BBA 75974

INVESTIGATION OF SPECIFICITY IN MEMBRANE BREAKAGE OCCURRING DURING SONICATION OF ROUGH MICROSOMAL MEMBRANES

HANS SVENSSON, GUSTAV DALLNER AND LARS ERNSTER

Institute of Biochemistry, University of Stockholm, and Institute of Pathology, Sabbatsberg Hospital, Karolinska Institutet, Stockholm (Sweden)

(Received February 14th, 1972)

SUMMARY

Rough microsomes were sonicated in a chamber designed to establish full temperature control. The submicrosomal particles obtained in this way varied in size and density. Utilizing these differences, zone and density gradient centrifugations were employed to investigate the distribution of microsomal enzymes. NADH–, NADPH–cytochrome *c* reductase, and glucose-6-phosphatase activities showed heterogeneous and, relative to each other, different distribution patterns, particularly on density gradient centrifugation. The localization of cytochrome b_5 and cytochrome P-450 exhibited similar tendencies to those found for the corresponding cytochrome *c* reductases, though the patterns were somewhat different. Resonication and repeated density gradient centrifugation experiments indicate that the heterogeneity of enzyme distribution among particles of rough microsomal membranes, absent in non-treated rough microsomes, is caused by the breakage of membranes, possibly at specific points. The resulting fragments differ in physicochemical properties and house probably not more than 15 to 30 electron transport chains.

INTRODUCTION

The rough microsomes of rat liver are particularly suited for study of the structural and molecular organization of endoplasmic reticulum membranes, mainly because of the ribosomes bound to them: the ribosomes provide a built-in marker for the common origin[1]. The study of this fraction is relatively easy, since pure rough microsomes can be isolated by a rapid and simple procedure[2]. Also, the presence of bound ribosomes both stabilizes the membrane structure and counteracts irreversible aggregation[3,4].

There is evidence indicating that the endoplasmic reticulum system, including rough microsomes, does not constitute a homogeneous system with random distribution of its enzymes but contains specialized membrane sections with enzymes or enzyme systems devoted to the same function[5,6]. On the other hand, Leskes *et al.*[7] could not find a heterogeneous distribution of glucose-6-phosphatase activity in rough microsomes using a histochemical method. In a previous investigation, sonication

was found to be one of the few available methods for breaking up the microsomal vesicle into smaller units suitable for the study of membrane composition[8]. The aim of the present study has been to extend this investigation and try to answer questions regarding the validity of membrane heterogeneity.

The first problem is to apply an efficient sonication procedure which is not limited by heat generation. Second, presupposing a specific break-up of membranes resulting in particles with a heterogeneous enzyme composition, an adequate type of subfractionation procedure must be determined. The possibility of artifact production is a very serious consideration, involving problems such as enzyme inactivation, enzyme solubilization, and coalescence of smaller membrane components. If breakage occurs at specific points and the released membrane fragments with different enzyme patches exhibit different physicochemical properties, membrane segments with high specific activities could be obtained. To this end, repeated treatments and gradient separations might result in a successful approach.

This paper describes a procedure for effective sonication of rough microsomes, the subfractionation of resultant fragments, and an investigation of the extent of heterogeneity in the isolated subfractions. Preliminary reports of a part of this work have already appeared[9,10].

MATERIALS AND METHODS

Animals

Adult male albino rats weighing 120–180 g were used. The animals were starved 20 h before sacrifice.

Fractionation

Separation of rough and smooth microsomes was performed as described previously[11]. After centrifugation in the 40.2 rotor, the upper phase containing smooth microsomes and a larger part of the 1.3 M sucrose phase were sucked off. The tight pellet from two tubes together with the loose part just above them were suspended in 11 ml cold distilled water. The rough microsomes were pelleted by centrifugation at 105000 × *g* for 60 min. The pellet was rinsed with 0.22 M sucrose and suspended in the same medium to a final concentration of rough microsomes from 1.4 g liver in 1 ml in the case of zone centrifugation experiments, and of microsomes from 1 g liver per ml when subfractionation was performed in a density gradient system. After sonication, the submicrosomal particles were subfractionated either by zone or density gradient centrifugation.

In the case of zone centrifugation, 1 ml of sonicated rough microsomes (from 1.4 g liver) was layered on 11.8 ml of a continuous sucrose gradient ranging from 0.29 to 0.58 M. The linear sucrose gradient was prepared with a mixing chamber[12]. The subfractionation was performed in an SW 40 rotor (Beckman L2-65B ultracentrifuge) at 198000 × *g* for 60 min excluding acceleration and deceleration. Fractions were collected by using an ISCO density gradient fractionator, Model D. Since the fractions are removed by pressing sucrose near the bottom of the centrifuge tube, Fraction 1 represents the very top of the gradient including the volume present in the origin. The small amount of sucrose still remaining on the wall of the centrifuge tube was wiped off with a filter paper. The pellet was suspended in 0.25 M sucrose using the same volume as the volumes of the individual subfractions.

When density gradient centrifugation was applied, 2 ml sonicated microsomes (from 2 g liver) was layered on the top of a continuous sucrose gradient with a volume of 35.8 ml and a concentration ranging from 0.29 to 1.32 M. Centrifugation was performed in an SW 27 rotor at 95000 × *g* for 16 h. Removal and further treatment of subfractions were performed in the same way as described for zone centrifugation.

Sonication

A special chamber of soft polythene was designed for the continuous sonication of rough microsomes (Fig. 1). The side tube housing the thermistor was constructed of PVC tubing screwed into the chamber. The inner diameter of the chamber was 10 mm and the length up to the lower edge of the funnel 54 mm. The tip of the sonicator (Branson fine tip, 14 mm) was inserted more or less in the center of the chamber 5 mm from the bottom. A long needle connected to a syringe was inserted along one side of the chamber with its tip close to the bottom. The outer end of the side tube was fitted with a rubber stopper, holding the thermistor (Philips 205 CEP/100 KS) firmly in place. Since the resistance of the thermistor is dependent on the temperature at its very tip, the slope of the side tube was adjusted so that the tip of the thermistor was located 2–3 mm from the sonicator tip. The resistance in the thermistor was converted to temperature scale by the circuit shown in Fig. 2.

