

ANALYTICAL STUDY OF MICROSOMES
AND ISOLATED SUBCELLULAR
MEMBRANES FROM RAT LIVER

II. Preparation and Composition of
the Microsomal Fraction

ALAIN AMAR-COSTESEEC, HENRI BEAUFAY, MAURICE WIBO,
DENISE THINÈS-SEPOUX, ERNEST FEYTMANS,
MARIETTE ROBBI, and JACQUES BERTHET

From the Laboratoire de Chimie Physiologique, Université de Louvain, Louvain, Belgium

ABSTRACT

Liver homogenates have been submitted to quantitative fractionation by differential centrifugation. Three particulate fractions: N (nuclear), ML (large granules), and P (microsomes), and a final supernate (S) have been obtained. The biochemical composition of the microsomal fraction has been established from the assay and distribution pattern of 25 enzymatic and chemical constituents. These included marker enzymes for mitochondria (cytochrome oxidase), lysosomes (acid phosphatase and *N*-acetyl- β -glucosaminidase), and peroxisomes (catalase). The microsomal preparations were characterized by a moderate contamination with large cytoplasmic granules (only 6.2% of microsomal protein) and by a high yield in microsomal components. Enzymes such as glucose 6-phosphatase, nucleoside diphosphatase, esterase, glucuronyltransferase, NADPH cytochrome *c* reductase, aminopyrine demethylase, and galactosyltransferase were recovered in the microsomes to the extent of 70% or more. Another typical behavior was shown by 5'-nucleotidase, alkaline phosphatase, alkaline phosphodiesterase I, and cholesterol, which exhibited a "nucleomicrosomal" distribution. Other complex distributions were obtained for several constituents recovered in significant amount in the microsomes and in the ML or in the S fraction.

INTRODUCTION

The prerequisite conditions to an analytical study of microsomes were to obtain a representative fraction containing the bulk of microsomal components with little contamination by other subcellular organelles and to establish its biochemical composition. Rather than to prepare in one step a postmitochondrial supernate from which micro-

somes are sedimented, it was found more suitable to sediment first successively a nuclear (N) fraction and a large granule (ML) fraction. This paper describes the fractionation method followed, which is similar to that used by de Duve et al. (11) except that the large granules were pelleted in a single fraction equivalent to the sum of the M

and L fractions of these authors. The microsomal fraction has been characterized by establishing the distribution patterns of 25 enzymatic and chemical constituents. The yield appears much higher than that reported for microsomes obtained by other methods described in the literature, and the large granules represent no more than 6% of the microsomal protein.

MATERIALS AND METHODS

Female rats of the Wistar strain, fed with rat food no. 515 (Aliments Protector S.A., Brussels, Belgium), were used throughout this work. In each experiment, four to five animals, weighing about 160 g and fasted for 18 h with water *ad libitum*, were killed by decapitation. The livers were quickly taken out, immersed, and weighed in a tared beaker containing ice-cold 0.25 M sucrose buffered at pH 7.4 with 3 mM imidazole-HCl (designated henceforth as buffered sucrose). All the subsequent operations were performed at about 2°C. Homogenization and quantitative fractionation by differential sedimentation (11) were carried out as follows.

Each liver, cut into 10–15 pieces, was put into the smooth-walled vessel of the tissue grinder model C (Arthur H. Thomas Co., Philadelphia, Pa.), containing 3 ml of buffered sucrose per g of wet tissue. The Teflon pestle was driven at 1,300 rpm while the grinding vessel, maintained in a plastic beaker filled with crushed ice and water, was pushed upwards once to force the tissue through the clearance of grinder. Passage of the whole tissue was accomplished in about 15 s. Each homogenate (15–20 ml) was poured into a separate tube (rotor no. 252, International Centrifuge, model PR-2, International Equipment Co., Needham Heights, Mass.). After 10 min of centrifugation at 1,700 rpm the supernates were decanted, and the pellets were homogenized in the same volume of buffered sucrose. The nuclear pellets were sedimented again at 1,400 rpm for 10 min. The washing process was repeated once more. In the decantation step, the whole loosely packed material was left with the pellet fraction which was finally resuspended in buffered sucrose and referred to as the N fraction.

The supernatant fluids were pooled to give the cytoplasmic extract (E), and their total volume was recorded. After mixing, a small sample was kept for analysis and the remainder was used for sedimenting an ML fraction at 25,000 rpm (rotor no. 40, ultracentrifuge model L2-65 or L3-50, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), by a time integral of the squared angular velocity ($W = \int_0^t \omega^2 dt$) of $2.5 \times 10^9 \text{ rad}^2 \text{ s}^{-1}$. The fluffy layer was removed with the supernatant fluid. The ML pellet

was washed¹ twice in 3 ml of buffered sucrose per g of liver and resuspended in the same medium. The microsomal (P) fraction was then separated from the postmitochondrial supernate (washings included) at the maximal speed of the no. 40 rotor ($W = 3 \times 10^{10} \text{ rad}^2 \text{ s}^{-1}$),² washed once in about 4 ml of buffered sucrose per g of liver, and resuspended carefully by means of the tight pestle of a Dounce homogenizer (Kontes Glass Co., Vineland, N. J.) with buffered sucrose, up to a volume of 1 ml/g of tissue. The high-speed supernates were pooled within a single S fraction.

Some experiments were performed on microsomal fractions prepared by centrifuging the postmitochondrial supernate at $W = 9 \times 10^{10} \text{ rad}^2 \text{ s}^{-1}$. In particular these more complete microsomal fractions were used for subfractionation by differential sedimentation in a stabilizing gradient. They will be referred to as P' fractions, and S' will designate the corresponding final supernates.

Electron microscopy of subcellular fractions was performed as described by Wibo et al. (52). Biochemical methods were described in the preceding paper (3).

RESULTS

In Table I are listed the distribution patterns and the recoveries obtained in the fractionation of rat liver tissue. These data were corrected for recovery to draw the distribution profiles of Fig. 1 as described by de Duve et al. (11). In that representation, the specific activity (or concentration) of all constituents is taken to be 1 in the sum of N, ML, P, and S fractions; the height of the blocks gives the extent of purification achieved over the homogenate and the surface area of the blocks (relative specific activity \times percent of protein) is the percentage of constituent recovered in the corresponding fraction. The content of the fractions in absolute values can be computed from the data of Table II.

Several patterns of distribution are provided by the constituents studied. Some enzymes are recovered in the P fraction to the extent of about 70% or more and with a relative specific activity approaching 4. These enzymes are glucose 6-phos-

¹ By "washed" we mean resuspension of pellets in buffered sucrose, followed by recentrifugation at the force used previously.

