regions containing phosphatase activities from each other. Increasing the deoxycholate concentration results in a dissection of these regions into smaller units, such as membrane particles specialized for NADH or NADPH oxidation. A complete characterization of the subunit of the microsomal membrane is, however, hindered at present by two factors. The first is that deoxy-

cholate inactivates several of the enzymes, and the second is the relative unspecificity of the method used for isolation of these subunits.

The finding that the microsomal membrane contains enzyme complexes is not surprising. It is improbable that functionally related enzymes involved in a large number of catabolic and metabolic reactions are completely randomly distributed in the same membrane. Subfractionation studies reveal some enrichment of specific enzymes in subfractions, which also speaks against a random distribution. The particles isolated in this investigation, like those of microsomal subfractions, do not have purified enzyme systems, but they are enriched in certain enzymes in a manner which is never found after isopycnic equilibrium centrifugation. The mechanism for formation of these particles is both an intriguing and important question. The possibility that the gradient centrifugation collects solubilized enzyme molecules into bands is not supported by the results from Millipore filtration, the density distribution, the visual appearance of the bands, and by the fact that the particles in the bands can be pelleted by centrifugation for 4 h at $105\,000 \times g$. Thus, the components of the bands are vesicles, large fragments or large aggregates.

The bands may contain vesicles but it is improbable that our density gradient centrifugation in the presence of deoxycholate simply effectively isolates intact microsomal vesicles with specialized composition. Experiments with rough and smooth microsomes gave practically identical results. Available electron microscopic data indicate that fragmentation of microsomes occurs in the presence of deoxycholate [25]. A large number of fractionation experiments have concluded that the heterogeneity of intact microsomal vesicles is limited, since an enzyme in a subfraction is generally not enriched more than 2 or 3 times above that of the total [1]. Also, histochemical reactions [26] and antibody-ferritin labelling [27, 28] show the presence of glucose-6-phosphatase and cytochrome b_5 in all intact microsomal vesicles. Detergents are used for solubilization of various membranes (29) and successful reconstitution can be achieved from such solutions [30]. The basic requirement for such reconstitution is removal of the detergent with the simultaneous addition of divalent cations, neither of which conditions are fulfilled in our centrifugations.

Most probably, deoxycholate liberates membrane fragments that subsequently equilibrate in the different bands. A fragmentation of rough microsomes into smaller vesicles was observed by Kreibich et al. using 0.05 % deoxycholate [25]. Theoretically the material in the band might originate directly from the fragment itself, which might take the form of a vesicle or a non-vesicular aggregate. In the cases of bands 2 and 3, the particles are smaller than microsomal vesicles, and they may therefore represent single fragments from intact microsomes. On the other hand, bands 4 and 5 are, in part, larger than microsomal vesicles but the lipid protein ratio and the enzyme composition are very different from any microsomal subfraction. They probably represent aggregates of fragments with similar composition. It is not known whether the particles in bands 2 and 3 are also the result of a similar co-aggregation. To analyze these questions it will be necessary to apply new approaches for estimation of the original size of the fragments. If aggregation takes place, the gradient centrifugation may play an important role in bringing similar fragments together at a high concentration.

An interesting finding is the great variation of phospholipid to protein ratio in the bands. The amount of lipid present probably cannot be explained as the amount

of lipid necessary for enzyme function, since reactivation of isolated enzyme proteins occurs with small amounts of phospholipid [31]. Lipids are asymmetrically distributed in microsomal membranes in the transverse plane [32]. There is also lateral asymmetry; patches of phospholipids are formed which differ in thier fluidity from the bulk phase [33]. Liberation of a protein complex should be accompanied by a simultaneous liberation of those lipids which are localized in or around this complex. Therefore, the amount of phospholipid per protein can be different in different complexes.

The complexes isolated in this study seem to have an enzymatic pattern which is reasonable from a functional point of view. The NADPH-oxidizing system has the well-investigated function of hydroxylation [7] and its components may be collected together in a specific region of the membrane. The situation is similar with the components of the NADH chain, which has at least one estabished function, i.e., fatty acid desaturation [34]. The phosphatase complex in band 1 may participate in interrelated reactions. Such a possibility would be phosphorylation of glucose, which may involve two enzyme components, nucleoside diphosphatase and glucose-6-phosphatase, since the transferase reaction involves both the hydrolysis of a nucleotide and the phosphorylation of a sugar [35]. According to this view, the function of glucose-6-phosphatase in this complex is not hydrolysis of the sugar phosphate but participation in the sugar-phosphotransferase reaction. This reaction is regarded as of great physiological importance and seems to be indispensable in diabetic conditions [35]. The presence of only glucose-6-phosphatase in the high density bands may be explained by the fact that this is the only enzyme in the final stages of glycogen breakdown which is membrane-bound.

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