ISOLATION OF SMOOTH VESICLES AND FREE RIBOSOMES FROM RAT LIVER MICROSOMES

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ABSTRACT

Microsomes, isolated from rat liver homogenate in 0.88 M sucrose, have been fractionated by differential centrifugation. The 2nd microsomal fraction, sedimented between 60 minutes at 105,000 *g* and 3 hours at 145,000 *g*, consists mainly of smooth vesicles, free ribosomes, and ferritin. By utilizing the differences in density existing between the membranes and the granular elements it has been possible to separate the smooth membranes from the free ribosomes and ferritin. The procedure is to resuspend the 2nd microsomal fraction in a sucrose solution of 1.21 or 1.25 density and centrifuge it at 145,000 *g* for 20 or 40 hours. A centripetal migration of membranes and a centrifugal sedimentation of granular elements are obtained. Phospholipids, as well as the enzymatic activities DPNH–cytochrome *c* reductase, glucose-6-phosphatase and esterase are localized in the membranes. The free ribosomes have been purified by washing. A concentration of 200 μg RNA per mg nitrogen has been reached. RNA is also present in the membranes. These results are discussed in relation to current views on microsomal structure and chemistry.

It is well known that the microsomes, isolated by differential centrifugation, are rich in ribonucleic acid and phospholipids; biochemically this property serves to identify the microsomal fraction (1, 2).

From a morphological point of view, the microsomes are essentially composed of structures which arise through fragmentation of the endoplasmic reticulum existing in the cell *in situ* (1, 3). In rat liver mainly the following structures are found: "rough" vesicles characterized by dense particles, or ribosomes, attached to the outer surface of their membranes, "smooth" vesicles lacking ribosomes, free ribosomes, and ferritin (1–5).

The importance of microsomes in cellular metabolism is known, particularly during the biosynthesis of proteins (6) and fatty acids (7, 8), and in the processes of detoxification (9–11); but although light is shed at times on the function of each microsomal structure, one aspect of the problem remains almost unknown: that of the physiological relationship between the different components.

In order to approach this problem one indispensable step lies in the separation of the various structures composing the microsomes of rat liver, in defined fractions and in a pure and unaltered state. This consideration stems moreover from a more general approach which has asserted itself for several years and which attempts to correlate the biochemistry and morphology of the subcellular components (1, 2, 4, 12–16).

In an earlier investigation we have defined the limits of the microsomal population of rat liver; also we have shown that it can be separated into two subfractions so that the 1st microsomal fraction contains the "rough" vesicles while the 2nd microsomal fraction corresponds to the "smooth" forms, free ribosomes, and ferritin (2). The separation of the smooth membranes from the other microsomal structures of normal liver was a problem which had to be resolved. Up to now,

to our knowledge, the recent report of Rothschild is the only one on this question (17).

In this work we present a method of centrifugation which enabled us to obtain from the 2nd microsomal fraction the smooth membranes on one hand, and the free particles and ferritin on the other. The isolated fractions were studied from a morphological, biochemical, and enzymological point of view. The results and the conclusions which they imply will be discussed. This work has already been the subject of a preliminary communication (18).

EXPERIMENTAL

Animals

Male Wistar rats (Commentry strain) weighing from 230 to 300 gm were fed on the standard diet of Le Breton (19). The animals were regularly fasted 15 to 18 hours before being killed by decapitation. The liver was promptly removed and chilled. All the subsequent operations were performed at 4°C.

Cell Fractionation

a) Isolation of the 2nd Microsomal Fraction

The liver was homogenized in 0.88 M sucrose (10 per cent homogenate) with a homogenizer of the modified Potter type (20). Centrifugations were carried out in the Spinco model L centrifuge and the centrifugal force was calculated for the bottom of the tube.

The homogenate was fractionated according to the procedure previously described (2). The principle was as follows: after elimination of the nuclei, cellular debris, and mitochondria, a centrifugation at 105,000 *g* (30,000 RPM—rotor 30)[1] for 60 minutes resulted in the sedimentation of the 1st microsomal fraction containing the rough membranes. The supernatant was removed and centrifuged at 145,000 *g* (40,000 RPM—rotor 40) for 3 hours. A sharp, dark-red sediment was obtained which corresponded to the 2nd microsomal fraction and contained essentially smooth forms, free particles, and ferritin.

The final supernatant corresponds to the cell sap.

b) Fractionation of the 2nd Microsomal Fraction

The sediment was resuspended in a concentrated sucrose solution of determined density. The following solutions were used:

40	per	cent	sucrose	(*w*/*v*):	density	1.15 at 20°C
48	"	"	"	"	"	1.18
56	"	"	"	"	"	1.21
67	"	"	"	"	"	1.25

In general, the suspension was brought to 25 ml for a quantity of liver weighing 30 to 40 gm and centrifuged at 145,000 *g* (40,000 RPM—rotor 40) for a time which varied between 20 and 40 hours depending on the density of the medium. At the end of the centrifugation three fractions were separated: the pellicle, which was relatively firm, was carefully detached from the tube, then removed with a fine spatula; the intermediate liquid was collected by means of a pipette with automatic aspiration; and the tube was cut above the pellet. The samples for biochemical analysis were stored at −40°C.