A vessel containing dry ice and alcohol was required for maintaining steady temperature.

For sonication, 3.5 ml of suspension was pipetted into the chamber. For routine experiments, a setting of 1.5 A was applied. The sonication time was 5 min (Branson sonifier Model S-125, Branson Instruments Inc., Stamford, Conn.). Sonication was interrupted for a few seconds twice every minute in order to mix the whole suspension thoroughly with syring and needle. The temperature was held at +2 °C during the whole sonication procedure.

Determination of sedimentation coefficients

Aliquots of subfractions after zone centrifugation were diluted with water to a final sucrose concentration of 0.08 M and 0.3 ml of this suspension was placed

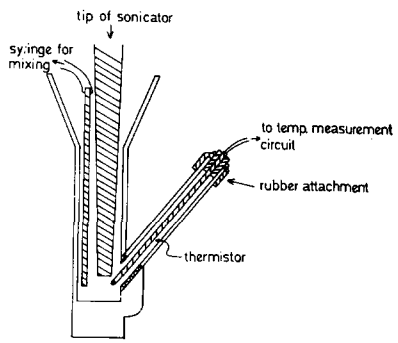


Fig. 1. Chamber for continuous sonication.

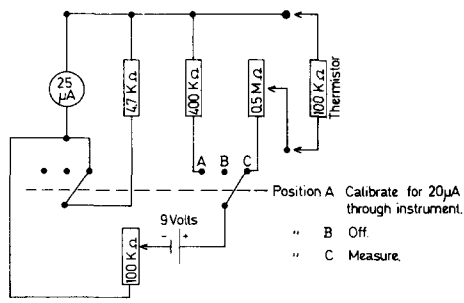


Fig. 2. Circuit for measurement of resistance in thermistor and for its conversion to temperature scale. The switch in the circuit has three positions: (1) for temperature measurements, (2) for battery check, and (3) for off position.

in a band forming cap of a Spinco SW 40 rotor. The particles were subjected to centrifugation on an isokinetic sucrose gradient according to Noll[13]. The convex gradient was calculated for an SW 40 rotor for particles with an equilibrium density around 1.20 and ranged between 0.145–0.464 M sucrose. After centrifugation the gradient was fractionated with the ISCO fractionator, Model D, equipped with an ISCO U2 ultraviolet analyzer for registration of absorbance at 280 nm. Calculation of $s_{20,w}$ was performed according to Noll[13].

Chemical analysis

Protein was measured according to Lowry *et al.*[14] with bovine serum albumin as standard. Phospholipid was separated from neutral lipids[15] and the amounts of phospholipids and cholesterol were determined as previously[16].

Enzyme assays

The activities of NADH– and NADPH–cytochrome *c* reductase, Mg^{2+}-ATPase, glucose-6-phosphatase and *p*-nitrophenylphosphatase activities were estimated as before[2]. In the case of glucose-6-phosphatase, 0.03 % deoxycholate was included in the incubation medium.

Cytochrome b_5 and cytochrome P-450 were determined spectrophotometrically as before with a Phoenix split-beam spectrophotometer (Phoenix Precision Instrument Company, Philadelphia, Pa.)[8]. NADH was used to reduce cytochrome b_5, to eliminate the error due to hemoglobin contamination which may arise when $Na_2S_2O_4$ is used as a reductant. For measurement of cytochrome P-450, both cuvettes were reduced with $Na_2S_2O_4$ and the sample cuvette was bubbled with O_2-free CO. Because interest was focused on the distribution of specific enzymes in relation to the membrane, enzyme activities were related to phospholipid rather than protein content. All the figures show representative results from at least five identical experiments.

Control of coalescence

[2-^{3}H]Glycerol (50 mCi/mmole) or DL-[1-^{14}C]leucine (55 mCi/mmole) from The Radiochemical Centre, Amersham, England, was injected intraperitoneally (20 μCi/100 g) in sterile Ringer solution. Rough microsomes from non-labeled rat and smooth II microsomes from labeled rat were isolated[11]. The isolated subfractions were washed first with 0.15 M Tris–HCl buffer, pH 8.0, in order to remove adsorbed protein, and subsequently were suspended in distilled water (1 g/10 ml) and incubated at 30 °C for 20 min in order to release the luminal content. Finally, the recentrifuged subfractions were again washed with alkaline Tris–HCl buffer. 3.5 ml (15 mg protein per ml) of rough microsomes and 3.5 ml (10 mg protein per ml) of smooth II microsomes were sonicated as described above. In a third sonication, 1.75 ml of both microsomes were sonicated together. Sonicated suspensions were layered above 2 ml 1.15 M sucrose–10 mM $MgCl_2$ in tubes of the 40.2 rotor: (a) 1 ml rough microsomes + 3.5 ml 0.25 M sucrose; (b) 1 ml smooth II microsomes + 3.5 ml 0.25 M sucrose; (c) 0.5 ml rough + 0.5 ml smooth II microsomes + 3.5 ml 0.25 M sucrose; (d) 1 ml from the suspension in which the two microsomal subfractions were sonicated together + 3.5 ml 0.25 M sucrose.

All four tubes were supplemented with 10 mM $MgCl_2$. After centrifugation at 102000 × *g* for 60 min, the upper sucrose layer was sucked off and discarded. The

interface layer was recentrifuged and suspended in 0.25 M sucrose. The lower 1.15 M sucrose was decanted, and the pellet was suspended in 0.25 M sucrose. Protein was measured with the procedure of Lowry *et al.*[14], and lipids were extracted as before[16]. The lipid extract was used for the determination of phospholipid and radioactivity in the case of [^{3}H]glycerol. The radioactivity was measured in a Beckman liquid scintillation counter (Beckman Instrument, Fullerton, Calif.), DPM-100, in a toluene scintillation mixture[17]. In the experiments with [^{14}C]leucine labeling, aliquots of washed subfractions were mixed with formic acid and supplemented with Bray solution[18].