² In some experiments the centrifugation was carried out in a rotor of greater capacity (Beckman-Spinco no. 42). W was then increased by a factor of 1.45 to compensate for the lower performance index of this rotor.

TABLE I
Distribution of Constituents after Fractionation of Liver by Differential Centrifugation

Constituent	No. of exps.	N	ML	P	S	Recovery
Protein	36	18.2 ± 4.1	22.5 ± 4.4	18.6 ± 1.8	39.5 ± 3.1	98.8 ± 3.9
Phospholipid	17	19.5 ± 3.8	25.0 ± 3.1	50.4 ± 3.3	7.8 ± 2.0	102.7 ± 10.5
Ribonucleic acid	32	19.0 ± 4.8	4.4 ± 2.1	54.9 ± 8.3	23.6 ± 9.8	101.9 ± 11.5
Cholesterol	16	26.8 ± 4.9	5.4 ± 1.7	56.0 ± 5.1	8.3 ± 2.3	96.5 ± 10.7
Glucose 6-phosphatase	38	14.5 ± 3.6	9.3 ± 5.5	75.5 ± 8.4	4.4 ± 2.6	103.7 ± 13.3
Nucleoside diphosphatase	20	13.5 ± 3.8	4.5 ± 2.9	65.6 ± 4.7	5.4 ± 1.8	89.0 ± 14.3
Esterase	13	13.4 ± 5.0	7.5 ± 1.7	68.9 ± 4.6	10.6 ± 3.4	100.4 ± 14.1
Glucuronyltransferase Acceptor: 4-methyl umbelliferone	6	14.0 ± 6.4	5.6 ± 1.8	75.8 ± 7.6	1.7 ± 1.6	97.1 ± 22.8
Acceptor: bilirubin	3	18.8 ± 2.4	9.4 ± 1.2	63.7 ± 8.6	0.3 ± 0.4	92.2 ± 8.5
Galactosyltransferase	5	4.6 ± 2.3	1.8 ± 1.0	72.9 ± 4.0	11.6 ± 1.0	90.9 ± 3.7
NADPH cytochrome <i>c</i> re- ductase	13	12.1 ± 2.8	11.1 ± 3.7	66.0 ± 6.7	11.1 ± 4.4	100.3 ± 12.6
Aminopyrine demethylase	3	10.3 ± 3.2	4.9 ± 4.7	69.1 ± 11.1	12.7 ± 4.4	97.0 ± 23.1
NADH cytochrome <i>c</i> re- ductase	30	13.0 ± 4.5	21.3 ± 6.1	58.4 ± 6.4	6.4 ± 2.2	99.1 ± 7.4
5'-Nucleotidase	32	34.0 ± 9.0	10.9 ± 3.8	49.9 ± 7.2	12.9 ± 2.7	107.7 ± 11.9
Alkaline phosphodiesterase I	4	41.6 ± 5.9	8.1 ± 6.0	51.7 ± 7.8	1.3 ± 0.6	102.7 ± 5.7
Alkaline phosphatase	4	27.6 ± 2.9	10.3 ± 4.4	37.1 ± 10.2	31.3 ± 4.6	106.3 ± 7.2
Cytochrome oxidase	34	20.2 ± 5.8	66.1 ± 6.1	4.2 ± 2.0		90.5 ± 10.1
Monoamine oxidase	37	18.3 ± 5.1	50.1 ± 7.1	19.9 ± 4.3	5.9 ± 3.0	94.2 ± 8.3
Acid phosphatase	31	12.1 ± 4.0	48.1 ± 7.9	16.7 ± 5.1	19.1 ± 4.5	96.0 ± 9.2
β -Glucuronidase	10	16.2 ± 4.0	41.7 ± 6.3	28.5 ± 6.0	15.4 ± 3.0	101.8 ± 21.2
<i>N</i> -Acetyl- β -glucosaminidase	4	13.6 ± 4.5	62.7 ± 9.5	8.5 ± 1.9	6.4 ± 3.1	91.2 ± 9.1
Catalase	32	8.8 ± 2.8	45.1 ± 6.4	5.7 ± 2.6	36.9 ± 5.1	96.5 ± 10.7
Fumarase	5	16.2 ± 1.4	28.0 ± 3.1	9.9 ± 3.2	49.1 ± 5.1	103.2 ± 9.4
Aldolase	10	19.6 ± 3.3	0.6 ± 0.3	33.5 ± 12.1	45.8 ± 14.2	99.5 ± 11.7
Glutamine synthetase	18	18.4 ± 6.0	1.0 ± 1.1	30.9 ± 7.6	61.2 ± 9.6	111.5 ± 16.0

Results are given as means \pm standard deviations, in percent of the sum of the absolute values found in fractions E and N.

phatase, nucleoside diphosphatase, esterase, glucuronyltransferase with 4-methylumbelliferone (4-MeU) or bilirubin as acceptor, NADPH cytochrome *c* reductase, aminopyrine demethylase, and galactosyltransferase. The distribution of the latter enzyme is characterized, however, by a lower yield in the N fraction: less than 5% instead of more than 14% on an average for the others. NADH cytochrome *c* reductase is also mainly recovered in microsomes, but to a lesser extent (60%), some 22% being found in the large granules.

Another characteristic pattern of distribution is that shown by alkaline phosphodiesterase I, 5'-nucleotidase, and alkaline phosphatase. They divide between fractions N and P with some advantage for microsomes with respect to both

the amount and the specific activity. This type of distribution has been designated as nucleomicrosomal in the sense that the amount recovered in the nuclear fraction is much higher than that observed for typical microsomal constituents (44). The three patterns differ, however, from one another by the amount of enzyme associated with the S fraction. Glutamine synthetase and aldolase are mainly recovered in the latter fraction, but the sedimentable activity is also divided between N and P.