Biochemical Methods

The different fractions were analyzed according to the modified Schmidt-Thannhauser method (21, 22). Ribonucleic acid was estimated with the orcinol reaction of Mejbaum (23) and expressed in ribonucleic acid phosphorus (RNA.P). Phospholipids were determined by the phosphorus of the lipid fraction (PL.P). Nitrogen (N) estimation was made by nesslerization on the alkaline digest of the Schmidt-Thannhauser method; the result corresponds to the sum (protein nitrogen + nucleic acid nitrogen). Total phosphorus was determined according to the Macheboeuf-Delsal technique after sulfuric acid digestion (24). In order to obtain the RNA and protein composition of the particles, calculations were made from the experimental values assuming that (*a*) ribonucleic acid nitrogen = RNA.P × 1.7; (*b*) protein = 6.25 × non-RNA nitrogen; (*c*) RNA = RNA.P × 10.7.

Treatment with Sodium Deoxycholate (DOC)

The sediments were resuspended in 0.4 per cent sodium deoxycholate in 0.88 M or 0.25 M sucrose adjusted to pH 7.6, so that the quantity of DOC equaled at least ¾ of that of the total proteins (25) The suspensions were rapidly homogenized and centrifuged at 145,000 *g* for 4 hours in 0.88 M sucrose or 1 hour in 0.25 M sucrose.

Enzymatic Assays

DPNH-cytochrome *c* reductase activity was determined spectrophotometrically at 20 to 22 °C by Hogeboom's technique (26). The increase of the optical density was read at 550 mμ against a blank containing all components of the reaction mixture except DPNH. Only the linear parts of the time and concentration curves were used for the quantitative determination.

Glucose-6-phosphate activity was assayed by a

[1] This corresponded to a centrifugation at 40,000 *g* for 2 hours in the multispeed attachment of the International centrifuge PR 1 (2).

modification of de Duve's method (27). The reaction mixture contained 0.01 M glucose-6-phosphate, 0.03 M histidine buffer pH 6.5 and 0.001 M Versene. The reaction was stopped by addition of perchloric acid, and the inorganic phosphorus liberated was determined.

Esterase activity was estimated according to a modification of the Nachlas and Seligman technique (28). The reaction mixture, containing 2 ml of 0.3 mM of α-naphthyl acetate buffered at pH 7.4 and enzyme, was incubated 30 minutes at 37°C. At this time, the reaction was stopped by the addition of 10 ml of 0.01 per cent Naphthanil diazo blue B in 0.5 per cent sodium dodecyl sulfate. After standing 30 minutes the optical density of the grey-green color was read at 600 mμ against blanks incubated without enzyme, and with enzyme added just before the diazo reagent (29).

Morphological Methods

Small pellets and thin pellicles were fixed *in toto* in a 1 per cent osmium tetroxide solution buffered at pH 7.3 (30). Sixty minutes later, they were washed with buffer, cut with a razor blade, dehydrated, and imbedded in *n*-butyl methacrylate and oriented. The ultrathin sections were made with the Servall microtome of Porter and Blum (31) through the entire depth of the sample in order to obtain a representative image of the specimen. Some preparations had been stained with a modified lead method (32). The sections were observed with both the RCA microscope EMU-2 and the Siemens Elmiskop I microscope.

Reagents

Glucose-6-phosphate and DPNH were obtained from Sigma Chemical Co. (St. Louis), deoxycholic acid from Hoffman Laroche (Paris), Cytochrome *c* from Boehringer and Soehne (Mannheim), and Naphthanil diazo blue B from Dajac Laboratory (Leominster, Mass.).

The α-naphthyl acetate was synthesized according to the technique of Nachlas and Seligman (33).

RESULTS

1. Subfractionation of the 2nd Microsomal Fraction

The 2nd microsomal fraction previously isolated is essentially composed of smooth membranes, free ribosomes, and ferritin; some additional components may also be found but in minor proportion (2). By differential centrifugation it is not possible to separate the membranes from the particulate elements.

Instead of utilizing the differences in rate of sedimentation of various cell components, we have used *the differences in density existing between the structures.* Indeed, the membranes rich in phospholipids should have a density lower than that of ribonucleoprotein particles and ferritin. Furthermore, this explains why structures so different in size as smooth vesicles (50 to 150 mμ), free ribosomes (10 to 15 mμ) and ferritin are able to sediment conjointly in the 2nd microsomal fraction. Thus, if this fraction is resuspended in a medium, which has a density lower than that of the particulate elements but greater than that of the vesicles, and centrifuged, it can be expected that a centripetal migration of membranes and a centrifugal sedimentation of heavy components will be obtained. In addition, the application of this principle of centrifugation is certainly one which would assure a minimum of contamination in the resulting fractions. This method differed from centrifugations executed with continuous or discontinuous density gradients by the fact that it was carried out in a medium of homogeneous density. An analogous method has already been employed for the preparation of nuclei of rat liver (34).