RESULTS

Mechanism of breakdown by sonication

The isolated rough microsomes, suspended in 0.25 M sucrose, were subjected to sonication at three energy levels and for various periods, using the sonication chamber described above (Fig. 3). The estimation of particle size by light-scattering shows that the breakdown of vesicles is rapid during the first minute. The slope of breakdown becomes steeper at higher energies. Between 1 and 5 min, the decrease in light-scattering is much slower, and after 5 min there is a certain stabilization in particle size. It is likely that the dominating factor during the first few minutes is a mechanical one, while in the later period a thermic effect may also influence the result. Owing to the efficiency of the system described, the temperature of the total suspension, at 1.5 A at least remained close to zero even after protracted sonication, though it is realized that the temperature at the compression center, *i.e.* under the tip, is probably higher. For this as well as other reasons described below, sonication was performed at 1.5 A for 5 min in all experiments.

There are some serious considerations against the extensive mechanical treatment of subcellular organelles in respect of possible artifacts. Three main possibilities were studied experimentally: (a) coalescence and/or secondary aggregation, (b) enzyme inactivation, and (c) solubilization of enzymes.

Theoretically it is conceivable that small dissociated membrane units are secondarily reaggregated but in a different order than in the natural condition. Such a new particle with components from different parts of the endoplasmic reticulum may also possess new physicochemical properties. In order to test this possibility, the property of non-identical cation sensitivity of microsomal subfractions was utilized.

On a discontinuous sucrose gradient containing Mg^{2+}, the membranes of rough microsomes aggregate and sediment. This does not occur with smooth II microsomes. Rough microsomes from unlabeled rats and smooth II microsomes from *in vivo* labeled rats (labeled phospholipid with [^{3}H]glycerol or labeled protein with [^{14}C]-leucine) were isolated, mixed, and sonicated under conditions used for the gradient experiments. If units released from rough and smooth microsomes were to reaggregate into a new type of vesicle, it might be expected that such vesicles were susceptible to aggregation by Mg^{2+} since they would contain parts of the rough endoplasmic reticulum system. Table I shows the distribution of radioactivity of phospholipids and protein in cation sensitive and insensitive particles. It is apparent that practically all the radioactivity is recovered at the interface layer, that is, in the particles that

are not aggregated by Mg^{2+}. The possibility that reincorporated rough units lose their cation sensitivity and remained in the interface layer is also unlikely, since the protein content of the interface layer after sonication remained constant.

In spite of the effective chilling described under Materials and Methods, some consideration must be paid to there being a higher temperature in the immediate surroundings of the sonicating tip than in other parts of the chamber. Also, the genera-

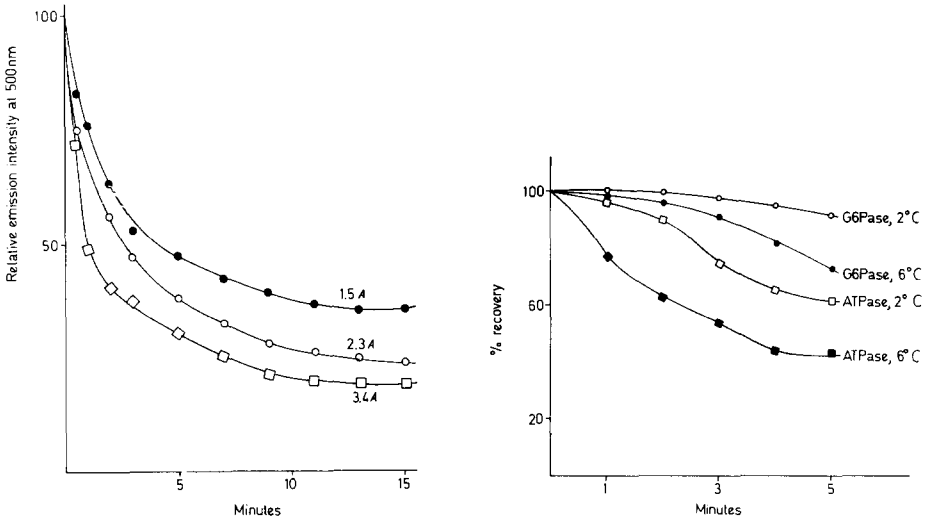


Fig. 3. Effect of time and energy output on the light-scattering capacity of rough microsomes during sonication. Batches of rough microsomal suspension (4 ml, from 4 g liver) were sonicated in the chamber described in Materials and Methods at various energy outputs. Sonication was interrupted for mixing twice a minute. 30 μl was removed and diluted with 3 ml 0.25 M sucrose. Light scattering was measured in a Hitachi Fluorimeter 204 at 500 nm.

Fig. 4. Effects of sonication on glucose-6-phosphatase (G6Pase) and ATPase activities of rough microsomes. Batches of rough microsomes (3.5 ml, from 3.5 g liver) were sonicated at 1.5 A in the chamber described, keeping the temperature at 2 or 6 °C. Sonication was interrupted every minute for mixing, and 0.1 ml was removed for enzyme activity measurements.

TABLE I

DISTRIBUTION OF PHOSPHOLIPID AND PROTEIN RADIOACTIVITY IN A DISCONTINUOUS GRADIENT AFTER SONICATION OF ROUGH AND SMOOTH II MICROSOMES

Details of the experiment are described in Materials and Methods.