Cytochrome oxidase, monoamine oxidase, catalase, acid phosphatase, β -glucuronidase, and *N*-acetyl- β -glucosaminidase are mainly associated with the large granules (ML). The distributions of these enzymes are in agreement with the data previously reported (1, 11, 45, 51). In particular,

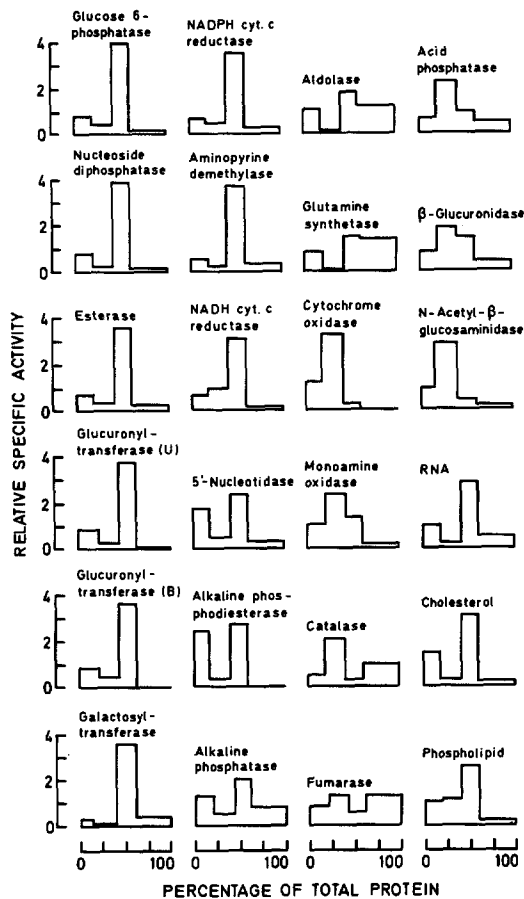


FIGURE 1 Distribution patterns of constituents after fractionation of liver by differential centrifugation. Graphs were constructed from the data given in Table I. Fractions are plotted in the order of the average coefficient of sedimentation of their subcellular components, i.e. from left to right: N, ML, P, and S. Each fraction is represented separately in the ordinate scale by the relative specific activity of the constituent (percentage of total amount/percentage of total protein). In the abscissa scale each fraction is represented cumulatively by its percentage of protein.

the rather high recoveries of monoamine oxidase (1, 9, 45) and β -glucuronidase (11, 17, 19, 20, 36) in microsomes are confirmed. The distribution of cytochrome oxidase expresses the behavior of mitochondria in the fractionation. In a similar way acid phosphatase and *N*-acetyl- β -glucosaminidase will serve to monitor the distribution of lysosomes, and catalase that of peroxisomes. The highest specific activity of fumarase is also observed in the ML fraction, but this enzyme is present in significant amounts in all fractions and especially in the supernate.

The microsomal fraction contains about 19% of the total protein, 49% of the phospholipid, and 55% of the RNA. Cholesterol has a typical nucleomicrosomal pattern of distribution, 28 and 58% being, respectively, associated with the N and P fractions.

Some enzymes were present in the S' fraction to the same amount as in the S fraction (Table III). Other constituents of the S fraction sedimented to a large extent with the microsomes of the P' fractions. Such is the case for RNA, glucose 6-phosphatase, galactosyltransferase, NADH cytochrome *c* reductase, and monoamine oxidase. The electron microscope appearance of the pellet obtained by centrifuging the S fraction for $W = 9 \times 10^{10} \text{ rad}^2 \text{ s}^{-1}$ (postmicrosomal fraction equivalent to the difference S-S' of Table III) is compared in Fig. 2 to that of the P fraction. The material sedimented from the S fraction shows essentially vesicular profiles of much smaller size than those of the P fraction, numerous free ribosomes, and ferritin molecules.

DISCUSSION

We have reported the distribution patterns of constituents obtained after fractionation of rat liver homogenates by differential centrifugation. At this stage, comparison will be made of our results with other data from the literature, and the chemical and enzymatic composition of the microsomal fraction prepared by our method will be considered. The intracellular localization of microsomal constituents will be discussed later in the light of information that will be brought out by the analytical subfractionation of microsomes, to be reported in the subsequent paper (4).

Subcellular Distribution of Chemical and Enzymatic Constituents

The microsomal character, previously established in rat liver by quantitative fractionation experiments for glucose 6-phosphatase (11, 23), nucleoside diphosphatase (51), and esterase (28, 33, 41, 46), has been confirmed. Aminopyrine demethylase (34) and glucuronyltransferase (14, 43) are commonly attributed to microsomes on the basis of their specific activity in various types of microsomal preparations. Although this kind of argument supports their occurrence in microsomal components, the quantitative method of tissue fractionation gives a comprehensive view of the subcellular localization of enzymes and may, for

TABLE II
Absolute Values of Measured Constituents

Constituent	EC	No. of exps.	Absolute values
Protein		43	219 ± 29
Phospholipid		18	45.6 ± 7.6
Ribonucleic acid		38	8.57 ± 1.90
Cholesterol		22	2.78 ± 0.50
Glucose 6-phosphatase	3.1.3.9	45	20.2 ± 4.6
Nucleoside diphosphatase	3.6.1.6	23	100 ± 20
Esterase	3.1.1.2	13	257 ± 60
Glucuronyltransferase	2.4.1.17		
Acceptor: 4-methylumbelliferone		6	2.38 ± 0.70
Acceptor: bilirubin		3	0.075 ± 0.004
Galactosyltransferase			
Acceptor: ovalbumin		5	0.013 ± 0.003
NADPH cytochrome <i>c</i> reductase	1.6.2.3	16	3.98 ± 1.83
Aminopyrine demethylase		3	0.079 ± 0.028
Cytochrome P 450		3	21.7 ± 3.0
NADH cytochrome <i>c</i> reductase	1.6.2.1	38	100 ± 34
Cytochrome <i>b</i> ₅		15	19.2 ± 3.0
5'-Nucleotidase	3.1.3.5	36	11.3 ± 3.3
Alkaline phosphodiesterase I	3.1.4.1	8	17.5 ± 4.6
Alkaline phosphatase	3.1.3.1	10	2.45 ± 0.57
Cytochrome oxidase	1.9.3.1	36	18.9 ± 5.2
Monoamine oxidase	1.4.3.4	46	0.507 ± 0.114
Acid phosphatase	3.1.3.2	31	5.67 ± 1.37
β-Glucuronidase	3.2.1.31	9	1.17 ± 0.14
<i>N</i> -Acetyl-β-glucosaminidase	3.2.1.30	4	6.88 ± 1.89
Catalase	1.11.1.6	30	47.6 ± 11.0
Fumarase	4.2.1.2	5	95.6 ± 9.4
Aldolase	4.1.2.13	10	7.96 ± 1.48
Glutamine synthetase	6.3.1.2	18	8.61 ± 1.90

Absolute values refer to 1 g fresh weight of liver. They are given as means ± standard deviations, in milligrams for protein, RNA, and cholesterol, in units for enzymes, in micromoles of organic phosphorus for phospholipid, in nanomoles for cytochromes. They are the sum of the amounts found in fractions E and N, with the exception of the values for cytochromes *b*₅ and P 450, which are the amounts found in the microsomal fraction.