The separations from the 2nd microsomal fraction were performed in sucrose media ranging in density from 1.15 to 1.25. Whatever the density, a prolonged centrifugation (the time of which depended on the adopted density) produced the following separation (Fig. 1):

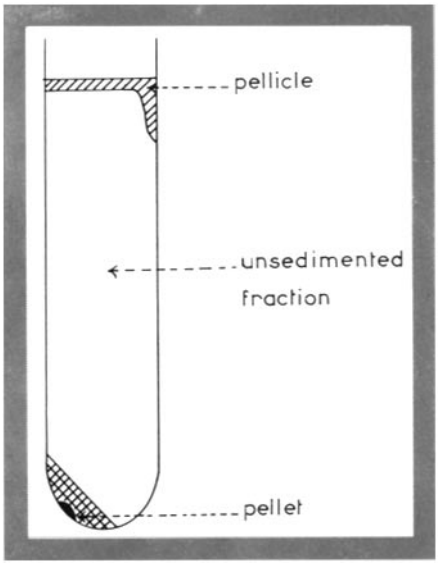
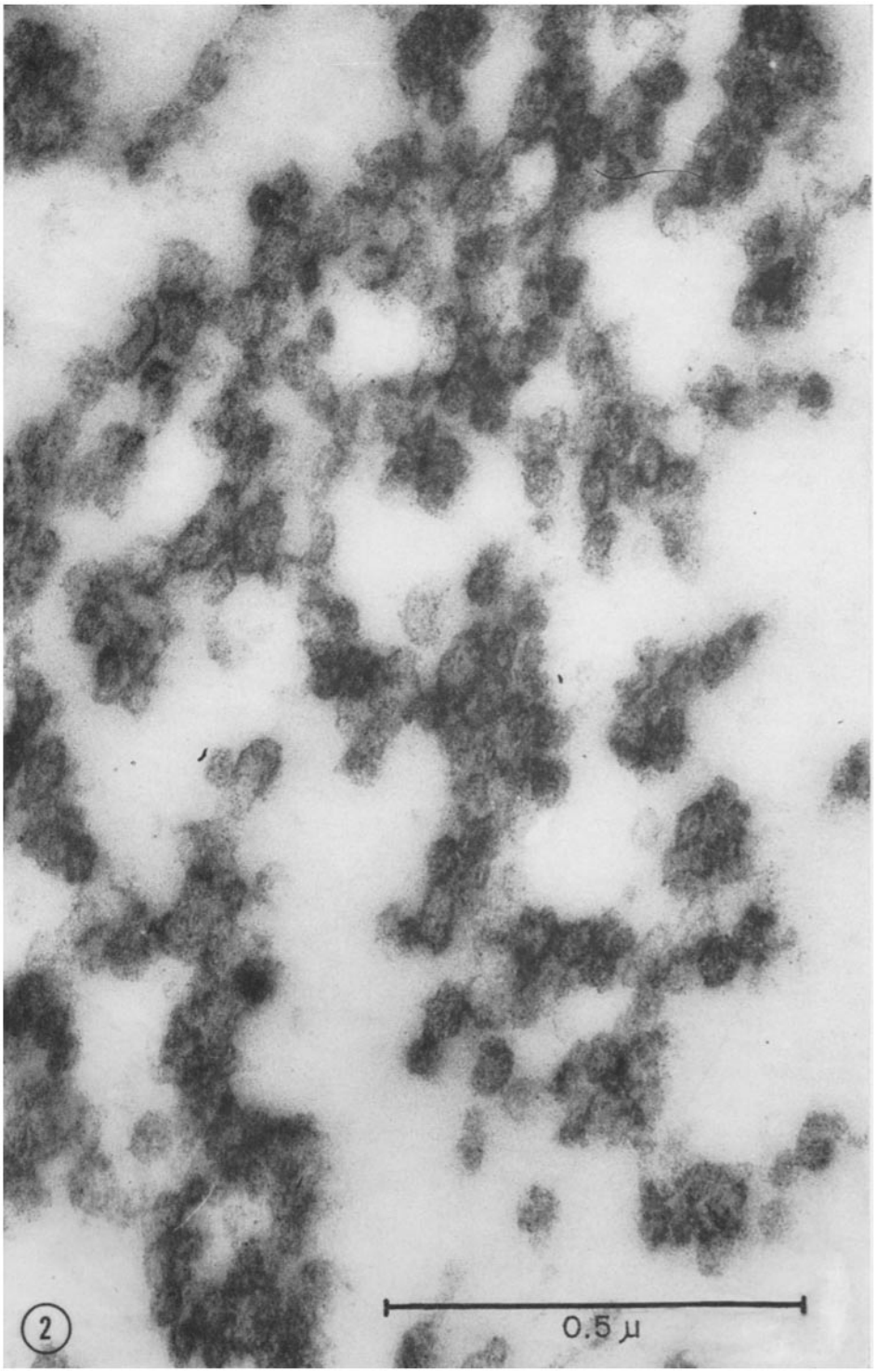


FIGURE 1
Illustration representing the separation of the 3 fractions after a prolonged centrifugation in concentrated sucrose medium.



Section of the pellicle, essentially composed of smooth membranes with a circular or oval profile. × 120.000.