Fraction	*Interphase (smooth II)*				*Pellet (rough)*			
	Phospholipid		*Protein*		*Phospholipid*		*Protein*	
	cpm	*% of total*	*cpm*	*% of total*	*cpm*	*% of total*	*cpm*	*% of total*
Rough, sonicated	43		25		0		0	
Smooth II, sonicated	14 069	97.8	4440	97.2	317	2.2	127	2.8
Rough + smooth II, mixed after sonication	13 880	96.9	4122	94.5	438	3.1	237	5.5
Rough + smooth II, sonicated together	15 447	97.3	4552	95.6	422	2.7	211	4.4

tion of a high local pressure may denature enzymes. If these factors should lead to an inactivation, the reliability of enzyme-topological studies would be diminished. The behavior of two phosphatases, glucose-6-phosphatase and ATPase, is shown in Fig. 4. In both cases, but particularly with ATPase, the extreme importance of temperature control is apparent. No more than a 4-degree increase in temperature results in a 20% decrease in enzyme activity after a few minutes sonication. Electron transport enzymes, measured either as activity or amount, display a high degree of stability after 5 min of sonication; a recovery of around 90% was obtained in all cases.

Finally, the question of solubilization by sonication must be taken into account, since this has been shown to occur in the case of at least one microsomal enzyme, namely, nucleoside diphosphatase[19]. As will be demonstrated later (*cf.* Table II), such solubilization does not occur with the enzymes under study.

Chemical composition of the subfractions

The submicrosomal particles obtained by 5 min of sonication of rough microsomes were subfractionated by both zone and density gradient centrifugations. Zone centrifugation on a shallow stabilizing gradient, 0.29-0.58 M sucrose, at 199000 × *g* for 60 min results in two protein peaks one for the pellet and one for the top (Fig. 5). When the steepness of the gradient is increased, particle density increasingly influences the sedimentation velocity and the small particles are no longer distributed over a broader range. The sedimentation coefficient of various submicrosomal particles is shown in Fig. 5, Expt a. The $s_{20,w}$ value is 290 ± 30 S for the top fraction and 620 ± 50 S for particles in the middle part of the gradient. Just above the pellet, the larger vesicles exhibit an $s_{20,w}$ value of 870 ± 55 S. Total rough microsomes, on the other hand, have a sedimentation coefficient between 1800 and 3400 S, that is, the decrease is highly significant.

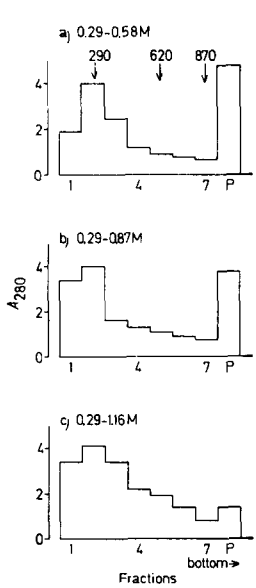
The amounts of protein, phospholipid and cholesterol in the subfractions after zone centrifugation indicate that the aim of obtaining a relatively broad distribution pattern was achieved. The phospholipid/protein and phospholipid/cholesterol ratios were quite even in all subfractions with the exception of the pellet, which was enriched in phospholipid on the basis of protein. A more even distribution of protein was observed when the particles were subjected to 16 h centrifugation on a density gradient, but this is not sufficient for the vesicles to reach complete equilibrium. Again, the phospholipid/protein and phospholipid/cholesterol ratios were similar in the various subfractions, and no marked enr ichment of phospholipid or cholesterol could be seen.

Enzyme distribution

The low phospholipid content of the various subfractions after gradient centrifugation prevented us, in general, from measuring more than two enzyme activities or amounts. For this reason, the phospholipid content of subfractions (removed as volume) varied. On the other hand, this difference in phospholipid distribution did not influence the general enzyme distribution pattern.

The distribution of electron transport enzymes after zone centrifugation is shown in Fig. 6. A certain degree of separation is apparent between NADH- and NADPH–cytochrome *c* reductase activities, the former being concentrated in the

high velocity fractions while the latter is enriched in the top fraction. The two cytochromes (cytochrome b_5 and cytochrome P-450) follow the pattern of their functional counterparts (NADH- and NADPH-cytochrome *c* reductases); cytochrome b_5 is found in somewhat higher concentration in the bottom part of the gradient, while the opposite is true for cytochrome P-450. The relatively low recovery of cytochrome P-450 is attributable to the unfavorable conditions during centrifugation; the major part of this cytochrome is situated in the upper part of the gradient, where the sucrose



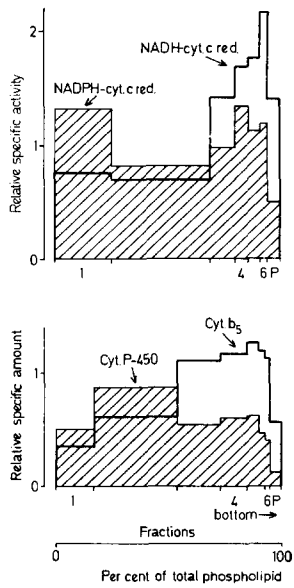


Fig. 5. Distribution of sonicated rough microsomes on various sucrose gradients after zone centrifugation. Zone centrifugation was performed as described in Materials and Methods. Linear sucrose gradients were in experiments (a) 0.29–0.58 M; (b) 0.29–0.87 M; (c) 0.29–1.16 M. $A_{280\ nm}$ was used for the estimation of protein distribution. The numbers in (a) give the sedimentation coefficients ($s_{20,w}$) in Svedberg's units. The individual subfractions, shown on the figure, were taken for determination of sedimentation coefficients which were made as described in Materials and Methods.

Fig. 6. Distribution of electron transport enzymes in submicrosomal particles after zone centrifugation. The plot is made in the manner adopted by de Duve *et al.*[20]. Relative phospholipid content of fractions is shown on the abscissa and the relative specific activity on the ordinata.

concentration is low. In the case of glucose-6-phosphatase, the specific activities of the subfractions on a phospholipid basis are higher in the middle and the bottom part than in the upper portion of the gradient (Fig. 7). *p*-Nitrophenylphosphatase activity was also determined in the subfractions in a few experiments and was found to be evenly distributed.

In the case of density gradient centrifugation, NADPH-cytochrome *c* reductase activity showed a clear tendency to be increasingly enriched in vesicles with decreasing density (Fig. 8). NADH-cytochrome *c* reductase activity, on the other hand, displayed two peaks, one in the middele part of the gradient and one in the pellet.