instance, exclude a possible association with particles other than microsomes. The present work shows that aminopyrine demethylase and glucuronyltransferase are true microsomal constituents, in the sense that they follow the same mode of distribution as glucose 6-phosphatase. Contradictory data have been reported for the subcellular distribution of NADPH cytochrome *c* reductase. According to Phillips and Langdon (38) and to Touster et al. (45) this enzyme is essentially located in microsomes. Present results of this laboratory lead to the same conclusion. Previously, however, de Duve et al. (11), in agreement with the observations made by Hogeboom and Schneider on mouse liver (24), have assigned

a dual localization to NADPH cytochrome *c* reductase, 68% of the particulate activity being associated with mitochondria (11). Another difference between earlier and present data of this laboratory concerns the level of NADPH cytochrome *c* reductase activity per gram liver, which was only 1.6 units vs. 3.98. It could thus be argued that this excess of activity is essentially localized in microsomes, concealing the mitochondrial enzyme. This point of view does not hold out against the partition of NADPH cytochrome *c* reductase activity between mitochondria and microsomes, as it can be computed from our data by equation 1 of de Duve et al. (11). The assumption supporting this computation is that a percent of NADPH

TABLE III
Comparison of the Content of S and S' Fractions in Various Constituents

	Fraction S*	Fraction S'	Difference S-S'
Protein	40 ± 3.1	37.1 ± 1.2 (7)	-2.9‡
Phospholipid	7.6 ± 2.0	8.4 ± 1.6 (2)	+0.8
Ribonucleic acid	23.2 ± 10.0	8.8 (1)	-14.4
Cholesterol	8.6 ± 2.4	5.5 ± 3.0 (5)	-3.1‡
Glucose 6-phosphatase	4.2 ± 2.5	1.5 ± 1.5 (7)	-2.7§
Nucleoside diphosphatase	6.1 ± 2.0	6.0 ± 0.6 (3)	-0.1
Esterase	10.6 ± 3.4	9.3 ± 1.5 (3)	-1.3
Galactosyltransferase	12.8 ± 1.3	6.8 ± 2.7 (3)	-6.0§
NADPH cytochrome <i>c</i> reductase	11.1 ± 4.4	9.0 ± 2.5 (3)	-2.1
NADH cytochrome <i>c</i> reductase	6.5 ± 2.2	1.3 ± 0.2 (7)	-5.2
5'-Nucleotidase	12.0 ± 2.5	9.7 ± 1.5 (5)	-2.3
Alkaline phosphodiesterase I	1.3 ± 0.6	1.7 ± 1.2 (4)	+0.4
Alkaline phosphatase	29.4 ± 4.3	28.9 ± 5.4 (6)	-0.5
Monoamine oxidase	6.3 ± 3.2	1.8 ± 0.6 (7)	-4.5

Results are given as means ± standard deviations, in percent of the sum of the absolute values found in fractions N, ML, P or P', and S or S'. Figures in parentheses refer to numbers of experiments.

* Data of Table I corrected for recovery.

‡ Difference from control significant at $P < 0.05$.

§ Difference from control significant at $P < 0.01$.

|| Difference from control significant at $P < 0.001$.

cytochrome *c* reductase follow exactly the distribution of cytochrome oxidase, taken as a mitochondrial marker, and that *b* percent of NADPH cytochrome *c* reductase follow the distribution of glucose 6-phosphatase, taken as a reference enzyme for microsomal components. Thus, in each fraction:

$$\% \text{ reductase} = \frac{a \times \% \text{ cytochrome oxidase} + b \times \% \text{ glucose 6-phosphatase}}{100}$$

In our case, the computation from fractions ML and P gives $a = 4.1\%$ and $b = 90.1\%$. The assumption seems to fit the data satisfactorily since $a + b$ accounts for 94.2% of the NADPH cytochrome *c* reductase activity. According to this calculation 0.16 and 3.58 units per g liver are associated with mitochondria and microsomes, respectively. The corresponding values found previously (11) were 1.09 and 0.34. Obviously, even when expressed in units per gram liver, much less activity follows the pattern of cytochrome oxidase in the present experiments. We have no simple explanation for the differences reported above. Both groups of experiments used essentially the same assay method, though on different strains of animals. The NADP used in the early experiments

was prepared and reduced in the laboratory. The present data agree well with the results of Phillips and Langdon (38) and of Touster et al. (45) for the absolute value of activity as well as for the distribution.

Since the autoradiographic study of Neutra and Leblond (32) indicating that, after injection of [³H]galactose, synthesis of glycoproteins takes place in the Golgi region of secretory cells, subcellular preparations of Golgi material from rat liver have been shown to exhibit glycosyltransferase activities (18, 30, 39, 48). To our knowledge the distribution pattern of galactosyltransferase has never been established quantitatively in differential centrifugation. It appears from the data presented here that this enzyme sediments with microsomes in a proportion slightly larger than the other microsomal constituents. A noticeable peculiarity of its distribution is the low activity found in the N fraction. The microsomal behavior of galactosyltransferase is not incompatible with the isolation of galactosyltransferase-rich Golgi fractions from low-speed sediments of rat liver homogenates (30), since in the latter procedure the tissue is homogenized under very mild conditions in a medium which maintains the cohesion of the Golgi apparatus. Besides, others have succeeded