TABLE I

Distribution of Nitrogen, PL.P, and RNA.P between the 3 Fractions Isolated from the 2nd Microsomal Fraction

Exp. No.		Density of the medium	Values per gm of wet liver			Per cent recovery		
			N	PL.P	RNA.P	N	PL.P	RNA.P
			μg	μg	μg			
50	12 hrs. at 145,000 *g*	1.21						
	membranes		536.5	107.6	13.3	36.5	72.7	18.2
	unsedimented fraction		731	38.1	30	49.6	25.8	40.8
	particles		205.5	2.2	30.1	13.9	1.5	41
	20 hrs. at 145,000 *g*	1.21						
	membranes		620	125	16.3	39.3	79.2	18.1
	unsedimented fraction		604.5	28.5	22.3	38.2	18.1	24.7
	particles		353	4.3	51.6	22.4	2.7	57.2
53	20 hrs. at 145,000 *g*	1.21						
	membranes		726	104.5	14.1	45.2	83.1	14.6
	unsedimented fraction		501	19.2	25.1	32.2	15.3	26.1
	particles		380	2.2	57.2	23.6	1.7	59.3
	40 hrs. at 145,000 *g*	1.21						
	membranes		685	104.5	14.4	42	85.8	13.9
	unsedimented fraction		417	13.7	16.1	25.6	11.2	15.5
	particles		525	3.5	73.1	32.3	2.9	70.5
54	40 hrs. at 145,000 *g*	1.25						
	membranes		886	113.7	23	49.8	95.1	22.5
	unsedimented fraction		615	5.2	40.3	34.6	4.3	39.4
	particles		278	0.6	39	15.6	0.5	38.1
85*	40 hrs. at 145,000 *g*	1.25						
	membranes		814	133.5	22.6	58.8	97.5	34.1
	unsedimented fraction		350	2.9	14.2	25.3	2.1	21.5
	particles		219	0.5	29.4	15.8	0.4	44.4

The 2nd microsomal fraction corresponds to an average value of 1,500 μg N, 90 μg RNA.P, and 170 μg PL.P per gm of wet liver. Each value represents about one third of the quantity found in the total microsomes (2).

* Experiment 85: in respect to the initial 2nd microsomal fraction, the total recoveries are 97 per cent for the nitrogen, 96 per cent for PL.P, and 89 per cent for RNA.P.

(*a*) at the surface, a deep rose, thin "pellicle," well separated from the liquid fraction situated beneath.

(*b*) at the bottom, a well packed red pellet with a darker center.

(*c*) in between, a clear, non-sedimented fraction, the yellow color of which became more intense towards the lower part of the tube. This color gradient was probably due to incomplete sedimentation of ferritin.

The higher the density of the solution, the greater is the difference in density between the membranes and the solution and the firmer the consistency of the pellicle. With appropriate care the latter could be quantitatively lifted off in 1.25 and 1.21 density media, while in 1.18 density medium it was very easily torn. Conversely, when the sucrose concentration was weaker, the sediment was larger and the unsedimented fraction clearer.

Morphology

PELLICLE: It contains numerous smooth vesicles with circular or oval profiles, varying in length from 60 to 120 mμ, totally free of attached dense particles. There are no visible free ribosomes or ferritin (Fig. 2). Similar results were obtained on preparations stained with a modified lead method.

PELLET: It contains granular elements, 12 to 20 mμ in diameter (Figs. 3 and 4). The density

TABLE II

RNA.P and PL.P Content of the 3 Fractions

Density of the medium	1.21		1.25	
Centrifugation time	20 hours		40 hours	
	PL.P/N	RNA.P/N	PL.P/N	RNA.P/N
Membranes	173.5	26.5	164	29
Unsedimented fraction	41.6	51.3	20	53.7
Particles	8.2	148.5	3.1	147
	8 experiments		6 experiments	

RNA.P and PL.P are expressed in μg; N in mg.
The average composition of the 2nd microsomal fraction is:
μg PL.P/mg N = 110 (25 experiments).
μg RNA.P/mg N = 60 (25 experiments).

of these particles to the electron beam is weaker than that of ribosomes of the liver cell fixed *in toto* or ribosomes of rough vesicles of the 1st microsomal fraction with a same fixation procedure (compare Fig. 3 in this paper with Fig. 4 in (2)). Ferritin is always found (Fig. 3). The examinations made from several preparations always exhibited an absence of microsomal membranes.

Thus, it is clear that morphologically the centrifugation has separated the smooth membranes from the granular elements.

Chemistry of the Subfractions

(*a*) The *distribution* of PL.P, RNA.P, and N showed that the percentages found in the 3 fractions varied according to the density and the centrifugation time (Table I). But whatever the conditions, the following facts were quite clearly established:

The great majority of phospholipids was contained in the pellicle, that is, in the membranes; thus more than 95 per cent of the PL.P in 1.25 medium was localized in this fraction.

The ribonucleic acid was always distributed between the 3 fractions; when the density was changed from 1.21 to 1.25 the quantity of RNA present in the pellet was decreased while that in the unsedimented fraction was increased. A medium of 1.25 density retards the sedimentation of lighter and/or smaller particles. The change of density did not noticeably affect the total amount of RNA.P in the pellicle.

As far as the nitrogen distribution is concerned, the longer the centrifugation continued, the smaller the nitrogen content of the non-sedimented fraction became. Nevertheless, there was always at least 25 to 30 per cent of the initial proteins which remained in the soluble fraction.

An extension of the centrifugation from 20 to 40 hours in 1.21 medium offered only little advantage; routinely the conditions adopted were the following:

1.21 density medium: 20 hours at 145,000 *g*
1.25 density medium: 40 hours at 145,000 *g*

In spite of the inconvenience of a much longer centrifugation, the utilization of 1.25 medium presented an advantage which justified its use: in effect, it enabled the separation of practically all of the microsomal membranes.

