ULTRASTRUCTURAL LOCALIZATION OF CYTOCHROME $b_5$
ON RAT LIVER MICROSOMES BY MEANS OF
HYBRID ANTIBODIES LABELED WITH FERRITIN

JOSÉ REMACLE, STANLEY FOWLER, HENRI BEAUFAY, and JACQUES BERTHET. From Laboratoire de Chimie Physiologique, Université de Louvain, Louvain, Belgium

INTRODUCTION
Subfractionation by density equilibration and by differential sedimentation in density gradients has resolved rat liver microsomes into several populations of subcellular components characterized by specific marker enzymes (1, 7). Vesicles derived from endoplasmic reticulum (ER) were found to be heterogeneous, both in their physical characteristics and in their enzyme content. On the basis of these results, ER enzymes have been classified into two groups. Group b includes cytochromes $b_5$ and P 450, and other oxidoreductases related to these hemoproteins; group c includes glucose 6-phosphatase, several other microsomal hydrolases, and glucuronyltransferase. With respect to enzymes of group b, those of group c sediment faster and equilibrate at higher densities in various gradients (1, 7). Furthermore, their equilibrium density is reduced more markedly by treatments which detach ribosomes from microsomal vesicles (3). Since these differences never allowed a true separation, two alternative explanations have been envisaged. Either the two groups are associated together in the same membranes, but in such a manner that the ratio of group c to group b enzymes increases with increasing ribosome load; or each group is associated with a different part of the ER, that containing group c being on an average richer in ribosomes than that containing group b. We report here the results of a cytoimmunological study showing that cytochrome $b_5$ (Group b) is present in essentially all microsomal vesicles derived from ER. Together with the cytoenzymological data showing the widespread distribution of glucose 6-phosphatase (group c) throughout the ER (8), these results support the existence of a single ER system in hepatocytes.

MATERIALS AND METHODS
Highly purified cytochrome $b_5$ was prepared from rat liver as described by Omura et al. (10). Analysis
by immuno-diffusion showed the preparations to be immunologically pure. Ferritin (purchased from Fluka AG, Basel, Switzerland), was reerystallized six times in the presence of CdSO4 (4). Rabbit antibodies were purified by affinity chromatography on cytochrome b$_5$ or ferritin, coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden) by means of cyanogen bromide (5), and were eluted by 2% formic acid. Hybrid molecules anticytochrome b$_5$/anti-ferritin were made from purified antibodies by the method of Nisonoff and Palmer (9), with minor modifications (11). The hybrid antibodies were isolated by successive immunoadsorbent chromatography on Sepharose-cytochrome b$_5$ and Sepharose-ferritin. An antigen-antibody complex between ferritin and ab$_5$/aF hybrid was prepared by slowly adding a dilute solution of ab$_5$/aF hybrid to a rapidly mixing solution of ferritin at neutral pH. The ferritin was in a fivefold molar excess over the amount of hybrid antibody added. Excess ferritin (about 60 mg) was separated from the ab$_5$/aF-ferritin complex (containing 10 mg of antibody protein) by adsorption of the complex onto a 2.5 cm X 8.5 cm column of SP-Sephadex equilibrated with 0.05 M acetate, pH 4.75. After a careful washing out of unreacted ferritin, the ab$_5$/aF-ferritin complex was eluted from the column with 0.1 M Tris-HCl, pH 7.4. Aggregates were eliminated by centrifugation (15 min at 40,000 rpm).

Microsomes (fraction P) were prepared from rat liver (2) and subfractionated by density equilibration in a sucrose gradient (7). Unfractionated microsomes (P), a light subfraction (P$_L$; density 1.123-1.140), and a heavy subfraction (P$_H$; density 1.245-1.267) were first treated with 25 mM Na-pyrophosphate, pH 7.4, in 0.25 M sucrose for detachment of the ribosomes (12), separated by chromatography on Biogel A-150m (Bio-Rad Laboratories, Richmond, Calif.), and then incubated with ab$_5$/aF-ferritin for 12 h at 2°C. The hybrid reagent was adjusted to provide a sixfold excess of cytochrome b$_5$ binding activity over the amount of membrane-bound cytochrome b$_5$ present in the fraction. After incubation the excess hybrid antibodies were removed by chromatography on Biogel A-150m. Controls were handled identically, but with an ab$_5$/aF-ferritin complex precubated with a 12-fold excess of purified cytochrome b$_5$. Electron microscopy was performed as described by Wibo et al. (13). Enzyme assays were done according to published methods, with minor modifications (6).

**RESULTS**

Some biochemical properties of the subcellular preparations submitted to morphological examination after reaction with the ab$_5$/aF-ferritin are given in Table I. The enzymatic heterogeneity of microsomes is evidenced by the 4.7-fold higher ratio of glucose 6-phosphatase to NADH cytochrome c reductase in P$_2$ with respect to P$_1$ fraction. The activity of alkaline phosphodiesterase I, galactosyltransferase, and monoamine oxidase indicates that P$_1$ fraction consists essentially of elements derived from ER, whereas ER elements are markedly contaminated by other structures in P and P$_1$ fractions.