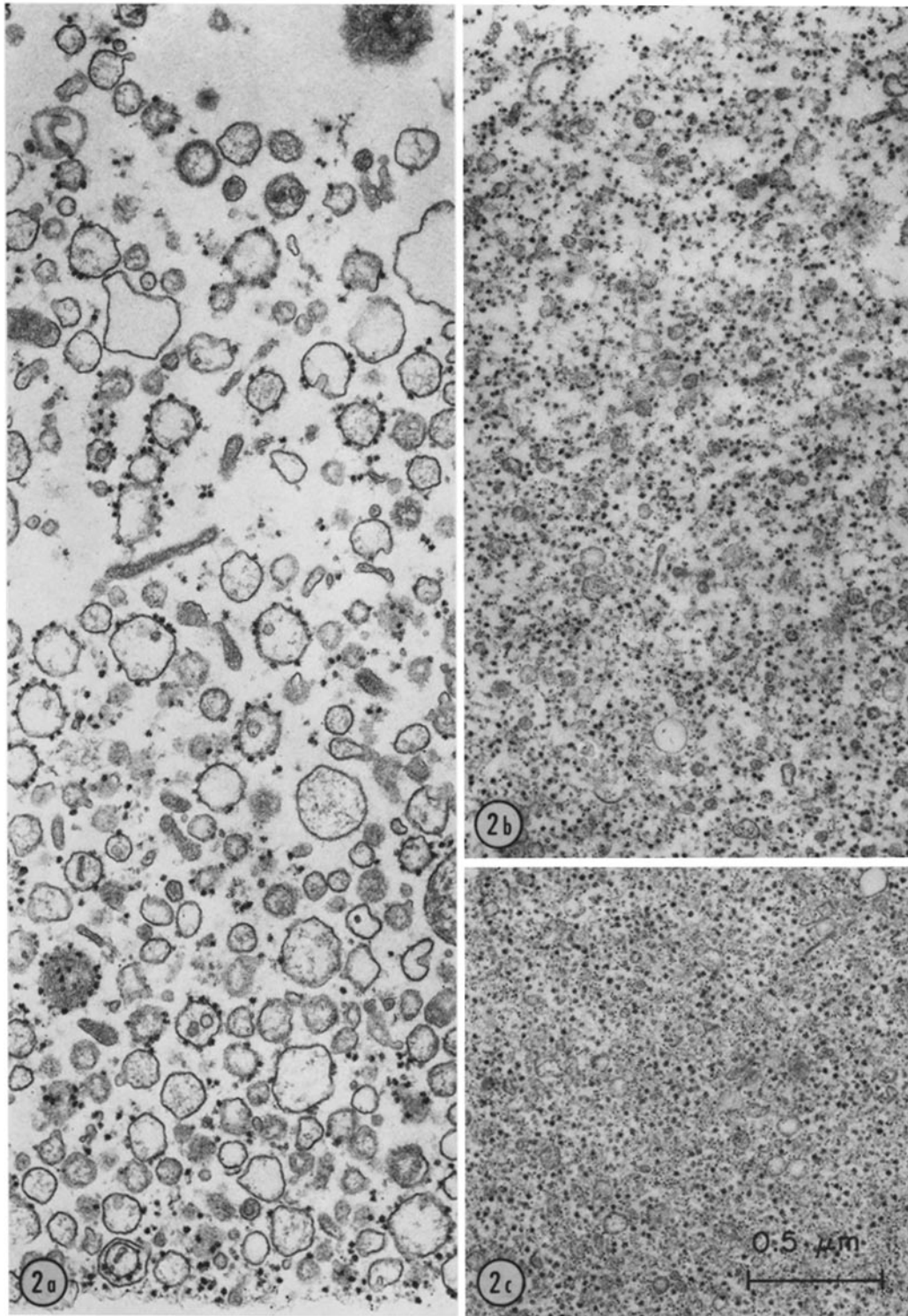


FIGURE 2 Morphology of microsomal (P) and postmicrosomal fractions. Fig. 2 *a* shows the appearance described previously (52) in a P fraction. Note that most ribosomes are attached to vesicles seen in transverse or in polar sections. Figs. 2 *b* and 2 *c* show, at the same magnification, the upper (2 *b*) and the lower (2 *c*) parts of a pellicle obtained from a postmicrosomal fraction. Note small vesicular profiles, ferritin molecules, and ribosomes which appear mainly as free particles.

in obtaining galactosyltransferase-containing Golgi preparations from microsomal fractions (15, 18).

Four constituents: 5'-nucleotidase, alkaline phosphodiesterase I, alkaline phosphatase, and cholesterol exhibit a bimodal distribution characterized by specific activities in both N and P fractions increased over that of the homogenate. Attention has been focused on this peculiar behavior (44). Our results are in good agreement with the distributions observed in other laboratories for alkaline phosphodiesterase (13, 45) and 5'-nucleotidase (12, 45, 51). The four distribution patterns, however, are far from being identical. Alkaline phosphodiesterase has the most typical nucleomicrosomal distribution. The three other distributions come closer to, but do not fit exactly, that of alkaline phosphodiesterase, when the activities recovered in the final supernate are not taken into consideration. These activities, which are truly unsedimentable (Table III), could be due to enzymes released from subcellular particles or to distinct phosphatases, reported to occur in the soluble fraction (31).

Other enzymes exhibit complex patterns of distribution. NADH cytochrome *c* reductase, although largely microsomal, occurs in the ML fraction to an extent that cannot be accounted for by contamination with microsomal elements (11). The reductase measured in the ML fraction represents partly the activity of the system associated with the outer mitochondrial membrane, which in many respects resembles its microsomal homologue (42), and partly that of the electron transport chain of the inner membrane, since no inhibitor was added to the assay mixture. The partition between mitochondria and microsomes, computed by the method applied to NADPH cytochrome *c* reductase, is similar to that found previously: 19.6 and 79.7%, respectively, vs. 24.9 and 72.2% reported by de Duve et al. (11). In terms of units per gram liver our activities are identical in mitochondria and slightly higher in microsomes (79.7 vs. 55.2).

Monoamine oxidase offers the reverse situation. It is mainly recovered in the ML fraction, in which it is carried by mitochondria (1), but some 25% occur in microsomes (*P'* fraction). As for the rotenone-insensitive NADH cytochrome *c* reductase, the submitochondrial localization of monoamine oxidase has been found on the outer membrane (40). It could thus be that microsomal monoamine oxidase is of mitochondrial origin, being either bound to small mitochondria or to frag-

ments of external membrane, detached as a result of the tissue homogenization or of the action of centrifugal pressure upon mitochondria (49, 50). The former possibility is unlikely in view of the low content in cytochrome oxidase of the microsomal fraction. The latter will be discussed in the light of the subfractionation results reported in the subsequent paper (4).

Another puzzling behavior is that exhibited by β -glucuronidase which is recovered in microsomes to a greater extent (29%) than any other lysosomal enzyme. Our data agree with previous findings on the distribution of this enzyme, which has been discussed extensively in numerous papers (see, for instance, 11, 17, 19). Most reports indicate that we are dealing with the same enzyme: β -glucuronidase from the ML fraction and that from microsomes have similar kinetic properties (27) and are decreased to the same extent by mutation in C3H mice (36). However, reports on the electrophoretic mobility of the purified enzyme are controversial. Some authors (47) have not detected any difference, whereas others (29) have observed a faster migration rate for the lysosomal enzyme. The difference may be due only to the mode of purification of the protein. The problem of the association of some β -glucuronidase activity with endoplasmic reticulum (ER) will be considered later on the basis of its distribution pattern in microsomal subfractionation.