(*b*) The *composition* of the two morphologically identified fractions (membranes of the pellicle and particles of the pellet) was largely independent of the density of the solution—which is an argument in favor of the isolation of defined fractions (Table II). The separation of distinct structures allows one to grasp their differences in composition.

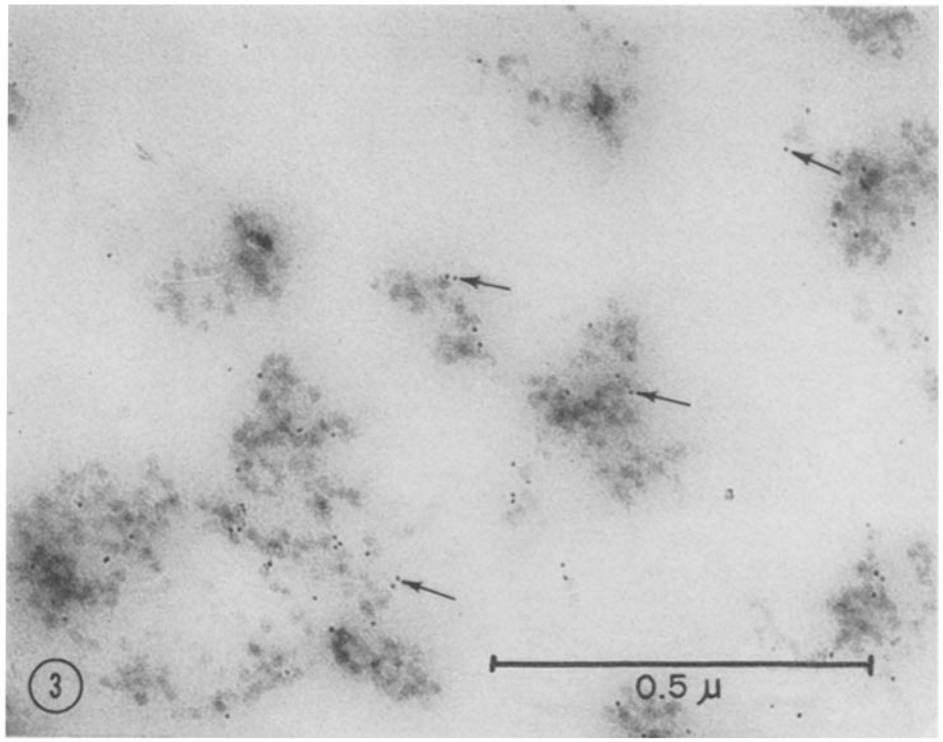
The high concentration of PL.P in the membranes is clearly revealed; although the microsomal fraction contains 110 μg PL.P/mg N (2), the pellicle has always a ratio which ranges between 160 and 170. Until now such a concentration had never been reached in a microsomal fraction; this corresponds to an amount of 40 per cent phospholipids in proportion to dry weight. The membranes always contained RNA, in the average of 27 μg RNA.P/mg N, whatever the density of the solution. The significance of the presence of this RNA will be considered later.

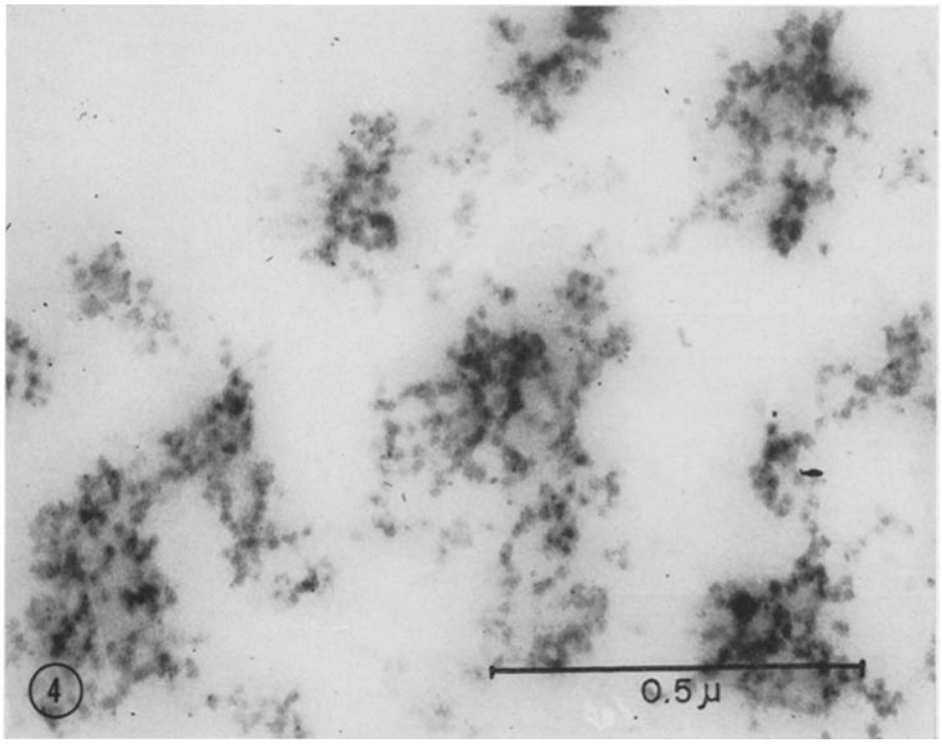
FIGURE 3

Section of the pellet, composed of granular elements 12 to 20 millimicrons in diameter. Arrows: particles of ferritin. × 100,000.