The morphological aspect of these preparations after incubation with the ab$_5$/aF-ferritin is shown in Fig. 1 a-c, that of one control (P$_1$ fraction) in Fig. 1 d. The latter is also representative of the controls made on P and P$_1$ fractions. In similar experiments not presented here, labeling in the controls was the same after incubation with aF/aF-ferritin complexes as after incubation with ab$_5$/aF-ferritin complexes pretreated with cytochrome b$_5$. The outer surface of many profiles is dotted with ferritin grains in all tests. On a quantitative basis, 96, 81, and 73% of the profiles were found specifically labeled in P$_2$, P, and P$_1$ fractions, respectively. Unlabeled profiles were usually of smaller size than the labeled ones in P$_1$ fraction; the reverse relationship between size and labeling occurred in P and P$_1$ fractions. Fractions P and P$_1$ contained some open membranes and these did not react with the ab$_5$/aF-ferritin. Some of the negative structures were identified as deriving from the Golgi or from the plasma membrane.
FIGURE 1. Electron micrographs of microsomes (a) and microsomal subfractions P₁ (b) and P₂ (c) incubated with the α₅/α₁F-ferritin complex after detachment of ribosomes by Na-pyrophosphate, and (d) control of the P₂ subfraction. Many profiles are ferritin-free in the control. In contrast, most profiles are labeled with ferritin grains in the three test preparations, except for some large profiles and one open membrane fragment (arrows). × 47,000.
DISCUSSION

The occurrence of ferritin grains on almost all profiles in the P2 microsomal subfraction shows that cytochrome b₄ is present in all the ER elements equilibrating between 1.245 and 1.267 in sucrose gradients. It is likely indeed that the few unlabeled profiles (4% of the total) derive from vesicles labeled in the neighboring sections. The smaller size of the unlabeled profiles is consistent with this interpretation. In view of the observations made on the P and P1 fractions, we may extend this conclusion to the whole ER, since the proportion of unlabeled vesicles in these fractions is readily accounted for by the presence of non-ER contaminants. Detailed quantitative calculations supporting this conclusion will be presented in a subsequent publication.

The authors wish to thank Dr. C. de Duve for many helpful discussions and his help in preparing the manuscript. The contribution of Dr. A. Amar-Costesc in the biochemical analyses and of Dr. M. Wibo in the morphological examinations is gratefully acknowledged. The authors are also indebted to Dr. A. Trouet for valuable suggestions during this work.

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. J. Remacle is Aspirant of the F.N.R.S. Dr. S. Fowler was supported by the Helen Hay Whitney Foundation.

Received for publication 13 August 1973, and in revised form 8 November 1973.

REFERENCES

1. AMAR-COSTESEC, A., H. BEAUFAY, E. FEYTMANS, D. THINES-SEMPoux, and J. BERTHET. 1969. Subfractionation of rat liver microsomes. In Microsomes and Drug Oxidations. J. R. Gillette, A. G. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts, and G. J. Manninger, editors. Academic Press Inc., New York. 41.

2. AMAR-COSTESEC, A., H. BEAUFAY, J. BERTHET, D. THINES-SEMPoux, M. Wibo, E. FEYTMANS, and M. ROBB. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparations and composition of the microsomal fraction. J. Cell Biol. 61:209.

3. AMAR-COSTESEC, A. 1973. Analytical study of rat liver microsomes treated by EDTA or pyrophosphate. Arch. Int. Physiol. Biochim. 81: 358.

4. ANDRES, G. A., K. C. HSU, and B. C. SEEGAL. 1967. Immunoferitin technique for the identification of antigens by electron microscopy. In Handbook of Experimental Immunology. D. M. Weir, editor. Blackwell Scientific Publications Ltd., Oxford, England. 527.

5. AXEN, R., J. PORATH, and S. ERNBÄCK. 1967. Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. Nature (Lond.). 214:1302.

6. BEAUFAY, H., A. AMAR-COSTESEC, E. FEYTMANS, D. THINES-SEMPoux, M. Wibo, M. ROBB, and J. BERTHET. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. I. Biochemical methods. J. Cell Biol. 61:188.

7. BEAUFAY, H., A. AMAR-COSTESEC, D. THINES-SEMPoux, M. Wibo, M. ROBB, and J. BERTHET. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. III. Subfractionation of the microsomal fraction by isotopic and differential centrifugation in density gradients. J. Cell Biol. 61:213.

8. LESKES, A., P. SIEKEVITZ, and G. E. PALADE. 1971. Differentiation of endoplasmic reticulum in hepatocytes. I. Glucose 6-phosphatase distribution in situ. J. Cell Biol. 49:264.

9. NISONOFF, A., and J. L. PALMER. 1964. Hybridization of half molecules of rabbit gammaglobulin. Science (Wash. D. C.). 143:376.

10. OMURA, T., P. SIEKEVITZ, and G. E. PALADE. 1967. Turnover of constituents of the endoplasmic reticulum membranes of rat hepatocytes. J. Biol. Chem. 242:2389.

11. REMACLE, J., S. FOWLER, and A. TROUET. 1973. Préparation et purification d'anticorps hybrides anti-ferritine, anti-cytochrome b₄. Arch. Int. Physiol. Biochim. 81:197.

12. SACHS, H. 1958. The effect of pyrophosphate on the amino acid incorporating system of rat liver microsomes. J. Biol. Chem. 233:650.

13. Wibo, M., A. AMAR-COSTESEC, J. BERTHET, and H. BEAUFAY. 1971. Electron microscope examination of subcellular fractions. III. Quantitative analysis of the microsomal fractions isolated from rat liver. J. Cell Biol. 51:52.