Aldolase, glutamine synthetase, and fumarase occur half in particulate fractions, half in the final supernate. The distribution pattern obtained by de Duve et al. (11) for fumarase is somewhat different from ours; more activity was found in microsomes (31% instead of 10%) at the expense of the supernate. It will be shown later that fumarase is easily released from the microsomes into the supernate, as already reported (25). It may be that our homogenization medium, which differs from the one used previously by the absence of EDTA and the addition of imidazole buffer, is responsible for the greater yield in the final supernate.

Composition of the Microsomal Fraction

The content of our microsomal fraction in chemical constituents—protein, phospholipid, and RNA—is compared in Table IV with values given in the literature. The cholesterol content, which in our case amounts to 1.61 mg/g liver (39 μ g/mg

TABLE IV
Chemical Composition of Rat Liver Microsomal Fractions Reported by Various Authors

Reference and Table no.	No. of exps.	Centrifugal field used for pelleting* of		Protein	Phospholipid	RNA	Phospholipid-Protein	RNA-Protein
		large granules	microsomes					
		<i>g-min</i> × 10 ⁻⁶			<i>mg/g of liver</i> ‡			
(37) I	10	0.6	6.3	19.3 ± 1.63	12.18 ± 1.33	3.46 ± 0.31	0.63	0.18
(16) III		0.1	6.3	20.6	12.5	4.23	0.61	0.21
				32.8		2.23		0.07
				26.7	16.0	2.80	0.60	0.10
				28.2	10.8		0.38	
(7) I		0.1	6.3	18.8		3.53		0.19
IX (Mg)				34.2		9.05		0.26
X (Mg)				28.2	7.47	5.41	0.26	0.19
XVI (Cs)				26.6		7.10		0.27
XVII (Cs)				22.2	5.32	4.65	0.24	0.21
XX (Cs Mg)				21.4	4.18	3.30	0.20	0.15
(22) I		0.06	6.3	20.5	9.9	2.5	0.48	0.12
(35) Fig. 7§	6	0.1	6.3	24.1 ± 1.46	6.75 ± 1.27	4.82 ± 0.38	0.28	0.20
(8) I	8	0.48	27.0	18.2 ± 3.84	8.48 ± 1.81	2.12 ± 0.40	0.47	0.12
(21) I		0.1	6.3	18.6	5.10	4.11	0.27	0.22
This work	36	0.25	3.1	41.2 ± 6.2			0.54	0.11
	17				22.4 ± 3.18			
	32					4.62 ± 1.12		

* Usually in 0.25 M sucrose and in Spinco rotor 40 excepted for references 8 and 37: 0.88 M sucrose; and reference 22: 0.35 M sucrose containing 0.01 M MgCl₂, 0.02 M K phosphate buffer pH 7.8, 0.03 M KHCO₃, 0.025 M KCl. In reference 8 sedimentation of large granules was performed in International PRI centrifuge, sedimentation of microsomes in rotor 50 Ti. Reference 21 should be consulted for experimental procedures.

‡ Statistics refer to average ± standard deviation. Data reported for reference 37 have been recalculated in milligrams per gram of liver.

§ 24 h after intraperitoneal injection of 0.9% NaCl.

microsomal protein), was 0.57 in reference 21. Our phospholipid-to-protein ratio falls in the middle range of other microsomal preparations, but our absolute values per gram of wet tissue appear to be two- to threefold higher for these two constituents. This difference is surprising, since in other laboratories the large granules are often separated by lower centrifugal fields and the microsomes by higher fields, than in our laboratory. Our yield should be smaller, not larger.

The higher content in microsomal protein and phospholipid obtained here is due to a better yield and not to a contamination by large granules. True microsomal enzymes (glucose 6-phosphatase, for instance) occur in our microsomes to an extent of more than 70% of their total activities, with specific activities greater than, or at least comparable to, those presented in other papers.

Moreover, the contamination by components of the ML fraction can be evaluated on the basis of the postulate of biochemical homogeneity (10), which assumes that a marker enzyme has the same specific activity throughout a given population of subcellular particles. Contamination by mitochondria, lysosomes, and peroxisomes is indicated by the percentage of total recovered activity of, respectively, cytochrome oxidase (4.6%), acid phosphatase and *N*-acetyl- β -glucosaminidase (17.4 and 9.3%, average 13.4%), and catalase (5.9%). These three populations of granules amount to 20% (26), 0.7% (2), and 2.5% (26) of total liver protein, respectively. From these values it is found that 2.55 mg of microsomal protein per g liver (or only 6.2% of the total microsomal protein) are represented by contaminating mitochondria (2.01 mg/g), lysosomes (0.21 mg/g), and peroxisomes

(0.33 mg/g). Corresponding values cannot be estimated for the other microsomal preparations of Table IV, since the activities of the suitable marker enzymes were not reported. This higher yield obtained in our experiments results from the mode of preparation of microsomes, especially from the stepwise removal of nuclei, gross debris, and large cytoplasmic granules and from the careful washing of the N and ML fractions. It provides more representative microsomal fractions, a point which deserves consideration when the aim is to perform analytical studies, even if the method of preparation is somewhat more tedious.

The amounts of RNA per milligram of microsomal protein recorded in Table IV vary between 0.07 (16) and 0.27 mg (7). Our present value (0.11 mg), which agrees with the one obtained earlier on the basis of a small number of experiments (0.10 mg [52]), is among the lowest reported in the literature. Nevertheless, this RNA content still exceeds slightly the amount that can be attributed to the ribosomes counted on electron micrographs (52). The larger amounts of RNA per milligram protein found in some microsomal preparations, in particular by Swedish authors (7, 35), reflect most probably the fact that they isolate more free ribosomes and less membranes, either rough or smooth, than we do. Their conditions of centrifugation are indeed sufficient to sediment a noticeable proportion of free ribosomes. On the contrary, at least 95% of the ribosomes were found membrane bound at the electron microscope in our microsomes (52). This view is corroborated here by the distribution patterns presented: RNA remains in the S fraction to the extent of 23%; a great deal of that RNA sediments by centrifuging 90 min at 40,000 rpm (Table III), and the pellet thus obtained from the S fraction contains mainly ribosomal material in the form of free particles (Fig. 2). The partition of RNA between nuclei (4–5%), membrane-bound ribosomes (60%), free ribosomes (20%), and non-sedimentable RNA (15%) has been determined (5) after fractionation of rat liver in sucrose solutions containing 50 mM Tris-HCl buffer, pH 7.5, 25 mM KCl, and 5 mM MgCl₂, to preserve the structure of ribosomes and their attachment to ER membranes. Except for a somewhat lower proportion of nonsedimentable RNA (9%) our distribution pattern of this constituent can be satisfactorily interpreted on the basis of the partition between subcellular components established by Blobel and Potter (5). It seems thus that the

fraction of ribosomes associated with subcellular membranes is not lower when rat liver is fractionated in the buffered sucrose used throughout this work.