FIGURE 4

Section of the pellet stained with a modified lead method (32) in order to increase the contrast of the granular elements. × 100,000.





The particles of the pellet were practically free of lipids; on the other hand, the amount of RNA was tripled in proportion to that of the original fraction. These particles containing 24 per cent RNA by dry weight with less than 1 per cent phospholipids probably correspond to the free ribosomes (see Discussion).

The composition of the supernatant did not have great significance in itself, being too influenced by incompletely sedimented particles.

The nucleotide composition determined on RNA isolated by NaCl extraction, followed by alkaline hydrolysis and electrophoresis according to the methods of Davidson and Smellie (35) and Markham and Smith (36), are reported in Table III.

Enzyme Distribution

The possibility of separating the membranes and the RNA-rich particles enabled the direct study of the localization of certain microsomal enzyme activities between the different structures. The results showed that the distribution of the assayed activities paralleled closely that of the phospholipids (Table IV), which constitutes direct experimental proof of the localization of these systems in the membranes. An additional argument gives weight to this conclusion, namely that the Activity/PL.P ratio in the different fractions showed much less variation than the specific activities expressed by mg nitrogen. Such a finding is in keeping with current views (1, 37–40), but until now the results have more often been obtained after the use of detergents (DOC, Lubrol, Tween, etc. . .) which dissolve the membranes. The results in regard to esterase are in total disagreement with those obtained by Takanami who found a localization of this enzyme in his RNA-rich particles (41).

TABLE III

Nucleotide Composition of RNA Isolated from Membranes, and RNA-Rich Particles Obtained in Sucrose d = 1.21, and from Cell Sap

	C‡	A‡	G‡	U‡
Membranes (12)	28.0	18.0	34.1	19.8
S.E.M.	0.5	0.3	0.4	0.3
Particles (12)	29.2	18.2	30.5	22.1
S.E.M.	0.4	0.2	0.3	0.2
Cell sap (7)	28.3	19.3	30.7	21.6*
S.E.M.	0.6	0.3	1.0	0.4

C, cytidylic acid; A, adenylic acid; G, guanylic acid; U, uridylic acid.

The figures in brackets represent the number of experiments.

* Uridylic acid + pseudouridylic acid.

‡ Moles per hundred moles of total nucleotides.

2. *Purification of the Isolated Fractions*

Washing the Fractions

Although the principle of separation based on differences in density greatly reduces contamination, purification attempts by washing were carried out for each of the 2 isolated fractions. The purpose of these experiments was to attain as nearly as possible the true composition of the microsomal structures.

PELLICLE: After isolation in 1.21 density sucrose medium, the pellicle was resuspended in the same medium and recentrifuged at 145,000 *g* for 20 hours. The results show that under these conditions the composition did not vary and that 83 per cent of the initial RNA was recovered in the washed pellicle (Table V). This indicates that the RNA present is not simply due to a mechanical effect of contaminating particles.

PELLET: The washes were carried out by the classical method of differential centrifugation by resuspending the pellet, centrifuging it, and then eliminating the supernatant. Successive centrifugation at 145,000 *g* for 30 minutes in phosphate −Mg buffer pH 7.5, increased the RNA concentration from 127 to 200 but the yield remained very low (Table V). If the centrifugation time was increased to 60 minutes at 145,000 *g* after each wash, then it was possible to attain the same concentration of RNA while greatly improving the yield. The use of 0.25 M sucrose produced the same results; use of the Hamilton and Petermann solution (0.5 mM K_2HPO_4; 0.5 mM KH_2PO_4; 0.5 mM $MgCl_2$) (42) would be less effective.

Thus, after one wash, particles containing around 200 μg RNA.P/mg N were obtained along with a recovery of 90 per cent of the tota RNA. It is noteworthy that one operation was sufficient and further washes did not sensibly increase the concentration of RNA but diminished the percentage of sedimentable RNA. The concentration of 200 μg RNA.P/mg N appears as the limiting value attained for a purification of this order.

TABLE IV

Distribution of Enzymic Activity between the 3 Fractions

		DPNH-cytochrome *c* reductase activity*			Esterase activity‡			Glucose-6-phosphatase activity§		
	PL.P	Activity	Activity/ mg PL.P	Activity/mg N	Activity	Activity/ μg PL.P	Activity/mg N	Activity	Activity/ μg PL.P	Activity/mg N
	per cent	*per cent*			*per cent*			*per cent*		
d = 1.21; exp. 67										
20 hrs. at 145,000 *g*										
membranes	57.3	60	100	19.6	45.1	25.2	3960			
unsedimented fraction	41.8	39	90	7.28	54	32.2	2690			
particles	0.8	1	120	1.19	0.8	24.5	247			
d = 1.25; exp. 82										
40 hrs. at 145,000 *g*										
membranes	89.8	89.3	130	28	88.3	15.8	3430			
unsedimented fraction	8.3	9.3	147	4.3	9.3	19.1	558			
particles	1.9	0.8	57	0.35	1.9	16.7	105			
d = 1.25; exp. 85										
40 hrs. at 145,000 *g*										
membranes	97.5	96.8	96	15.8				97	5.39	885
unsedimented fraction	2.1	3	139	1.1				2.9	7.6	64
particles	0.4	0.2	54	0.13				~0		

* Micromoles cytochrome *c* reduced per 10 minutes at 22°C. In respect to the initial 2nd microsomal fraction, the total activities recovered are respectively 77.5 per cent, 70.8 per cent, and 77 per cent for the experiments 67, 82, and 85.