Cytochrome b_5 and cytochrome P-450 do not follow the distribution pattern of the cytochrome *c* reductases exactly. High amounts of the former cytochrome are present in the high density fractions, while the majority of cytochrome P-450 is

found in the upper and middle part of the gradient. The peaks are more even and broader than in the case of cytochrome *c* reductase activities. The importance of this finding cannot be judged presently, since both cytochromes are present in microsomes in large excess and they probably participate in more than a single reaction. Removal of a larger part of the cytochrome b_5 does not influence NADH–cytochrome *c* reductase activity[21].

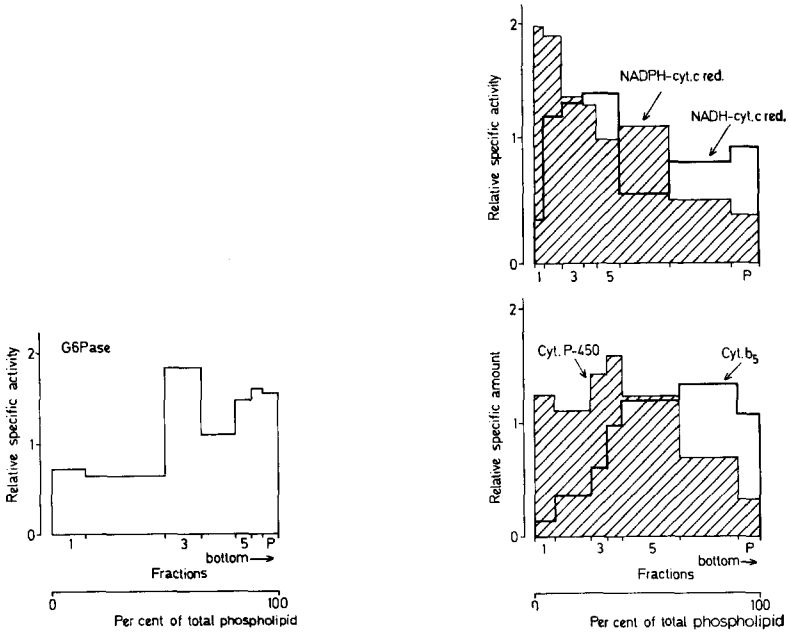


Fig. 7. Distribution of glucose-6-phosphatase in submicrosomal particles after zone centrifugation. Distribution is represented as in Fig. 6. The specific activity of glucose-6-phosphatase in total rough microsomes before sonication was 10.1 μmoles P_i per 20 min per mg phospholipid.

Fig. 8. Distribution of electron transport enzymes in submicrosomal particles after density gradient centrifugation. Distribution represented as in Fig. 6.

Further analysis of subfractions

In order to obtain more distinct results, both the top fractions and the pellet obtained in the zone centrifugation experiments were further treated and analyzed.

The enrichment of NADPH–cytochrome *c* reductase activity in the top of the gradient raises the possibility that the enzyme, known to be loosely attached to the microsomal membrane[21], becomes solubilized during intense sonication, which would result in a distribution pattern like that described for zone centrifugation. In order to put this possibility to test, the first 3-ml portion of the top of the gradient after zone centrifugation was removed, diluted, and centrifuged at 198000 × *g* for 7 h. The resultant pellet contained 87% of the total NADPH- and 99% of the NADH–cytochrome *c* reductase activity (Table II). Utilizing the equations deduced by De Duve *et al.*[22] it can be calculated that the small vesicles with a sedimentation coefficient ($s_{20,w}$) of 290 S are sedimented under the conditions used. On the other hand, solubilized NADPH–cytochrome *c* reductase ($s_{20,w} = 5.29 \cdot 10^{-13}$ s)[23] within the same period of time and in the same system should move not more than 1.5 cm. This

TABLE II

RECENTRIFUGATION OF SLOWLY SEDIMENTING SUBMICROSOMAL PARTICLES

The first fraction after zone centrifugation (Fraction 1) was diluted with water (1:4). 1 ml of the suspension was layered over 11.8 ml 0.15 M sucrose and centrifuged in an SW 40 rotor at 198000 × *g* for 7 h. After centrifugation, the supernatant was decanted and the pellet was suspended in 0.25 M sucrose.

	μmoles NADH or NADPH oxidized per min	
	Pellet	*Supernatant*
NADH–cytochrome *c* reductase	0.748	0.005
NADPH–cytochrome *c* reductase	0.153	0.022