The authors wish to thank Dr. C. de Duve for substantial improvements suggested for this paper.

This work has been supported by grants from the Belgian Fonds National de la Recherche Scientifique (F.N.R.S.), Fonds de la Recherche Fondamentale Collective and Ministère de la Politique et Programmation Scientifiques. Dr. A. Amar-Costesec was Chargé de Recherches à l'Institut National de la Santé et de la Recherche Médicale, France. Dr. M. Wibo is Chargé de Recherches of the F.N.R.S.

Received for publication 8 August 1973.

REFERENCES

1. BAUDHUIN, P., H. BEAUFAY, Y. RAHMAN-LI, O. Z. SELLINGER, R. WATTIAUX, P. JACQUES, and C. DE DUVE. 1964. Tissue fractionation studies. 17. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat-liver tissue. *Biochem. J.* 92:179.
2. BEAUFAY, H. 1972. Methods for the isolation of lysosomes. In *Lysosomes. A Laboratory Handbook*. J. T. Dingle, editor. North-Holland Publishing Co., Amsterdam. 1.
3. BEAUFAY, H., A. AMAR-COSTESESEC, E. FEYTMANS, D. THINÈS-SEMPOUX, M. WIBO, M. ROBBI, and J. BERTHET. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. I. Biochemical methods. *J. Cell Biol.* 61:188.
4. BEAUFAY, H., A. AMAR-COSTESESEC, D. THINÈS-SEMPOUX, M. WIBO, M. ROBBI, and J. BERTHET. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. III. Subfractionation of the microsomal fraction by isopycnic and differential centrifugation in density gradients. *J. Cell Biol.* 61:213.
5. BLOBEL, G., and V. R. POTTER. 1967. Studies on free and membrane-bound ribosomes in rat liver. I. Distribution as related to total cellular RNA. *J. Mol. Biol.* 26:279.
6. BRIGHTWELL, R., and A. L. TAPPEL. 1968. Subcellular distributions and properties of rat liver phosphodiesterases. *Arch. Biochem. Biophys.* 124:325.
7. DALLNER, G. 1963. Studies on the structural and enzymatic organization of the membranous elements of liver microsomes. *Acta Pathol. Microbiol. Scand. Suppl.* 166:1.

8. DECLOITRE, F., and J. CHAUVEAU. 1968. Fractionnement des microsomes de foie de rat. I. Caractères biochimiques et activités enzymatiques des subfractions obtenues sur gradient de densité. *Bull. Soc. Chim. Biol.* 50:491.
9. DE DUVE, C., H. BEAUFAY, P. JACQUES, Y. RAHMAN-LI, O. Z. SELLINGER, R. WATTIAUX, and S. DE CONINCK. 1960. Intracellular localization of catalase and of some oxidases in rat liver. *Biochim. Biophys. Acta.* 40:186.
10. DE DUVE, C., and J. BERTHET. 1954. The use of differential centrifugation in the study of tissue enzymes. *Int. Rev. Cytol.* 3:225.
11. DE DUVE, C., B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX, and F. APPELMANS. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* 60:604.
12. DE LAMIRANDE, G., C. ALLARD, and A. CANTERO. 1958. Intracellular distribution of 5'-nucleotidase in rat liver. *J. Biophys. Biochem. Cytol.* 4:373.
13. DE LAMIRANDE, G., S. BOILEAU, and R. MORAIS. 1966. Distribution of the nucleases of the microsomal fraction of rat liver between ribosomes and endoplasmic membranes. *Can. J. Biochem.* 44:273.
14. DUTTON, G. J., and I. D. E. STOREY. 1954. Uridine compounds in glucuronic acid metabolism. 1. The formation of glucuronides in liver suspensions. *Biochem. J.* 57:275.
15. EHRENREICH, J. H., J. J. M. BERGERON, and G. E. PALADE. 1970. A new procedure for the isolation of a Golgi fraction from rat liver. *J. Cell Biol.* 47(2, Pt. 2):55 a. (Abstr.).
16. ERNSTER, L., P. SIEKEVITZ, and G. E. PALADE. 1962. Enzyme-structure relationships in the endoplasmic reticulum of rat liver. A morphological and biochemical study. *J. Cell Biol.* 15:541.
17. FISHMAN, W. M., S. S. GOLDMAN, and R. DE LELLIS. 1967. Dual localization of β -glucuronidase in endoplasmic reticulum and in lysosomes. *Nature (Lond.)*. 213:457.
18. FLEISCHER, B., and S. FLEISCHER. 1970. Preparation and characterization of Golgi membranes from rat liver. *Biochim. Biophys. Acta.* 219:301.
19. GIANETTO, R. 1964. The intracellular distribution of rat liver β -glucuronidase. *Can. J. Biochem.* 42:499.
20. GIANETTO, R., and C. DE DUVE. 1955. Tissue fractionation studies. 4. Comparative study of the binding of acid phosphatase, β -glucuronidase and cathepsin by rat-liver particles. *Biochem. J.* 59:433.
21. GLAUMANN, H., and G. DALLNER. 1968. Lipid composition and turnover of rough and smooth microsomal membranes in rat liver. *J. Lipid Res.* 9:720.
22. HALLINAN, T., and H. N. MUNRO. 1965. A rapid method for preparing granular and agranular endoplasmic reticulum and free ribosomes from rat liver. *Q. J. Exp. Physiol. Cogn. Med. Sci.* 50:93.
23. HERS, H. G., J. BERTHET, L. BERTHET, and C. DE DUVE. 1951. Le système hexose-phosphatase. III. Localisation intracellulaire des ferments par centrifugation fractionnée. *Bull. Soc. Chim. Biol.* 33:21.
24. HOGEBOOM, G. H., and W. C. SCHNEIDER. 1950. Cytochemical studies of mammalian tissues. III. Isocitric dehydrogenase and triphosphopyridine nucleotide-cytochrome *c* reductase of mouse liver. *J. Biol. Chem.* 186:417.
25. KUFF, E. L. 1954. The distribution of fumarase activity in mouse liver homogenates. *J. Biol. Chem.* 207:361.
26. LEIGHTON, F., B. POOLE, H. BEAUFAY, P. BAUDHUIN, J. W. COFFEY, S. FOWLER, and C. DE DUVE. 1968. The large-scale separation of peroxisomes, mitochondria, and lysosomes from the livers of rats injected with Triton WR-1339. Improved isolation procedures, automated analysis, biochemical, and morphological properties of fractions. *J. Cell Biol.* 37:482.
27. LUCIER, G. W., and O. S. MACDANIEL. 1972. Alterations in rat liver microsomal and lysosomal β -glucuronidase by compounds which induce hepatic drug-metabolizing enzymes. *Biochim. Biophys. Acta.* 261:168.
28. LUDEWIG, S., and A. CHANUTIN. 1950. Distribution of enzymes in the livers of control and X-irradiated rats. *Arch. Biochem. Biophys.* 29:441.
29. MAMELI, L., M. POTIER, and R. GIANETTO. 1972. Difference in electrophoretic mobility between the lysosomal and the microsomal β -glucuronidase of rat liver. *Biochem. Biophys. Res. Commun.* 46:560.
30. MORRE, D. J., L. M. MERLIN, and T. W. KEENAN. 1969. Localization of glycosyl transferase activities in a Golgi apparatus-rich fraction isolated from rat liver. *Biochem. Biophys. Res. Commun.* 37:813.
31. NEIL, M. W., and M. W. HORNER. 1964. Studies on acid hydrolases in adult and foetal tissues. Acid *p*-nitrophenyl phosphate phosphohydrolases of adult guinea-pig liver. *Biochem. J.* 92:217.
32. NEUTRA, M., and C. P. LEBLOND. 1966. Radioautographic comparison of the uptake of galactose- H^3 and glucose- H^3 in the Golgi region of various cells secreting glycoproteins or mucopolysaccharides. *J. Cell Biol.* 30:137.