‡ Micromoles α-naphthol liberated per 30 minutes at 37°C. In respect to the initial 2nd microsomal fraction, the total activities recovered are respectively 88.3 per cent, and 93 per cent for the experiments 67 and 82.

§ Micrograms inorganic phosphorus liberated per 30 minutes at 37°C. In respect to the initial 2nd microsomal fraction, the total activity recovered is 54.5 per cent.

This value is very close to that obtained by Kuff and Zeigel for purified free ribosomes of the Novikoff hepatoma (211 μg RNA.P/mg N) (43).

Action of Sodium Deoxycholate (DOC)

As used by Palade and Siekevitz, the DOC dissolves the membranes, conserves the individual structure of the ribosomes, and permits the isolation of a pellet containing around 35 to 40 per cent RNA on a dry-weight basis (1). Furthermore, Petermann and Hamilton find that liver ribonucleoprotein particles obtained after DOC treatment and extensive purification contain 40 per cent RNA (44, 45). The DOC treatment of the pellets isolated in 1.21 and 1.25 media increased considerably the RNA concentration of the granular components with a recovery of 90 per cent RNA (Table VI). Nevertheless, the values of the μg RNA.P/mg N ratio obtained after DOC exhibited a certain variation between 230 and 288, but they were always greater than the limiting value of 200 found for washed particles. Furthermore, this limiting value could, in turn, be increased by treating the buffer-washed particles with DOC (Table VI, experiment 100). Here the results were in close agreement to those reported by Kuff and Zeigel; they raise the question of the action of DOC on the integrity of the ribosomes.

INTERPRETATION AND DISCUSSION

The aim of the separation of cell components is to further the analysis of cellular metabolism. One indispensable condition is to respect to the fullest extent possible the morphological integrity of the different structures in the course of their isolation. The participation of the microsomes in important metabolic pathways justifies attempts

TABLE V

Purification of the Pellicle and Pellet

		μg RNA.P/mg N	RNA.P per cent recovered
(1) Pellicle			
Exp. 53	Control	19.4	(100)
	Washed × 1	20.1	83
(2) Pellet isolated in medium d = 1.25			
1. Wash with phosphate—Mg buffer* (30 min. at 145,000 *g*)			
Exp. 92	Control	127	(100)
	Washed × 1	189	60
	Washed × 2	200	35.4
	Washed × 3	159	24.5
2. Wash with phosphate—Mg buffer* (60 min. at 145,000 *g*)			
Exp. 98	Control	148	(100)
	Washed × 1	199	87
	Washed × 2	202	76.4
	Washed × 3	209	65.4
3. Wash with Hamilton and Petermann buffer‡ (60 min. at 145,000 *g*)			
Exp. 100	Control	158	(100)
	Washed × 1	178	92.5
4. Wash with 0.25 M sucrose (60 min. at 145,000 *g*)			
Exp. 99	Control	147.5	(100)
	Washed × 1	204	100

The pellet was resuspended in buffer or sucrose solution and centrifuged at 145,000 *g* for 30 or 60 min.

* 5 mM sodium phosphate; 0.5 mM $MgCl_2$ pH 7.5.

‡ 0.5 mM K_2HPO_4; 0.5 mM $MgCl_2$; 0.5 mM KH_2PO_4.

TABLE VI

Effect of DOC Treatment on RNA-rich Particles Isolated in Sucrose Solutions

Experiment No.			μg RNA.P/mg N	Per cent of RNA.P
51	Pellet*	Control	160	(100)
		After DOC	280	88
76	Pellet*	Control	150	(100)
		After DOC	230	90
99	Pellet‡	Control	147.5	(100)
		After DOC	204	95.6
100	Pellet‡	Control	158	
		After DOC	288	
100	Pellet‡	Washed Control	178	(100)
		After DOC	242	89.3

* Pellets isolated in sucrose solution d = 1.21, resuspended in 0.4 per cent sodium deoxycholate in 0.88 M sucrose pH 7.6, and centrifuged at 145,000 *g* for 4 hrs.

‡ Pellets isolated in sucrose solution d = 1.25, resuspended in 0.4 per cent sodium deoxycholate in 0.25 M sucrose, and centrifuged at 145,000 *g* for 1 hr.

to fractionate the various morphological structures contained in them. In the case of liver the rough vesicles can be localized in one definite fraction (2), but until now the free particulate components (ribosomes and ferritin) could not be separated from the smooth membranes.[2] At most, the methods of differential centrifugation using a saline or 8.5 per cent sucrose medium led to a "post-microsomal fraction" having a RNA.P/N ratio greater than that of the total microsomes and composed of a mixture of membranes and many free ribosomes (5, 41, 47, 48).