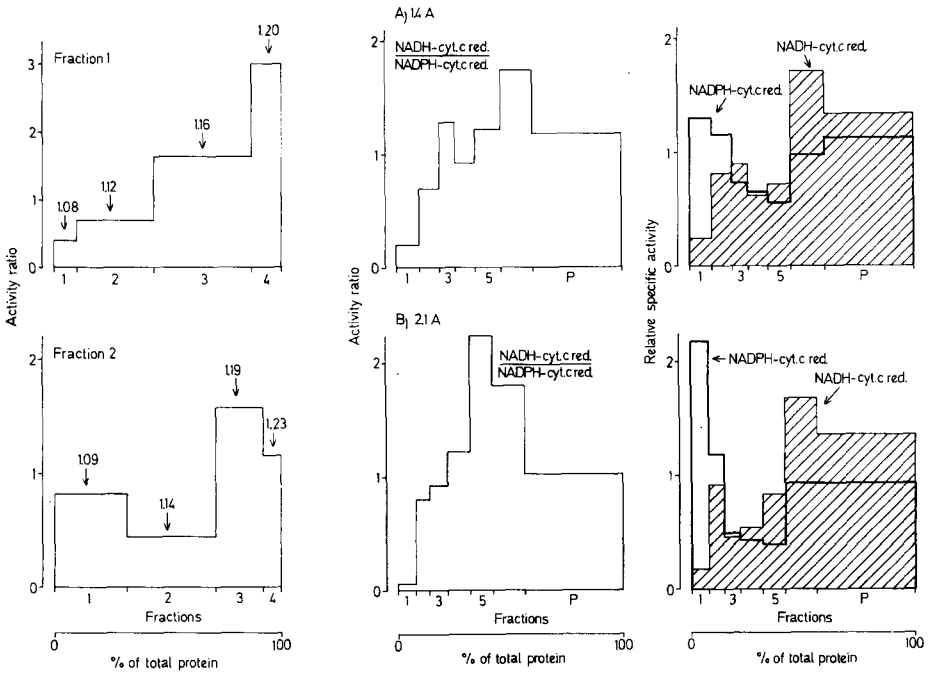


Fig. 9. NADH/NADPH–cytochrome *c* reductase activity ratio of slowly sedimenting submicrosomal particles after recentrifugation on a density gradient. After zone centrifugation the first fraction on the top (Fraction 1) was adjusted to a final sucrose density of 1.08 with the use of a concentrated sucrose solution; 1 ml of the suspension was layered on the top of a continuous sucrose gradient ranging from 0.73 to 1.61 M (Expt a). Fraction 2 was adjusted to a final sucrose density of 1.09, and 1 ml of this suspension was layered on a 0.8–1.90 M gradient (Expt b). The gradient volume was 3.1 ml. After centrifugation at 308000 × *g* for 16 h in an SW 56 rotor, the content of the centrifuge tubes was divided into 4 fractions. NADH/NADPH–cytochrome *c* reductase activity ratios (arbitrary units) are shown on the ordinata. Numbers above the individual subfractions represent the median density of the sucrose solution in that fraction.

Fig. 10. Cytochrome *c* reductases of the high sedimentation velocity submicrosomal particles after resonication and recentrifugation on a density gradient. Pellets after zone centrifugation were suspended in 1 ml H_2O per pellet and resonicated at 1.4 A (Expt a) and 2.1 A (Expt b) for 5 min. Subfractionations were performed as in other experiments. The first column shows NADH/NADPH–cytochrome *c* reductase activity ratios represented as in Fig. 9. The second column demonstrates the relative specific activity of the two cytochrome *c* reductases on a protein basis in the individual subfractions. Recoveries are around 90%, as is shown in the figure. Distribution represented as in Fig. 6.

demonstrates that the localization of the NADPH–cytochrome *c* reductase cannot be explained on the basis of solubilization.

The nature and possible heterogeneity of small vesicles in the top of the gradient were investigated by repeated subfractionation. Both slow sedimentation velocity Fractions 1 and 2 after zone centrifugation were layered on a sucrose gradient and centrifuged to isopycnic equilibrium density (Fig. 9). As is apparent from the ratio of the two cytochrome *c* reductase activities, all new subfractions contained both enzymes. However, the relative activities were very different, particularly in the case of Fraction 1. Particles with a median equilibrium density of 1.08 to 1.14 were much richer in NADPH–cytochrome *c* reductase activity than the particles in the density range of 1.16 to 1.23.

The pellet from zone centrifugation, which was enriched in NADH–cytochrome *c* reductase, was also further analyzed. Three questions were posed: (1) Is it possible to subdivide the vesicles in the pellet? (2) If so, is it possible to separate vesicles with different enzyme distribution? (3) If subdivision and separation are possible, which electron transport enzyme is released preferentially: NADH– or NADPH–cytochrome *c* reductases?

In order to approach the problem, the pellet fraction was sonicated at different energy outputs and subfractionated by density gradient centrifugation (Fig. 10). Resonication results in a 50 % release of the protein as well as lipid content into the gradient. Analyses of the ratios of the two cytochrome *c* reductase activities as well as the relative specific activities reveal a specialization among the particles on the gradient, particularly at higher energy level. Those with low density (upper part) are highly enriched in NADPH–cytochrome *c* reductase, while the high-density fraction (lower part of the gradient) exhibits a relatively high NADH–cytochrome *c* reductase activity.

DISCUSSION

The aim of this study was to apply an effective sonication procedure to rough microsomes in order to break them up into smaller units. Two types of heterogeneity were investigated in these particles: first the distribution of particle size and density, and second the enzymic organization of fragments.

The rough microsomes of rat liver are known to display a certain degree of heterogeneity from the morphological and enzymic points of view[5]. The vesicles of separated subfractions are found to differ with regard to size, the number of bound ribosomes, and the distribution of certain membrane bound enzymes. However, an investigation of total rough microsomes or subfractions thereof provides only limited information about the molecular organization of the endoplasmic reticulum membranes. Clearly, the membrane of a vesicle after homogenization is still complex and heterogeneous. In a first approach to this problem[8], rough microsomes were sonicated and subfractionated on a sucrose density gradient. These experiments have revealed a heterogeneity in the distribution of the two microsomal electron transport systems, indicating that the microsomal membrane, to a certain extent at least, contains specialized segments for limited functions.

The possibility that the two electron transport systems belong to separate membrane entities, *i.e.* are localized in spatially distinct parts of the endoplasmic

reticulum, cannot be excluded. In this case, the sonication energy employed may produce smaller fragments of vesicles rich in NADPH–cytochrome *c* reductase, leading to an overall decrease in size of these particles. The extent of information obtained in previous studies was limited by the techniques used for the disintegration of microsomes, since the generation of heat allowed only low energy output and a relatively short sonication period.

The sonication procedure described in this paper is simple and permits relatively protracted sonication with simultaneous temperature control. The particles obtained in this way appear to represent intact segments of the "*in situ*" membranes, without the occurrence of major artifacts. The control experiments seem to eliminate membrane reorganization by secondary distribution of the components; ribosomes were partially released but not tightly attached enzymes, which retained their original activities. When sonication was performed in a N_2 atmosphere, the isolated subfractions exhibited identical chemical and enzymic properties to those routinely obtained after sonication in the presence of air. This also excludes the influence of lipid peroxidation which, theoretically, could be mediated by the hemoglobin always present in microsomes prepared from a non-perfused liver[24].