33. OMACHI, A., C. P. BARNUM, and D. GLICK. 1948. Quantitative distribution of an esterase among cytoplasmic components of mouse liver cells. *Proc. Soc. Exp. Biol. Med.* 67:133.
34. ORRENIUS, S. 1965. On the mechanism of drug hydroxylation in rat liver microsomes. *J. Cell Biol.* 26:713.
35. ORRENIUS, S., J. L. E. ERICSSON, and L. ERNSTER. 1965. Phenobarbital-induced synthesis of the microsomal drug-metabolizing enzyme system and its relationship to the proliferation of endoplasmic membranes. *J. Cell Biol.* 25:627.
36. PAIGEN, K. 1961. The effect of mutation on the intracellular location of β -glucuronidase. *Exp. Cell Res.* 25:286.
37. PALADE, G. E., and P. SIEKEVITZ. 1956. Liver microsomes. An integrated morphological and biochemical study. *J. Biophys. Biochem. Cytol.* 2:171.
38. PHILLIPS, A. H., and R. G. LANGDON. 1963. Hepatic triphosphopyridine nucleotide-cytochrome *c* reductase: isolation, characterization, and kinetic studies. *J. Biol. Chem.* 237:2652.
39. SCHACHTER, H., I. JABBAL, R. L. HUDGIN, L. PINTERIC, E. J. MCGUIRE, and S. ROSEMAN. 1970. Intracellular localization of liver sugar nucleotide glycoprotein glycosyltransferases in a Golgi-rich fraction. *J. Biol. Chem.* 245:1090.
40. SCHNAITMAN, C., V. G. ERWIN, and J. W. GREENAWALT. 1967. The submitochondrial localization of monoamine oxidase. An enzymatic marker for the outer membrane of rat liver mitochondria. *J. Cell Biol.* 32:719.
41. SHIBKO, S., and A. L. TAPPEL. 1964. Distribution of esterases in rat liver. *Arch. Biochem. Biophys.* 106:259.
42. SOTTOCASA, G. L., B. KUYLENSTIERNA, L. ERNSTER, and A. BERGSTRAND. 1967. An electron-transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study. *J. Cell Biol.* 32:415.
43. STROMINGER, J. L., H. M. KALCKAR, J. AXELROD, and E. S. MAXWELL. 1954. Enzymatic oxidation of uridine diphosphate glucose to uridine diphosphate glucuronic acid. *J. Am. Chem. Soc.* 76:6411.
44. THINES-SEMPOUX, D., A. AMAR-COSTESECC, H. BEAUFAY, and J. BERTHET. 1969. The association of cholesterol, 5'-nucleotidase, and alkaline phosphodiesterase I with a distinct group of microsomal particles. *J. Cell Biol.* 43:189.
45. TOUSTER, O., N. N. ARONSON, J. T. DULANEY, and H. HENDRICKSON. 1970. Isolation of rat liver plasma membranes. Use of nucleotide pyrophosphatase and phosphodiesterase I as marker enzymes. *J. Cell Biol.* 47:604.
46. UNDERHAY, E., S. J. HOLT, H. BEAUFAY, and C. DE DUVE. 1956. Intracellular localization of esterase in rat liver. *J. Biophys. Biochem. Cytol.* 2:635.
47. VAN LANCKER, J. L., and P. L. LENTZ. 1970. Study on the site of biosynthesis of β -glucuronidase and its appearance in lysosome in normal and hypoxic rats. *J. Histochem. Cytochem.* 18:529.
48. WAGNER, R. R., and M. A. CYNKIN. 1969. Enzymatic transfer of ^{14}C -glucosamine from UDP-*N*-acetyl- ^{14}C -glucosamine to endogenous acceptors in a Golgi apparatus-rich fraction from liver. *Biochem. Biophys. Res. Commun.* 35:139.
49. WATTIAUX, R., and S. WATTIAUX-DE CONINCK. 1970. Distribution of mitochondrial enzymes after isopycnic centrifugation of a rat liver mitochondrial fraction in a sucrose gradient: influence of the speed of centrifugation. *Biochem. Biophys. Res. Commun.* 40:1185.
50. WATTIAUX, R., S. WATTIAUX-DE CONINCK, and M. F. RONVEAUX-DUPAL. 1971. Deterioration of rat-liver mitochondria during centrifugation in a sucrose gradient. *Eur. J. Biochem.* 22:31.
51. WATTIAUX-DE CONINCK, S., and R. WATTIAUX. 1969. Nucleosidediphosphatase activity in plasma membrane of rat liver. *Biochim. Biophys. Acta.* 183:118.
52. WIBO, M., A. AMAR-COSTESECC, J. BERTHET, and H. BEAUFAY. 1971. Electron microscope examination of subcellular fractions. III. Quantitative analysis of the microsomal fraction isolated from rat liver. *J. Cell Biol.* 51:52.