The 2nd microsomal fraction previously isolated is composed of elements existing in the *free state*: smooth membranes, ribosomes, ferritin. By utilizing the differences in density between them, it was possible to separate the morphological structures. It is clear that the biochemical results obtained for each of the fractions thus isolated approach more nearly the true composition of the components. Certain of the results are in agreement with ideas proposed for some years, such as the exclusive localization of phospholipids in the membranes, the richness of RNA in the ribosomes, and the presence of some microsomal enzymes in the membrane (1, 37, 38, 40). However, these conclusions were based on the results obtained after treatment of the microsomes with detergents which dissolve the membranes and may also affect the integrity of the particles. The experiments reported here bring forward additional evidence: *they constitute a direct experimental proof obtained from isolated and morphologically identifiable structures.*

The particles of the pellet have an identical concentration of RNA, whatever the density of the medium; the almost complete lack of phospholipids confirms the morphological examination regarding the absence of membranous structures. However during the initial centrifugation a simultaneous sedimentation of contaminating protein material occurs since subsequent purifications of the pellet by washing increase the RNA concentration from 148 to 200 μg RNA.P/mg N, *i.e.*, from 24 to 33 per cent RNA by dry weight. It does not appear that this last value can be surpassed by the method of purification employed. To our knowledge it is the first time that particles having such a concentration of RNA have been obtained from normal liver *without* using detergents.[3] Still we cannot state that this concentration represents the true value of RNA in these particles.

Morphologically, these RNA-rich particles appear in the form of granular elements comparable to the free particles isolated from onion roots (49) or brain (50). Their light density to the electron beam might be explained by the experimental conditions of isolation (duration of preparation time, absence of certain ions . . .). Nevertheless, the results reported here show that *without detergents free particles uncontaminated by membranes can be separated from the microsomes.* They have a high specific gravity (>1.25), are rich in RNA, and represent most probably the free ribosomes of the liver cell.[4]

The action of DOC on the isolated RNA-rich particles is always followed by an increase in the concentration of RNA even when starting with a purified preparation. It is possible that this increase may be due to the solubilizing of contaminating proteins which are not eliminated by washing; however we think rather, in accord with Kuff and Zeigel (43), that the DOC may remove a significant amount of protein material from the particles. Not only could this material have an important physiological significance but certain observations might show that it plays a role in the stability of the particles. Finally it is necessary to note that treatment of the RNA-rich particles by DOC leads to the recovery of at least 90 per cent of the initial RNA; on the other hand, when DOC acted on the microsomes only 50 to 60 per cent of the RNA was recovered in the pellet (2). This fact argues in favor of the heterogeneity of behaviour of the microsomal RNA.

The pellicle represents a *highly purified preparation of smooth membranes*, but obviously the purity of the preparation does not imply a unique origin. These vesicles come essentially from the endoplasmic reticulum but also from any other morphological structure arising through fragmentation of smooth-membraned vesicles (microvilli, cell wall, vacuoles . . .). One other fact to emphasize is

[2] For other mammalian tissues the work of Palade and Siekevitz on pancreas (12), and that of Hanzon and Toschi on brain (15, 46) should be cited.

[3] From Novikoff hepatoma which is characterized by an abundance of free ribosomes, Kuff and Zeigel isolated a pellet consisting of particles containing 37 per cent RNA after purification (43).

[4] An allowance must be made for the ribosomes of the rough vesicles which could be detached from the membranes in the course of the manipulations.

that more than 95 per cent of the membranes can be thus separated if we assume that all the phospholipids are present in membranes. From the chemical point of view, if the richness in phospholipids and the localization of certain enzyme activities are generally accepted features, the presence of ribonucleic acid in the membranes reflects a less widespread opinion. Can this presence be interpreted as proof of the existence of RNA in the membranes as we have previously advanced (2, 51)? A discussion of this hypothesis must take into account the following points:

The RNA concentration in the pellicle is always in the same range (~ 27 μg RNA.P/mgN), whatever the density of the medium, particularly in a 1.18 density medium. In addition, the washing of the membranes followed by a second centrifugation does not cause the elimination nor even a decrease in concentration of RNA in this fraction (Table V). If these experimental facts indicate that a mechanical transportation of free ribosomes does not occur, then the hypothesis of an adsorption phenomenon cannot be denied. Other results are more in favor of the existence of RNA in the membranes.

The nucleotide composition shows a significant difference for guanylic and uridylic acids between the RNA of the membranes and that of both the pellet and cell sap (Table III). The last two ribonucleic acids are very similar in composition, in agreement with the results of Elson and Chargaff (52) and Crosbie *et al.* (53).

A study of incorporation of P^{32} into the RNA of the membranes and the free ribosomes shows differences in the metabolic activity of the RNA of the two components. After DOC treatment, a "2nd DOC pellet" containing RNA is obtained from both the pellicle and the sediment. The specific activity of RNA for the free ribosomes is three times greater than that obtained for the membranes. This fact does not speak for a common origin of the RNA. Details of these experiments will appear in a future paper.

All these results tend to indicate that the RNA found in the membranes has a certain autonomy of behaviour; they draw attention to the probable existence of ribonucleic acid in the membranes, which physiologically could be very important.

Recent investigations have revealed the metabolic heterogeneity of the microsomal RNA in rat liver (41, 54–58), but without establishing a correlation between the biochemical and morphological results. It is now possible to divide the microsomal population into well defined fractions: rough membranes, smooth membranes, free ribosomes. It is obvious that the study of RNA metabolism or, more generally, any metabolic function in cytologically identified fractions is much more significant. This might well be a means of understanding the relationships which exist between the different structures.

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