The NADH–cytochrome *c* reductase bearing parts of the endoplasmic reticulum seem to have a higher density than the NADPH counterpart, which may allow the separation of the two electron transport enzymes, even in the case of a random breakage. Alternatively, breakage of the microsomal membranes during sonication probably occurs at preexisting weak points. Theoretically, there are several explanations for weak points:

(a) Lack of certain lipids or sudden changes in the organized sequence of the lipid layer might well be a possibility. An example would be the lack of cholesterol, which is known to stabilize membrane-like structures[25,26]. There are a number of experimental data demonstrating that phospholipid is involved in membrane rupture and resealing[27–30].

(b) If the protein cover of the unit membrane were interrupted, leaving the phospholipid layer uncovered, the continuity of the membrane at such empty "slots" would depend only on the cohensive forces within the lipid phase[31].

(c) The transformation of susceptible parts of the lipid layer into a new molecular arrangement could abolish strong interacting forces. The micellization of lipids in membranes is believed to be one of the actions of insulin and of phospholipase *c*[32,33]. Recent experiments of Redman indicate the involvement of phosphatidic acid and polyphosphoinositide metabolism in the leakiness and repair mechanism of erythrocyte membrane[34].

(d) Weak points in the membrane may arise from conformational changes of proteins brought about by sonication[35,36].

(e) Sonication might activate certain proteolytic or lipolytic enzymes which are present in the vesicles in a latent state. The activation of a degradative enzyme may be involved in the energy-dependent interaction of zymogen granules and plasma membrane in the exocrine pancreas cell[37].

Implicit in the present approach to the separation of microsomal membrane fragments specialized in certain enzymic functions by means of gradient centrifugation is that the fragments in question also differ physicochemically. Separation procedures by ultracentrifugation methods involve the utilization of differences in osmotic

behavior, surface charge, size and density, which, however, do not necessarily bear any close relationship to the enzymic composition[22,38–41]. Sucrose penetrates the microsomal membrane freely[42,43] and in experiments with [^{14}C]sucrose we also found that sonicated rough microsomes are completely permeable to sucrose. Therefore, osmotic change caused by the solute may not be a significant factor in these experiments. Also, because of the free permeability of the solute in the present case, surface charge differences in the innerside of the membranes cannot be titrated by cations[41,44] and consequently, it is not possible to produce differences in the characteristics of the particles. The two properties of size and density difference were employed in the separation experiments. Since the size of the particles after sonication exhibits broad variation, this property could be used with success. The particles also differ in density, but the reason for this is not quite clear. Sonication liberates most of the bound ribosomes, and therefore, they cannot be responsible for the difference in density. The phospholipid/protein ratio of the subfractions is quite similar and therefore does not appear to be an immediate reason for the density difference. On the other hand, we have no detailed knowledge of the intermolecular arrangement of the various chemical components, an arrangement which might be responsible for density variation by, for example, influencing the amount of hydration water[15].

Gradient centrifugation techniques did not give subfractions containing one single enzyme or enzyme system exclusively. However, comparing subfractions with the lowest and highest specific activities or specific amounts, an up to 5-fold enrichment of the enzymes of NADH and NADPH electron transport chain components as well as glucose-6-phosphatase were obtained. When the NADPH–cytochrome *c* reductase activity enriched top fraction was further subfractionated (Fig. 9), the 1.20-density bottom fraction exhibited 8 times more NADH– than NADPH–cytochrome *c* reductase activity. On the other hand, resonication of the pellet with 2.1 A after zone centrifugation and subsequent equilibration gave a top fraction displaying 10 times higher activity of NADPH-cytochrome *c* reductase in comparison with the NADH counterpart. Approximative calculations for the number of enzyme molecules present in the small vesicles studied (diameter about 300 Å) give the following results. It can be calculated that the amount of phospholipid is $6 \cdot 10^{-18}$ g and of protein is $9 \cdot 10^{-18}$ g per vesicle. Assuming that no structural protein is present and all the enzyme molecules in the 300 Å vesicle are those of NADPH–cytochrome *c* reductase or cytochrome b_5, and assuming that the molecular weight of the former enzyme is 68000[46] and of the latter 15000[47], the number obtained by using Avogadro's constant is 85 molecules NADPH–cytochrome *c* reductase or 300 molecules cytochrome b_5 per vesicle. Taking into consideration that these smallest vesicles (*cf.* Figs 9 and 10) contain components from both electron transfer systems, that the NADPH–cytochrome *c* reductase-rich particles also contain in 1:5 ratio cytochrome P-450 (M_r 50000)[48], and that the cytochrome b_5-rich particles contain in 10:1 ratio NADH–cytochrome *c* reductase (M_r 30000)[48], the number of electron transfer chains per vesicle can be calculated. The NADPH–cytochrome *c* reductase rich particle would contain 15 complete NADPH and 2 NADH electron transport chains, while the particles enriched in NADH–cytochrome *c* reductase would consist of 25 complete NADH and 3 NADPH electron transfer chains. These values, however, are very approximative since they do not take into account the probable occurrence of other enzymic or structural proteins in the membranes, the fact that the molecular weights

are not corrected to the hydration water, and the fact that enzyme activity is directly related to enzyme amount.

Regarding heterogeneity, it is of interest to calculate the space taken up in the endoplasmic reticulum by the 300-Å vesicle, whose surface area would be $2.5 \cdot 10^5$ Å^2. Assuming the endoplasmic reticulum to consist of large flattened sheets, it is necessary to cut a circular area with 3200 Å diameter in order to obtain a vesicle with a diameter of 1700 Å (surface area $90 \cdot 10^5$ Å^2), which is commonly present after homogenization. If the 300-Å vesicle derives from the same system, the diameter of the circular area on the sheet would be 560 Å, that is, a roughly 6 times shorter diameter than that for the ordinary microsomes.

It appears that a certain separation may be obtained between electron transport enzymes and phosphatases at the microsomal vesicle level[40]. This study indicates that separation of the two electron transport systems from each other requires the preparation of smaller particles: the smaller the size, the larger the enrichment. Consequently, a reconstruction of microsomal vesicles from particles after sonication should result in vesicles all containing a number of units of both electron transport systems.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Medical Research Council and the Magnus Bergvalls Foundation. The authors wish to thank Miss Elisabeth Pettersson for the valuable technical assistance.

REFERENCES

1 G. E. Palade and P. Siekevitz, *J. Biophys. Biochem. Cytol.*, 2 (1956) 171.
2 G. Dallner, *Acta Pathol. Microbiol. Scand. Suppl.*, 166 (1963).
3 G. Dallner and R. Nilsson, *J. Cell Biol.*, 31 (1966) 181.
4 P. H. Jellinck and G. Perry, *Biochim. Biophys. Acta*, 137 (1967) 367.
5 G. Dallner, A. Bergstrand and R. Nilsson, *J. Cell Biol.*, 38 (1968) 257.
6 A. Amar-Costesec, H. Beaufay, E. Feytmans, D. Thinès-Sempoux and J. Berthet, in J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts and G. J. Mannering, *Microsomes and Drug Oxidation*, Academic Press, New York. 1969, p. 41.
7 A. Leskes, P. Siekevitz and G. E. Palade, *J. Cell Biol.*, 49 (1971) 264 and 288.
8 P. R. Dallman, G. Dallner, A. Bergstrand and L. Ernster, *J. Cell Biol.*, 41 (1969) 357.
9 H. Svensson and G. Dallner, *Abstr. 6th Meet. Fed. Eur. Biochem. Soc., Madrid, 1969*, p. 272.
10 G. Dallner, H. Svensson and L. Ernster, in Å. Åkeson and A. Ehrenberg, *Structure and Function of Oxidation Reduction Enzymes*, Pergamon Press, Oxford, 1972, p. 567.
11 A. Bergstrand and G. Dallner, *Anal. Biochem.*, 29 (1969) 351.
12 R. V. Britten and R. B. Roberts, *Science*, 131 (1960) 32.
13 H. Noll, *Nature*, 215 (1967) 360.
14 O. H. Lowry, N. V. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
15 B. Borgström, *Acta Physiol. Scand.*, 25 (1952) 101.
16 H. Glaumann and G. Dallner, *J. Lipid Res.*, 9 (1968) 720.
17 D. S. Beattie and R. E. Basford, *J. Biol. Chem.*, 241 (1966) 1419.
18 G. A. Bray, *Anal. Biochem.*, 1 (1960) 279.
19 L. Ernster and L. C. Jones, *J. Cell Biol.*, 15 (1962) 541.
20 C. de Duve, B. C. Pressman, R. Gianetto, R. Wattiaux and F. Appelman, *Biochem. J.*, 60 (1955) 604.
21 L. Ernster, P. Siekevitz and G. E. Palade, *J. Cell Biol.*, 15 (1962) 541.
22 C. de Duve, J. Berthet and H. Beaufay, *Prog. Biophys. Biophys. Chem.*, 9 (1959) 325.
23 C. H. Williams and H. Kamin, *J. Biol. Chem.*, 237 (1962) 587.
24 E. D. Wills, *Biochem. J.*, 113 (1969) 315.

25 J. De Gier, in R. Eigenmann, *Proc. 4th Int. Congr. on Pharmacology, Basel, 1969*, Vol. IV, Schwabe and Co., Basel/Stuttgart, 1969, p. 102.
26 J. De Gier, J. G. Mandersloot and L. L. M. van Deenen, *Biochim. Biophys. Acta*, 173 (1969) 143.
27 M. L. Karnovsky and D. F. H. Wallach, *J. Biol. Chem.*, 236 (1961) 1895.
28 P. S. Sastry and L. E. Hokin, *J. Biol. Chem.*, 241 (1966) 3354.
29 H. S. Jacob and M. L. Karnovsky, *J. Clin. Invest.*, 46 (1967) 173.
30 M. Schramm, B. Eisenkraft and E. Barkai, *Biochim. Biophys. Acta*, 135 (1967) 44.
31 D. F. H. Wallach, in D. B. Davis and L. Warren, *The Specificity of Cell Surfaces*, Prentice-Hall, Englewood Cliffs, N.J., 1967, p. 129
32 M. Blecher, *Biochem. Biophys. Res. Commun.*, 23 (1966) 68.
33 M. Rodbell, *J. Biol. Chem.*, 241 (1966) 130.
34 G. M. Redman, *J. Cell Biol.*, 49 (1971) 35.
35 A. Geyer, *Arch. Microbiol.*, 46 (1963) 9.
36 I. E. Elpiner and L. L. Stekolinikov, *Biokhimiya*, 28 (1969) 801.
37 J. D. Jamieson and G. E. Palade, *J. Cell. Biol.*, 48 (1971) 503.
38 C. de Duve, *Harvey Lect.*, 59 (1965) 49.
39 D. F. H. Wallach and V. B. Kamat, *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 721.
40 G. Dallner and L. Ernster, *J. Histochem. Cytochem.*, 16 (1968) 611.
41 T. L. Steck and D. F. H. Wallach, in H. Busch, *Methods in Cancer Research*, Vol. 5, Academic Press, New York, 1970, p. 93.
42 L. Share and R. W. Hansrote, *J. Biophys. Biochem. Cytol.*, 7 (1960) 239.
43 R. Nilsson, E. Pettersson and G. Dallner, *FEBS Lett.*, 15 (1971) 85.
44 T. L. Steck, J. M. Straus and D. F. H. Wallach, *Biochim. Biophys. Acta*, 203 (1970) 385.
45 H. Beaufay and J. Berthet, *Biochem. Soc. Symp.*, 23 (1963) 66.
46 B. L. Horecker, *J. Biol. Chem.*, 183 (1950) 593.
47 P. Strittmatter, *J. Biol. Chem.*, 235 (1960) 2492.
48 T. Omura, *Abstr. 8th Int. Congr. Biochem. Switzerland, 1970*, p. 260.