

BIOGENESIS OF ENDOPLASMIC RETICULUM MEMBRANES

II. Synthesis of Constitutive Microsomal Enzymes in Developing Rat Hepatocyte

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ABSTRACT

The constitutive enzymes of microsomal membranes were investigated during a period of rapid ER development (from 3 days before to 8 days after birth) in rat hepatocytes. The activities studied (electron transport enzymes and phosphatases) appear at different times and increase at different rates. The increase in the enzyme activities tested was inhibited by Actinomycin D and puromycin. G-6-Pase and NADPH-cytochrome *c* reductase activities appeared first in the rough microsomes, and subsequently in smooth microsomes, eventually reaching a uniform concentration as in adult liver. The evidence suggests that the enzymes are synthesized in the rough part, then transferred to the smooth part, of the ER. Changes in the fat supplement of the maternal diet brought about changes in the fatty acid composition of microsomal phospholipids but did not influence the enzymic pattern of the suckling. Microsomes from 8-day-old and adult rats lose 95% of PLP and 80% of NADH-cytochrome *c* reductase activity after acetone-H₂O (10:1) extraction. However, one-half the original activity could be regained by adding back phospholipid micelles prepared from purified phospholipid, or from lipid extracts of heart mitochondria, or of liver microsomes of 8-day or adult rats, thus demonstrating an activation of the enzyme by nonspecific phospholipid. The results suggest that during development the enzymic pattern is not influenced by the fatty acid or phospholipid composition of ER membranes.

INTRODUCTION

In the preceding paper (1), we have described the rapid development of the ER¹ that occurs in rat

¹ Abbreviations used are: NAD and NADH, oxidized and reduced nicotinamide adenine dinucleotide; NADP and NADPH, oxidized and reduced nicotinamide adenine dinucleotide phosphate; ER, endoplasmic reticulum; DOC, deoxycholate; NT, neotetrazolium chloride; PCMB, *p*-chloromercuribenzoate; IDP, inosine diphosphate; ATP, adenosine triphosphate; G-6-Pase, glucose-6-phosphatase; PLP, phospholipid; FA, fatty acid; Tris, Tris(hydroxymethyl)aminomethane.

hepatocytes immediately before and after birth. In the fetus, the newly formed membranes are primarily of the rough type, while in the newborn the production of smooth membranes predominates. During this period, there are no changes in the phospholipid composition of microsomal membranes, but large changes in the fatty acid composition of phospholipids do occur.

The aim of this part of the investigation was to look into the production of membrane proteins, primarily constitutive microsomal enzymes, during the pre- and postnatal periods. The results

obtained indicate that these enzyme activities appear at different time points and increase at different rates. Experiments carried out with protein synthesis inhibitors and with various phosphatides, used to restore activity of lipid-extracted membranes, show that the enzymic activities emerging during differentiation are due to the synthesis of new enzymes, rather than the activation of preexisting, potentially enzymatic proteins. A brief report of this work has already appeared (2).

MATERIALS AND METHODS

Chemicals

All chemicals employed were standard commercial products. Neotetrazolium chloride and puromycin dihydrochloride were bought from Nutritional Biochemical Corp., Cleveland; Actinomycin D was obtained from Merck and Co., Rahway, New Jersey, and aolectin (purified soybean phosphatides) from Associated Concentrates, New York.

A sterile stock solution of Actinomycin D was prepared by dissolving 1 mg in 0.25 ml of 95% ethanol, and subsequently adding 0.75 ml of 0.1 M Tris¹ buffer, pH 7.5. This stock solution was stored in the refrigerator and diluted with 0.1 M Tris buffer, pH 7.5, under sterile conditions before use.

Puromycin dihydrochloride was dissolved in 1.8% NaCl. The pH was adjusted to 7 with NaOH and the solution diluted with H₂O, under sterile conditions, to a final NaCl concentration of 0.9%.

Fat Diets

In the fat diet experiments, pregnant rats (CFN strain) were fed ad libitum from the 7th day of pregnancy on, with one of the following diets:

- (a) Basic diet, which was Lab Chow (Ralston Purina Co., St. Louis). This diet contained 3.8% fat and 23.4% protein.
- (b) Basic diet supplemented with 20% corn oil.
- (c) Basic diet supplemented with 20% lard.

The mothers were kept on these diets after delivery until the end of the experiments.

General Procedures

Details concerning fractionation procedures, electron microscope techniques and various chemical analyses are given in (1). Protein was determined according to Lowry et al. (3), with bovine serum albumin as standard.

Enzyme Assays

DIAPHORASES: The assay system contained 0.1 mM NADH or NADPH, 6.6×10^{-4} M K₃Fe(CN)₆,

0.33 mM KCN, and 0.05 M potassium phosphate, pH 7.5, in a final volume of 3 ml. Oxidation of the reduced pyridine nucleotides was followed at 340 m μ in a Beckman spectrophotometer with a Gilford automatic positioner and recorder attachment. The extinction coefficient (mole/liter/cm) used was: 6.22×10^3 at 340 m μ for NADH or NADPH (4).

CYTOCHROME C REDUCTASES: The assay system contained 0.1 mM NADH or NADPH, 0.33 mM KCN, 0.05 mM cytochrome *c*, and 0.05 M potassium phosphate, pH 7.5, in a final volume of 3 ml (5). The reduction of cytochrome *c* was followed at 550 m μ . The extinction coefficient (mole/liter/cm) of 18.5×10^3 at 550 m μ for reduced minus oxidized cytochrome *c* (6) was used in the calculations.

NEOTETRAZOLIUM REDUCTASES: In a final volume of 1.5 ml, the assay system contained 0.8 mM NADH or NADPH, 0.75 mM NT, and 0.05 M potassium phosphate buffer, pH 7.5. After incubation at 37° for 10 min, the reaction was stopped by the addition of 1.5 ml of a Triton-formalin solution, pH 3.5 (7), which cleared the medium and made extraction of the Formazan unnecessary. The Formazan intensity was measured at 505 m μ ; NT reduced by ascorbic acid was used as a standard.

OXIDATIVE DEMETHYLATION: The incubation system contained 5 mM aminopyrine, 50 mM nicotinamide, 5 mM MgCl₂, 0.05 M Tris-buffer, pH 7.5, 1 mM NADP, and an NADPH-generating system consisting of 5 mM DL-isocitrate, 0.05 mM MnCl₂, and enough isocitric dehydrogenase to reduce 0.32 μ moles of NADP per min, all in a final volume of 2 ml (8). The microsomes to be assayed were resuspended in 0.15 M KCl rather than in 0.25 M sucrose. Incubation time was 20 min at 37°. The amount of formaldehyde formed was measured by the Nash reaction (9).

CYTOCHROME B₅: Since, in our case, removal of blood by perfusion of the liver was not possible, adsorbed hemoglobin, which interferes with the measurement of microsomal cytochromes, was removed by twice resuspending the microsomes in 0.3 M tris buffer, pH 7.9, and subsequently sedimenting them at 105,000 *g* for 60 min. The difference spectrum of the resulting microsomes was measured in a Cary model 14 MR recording spectrophotometer. The two cuvettes contained identical fractions in 0.05 M phosphate buffer, pH 7.5, in a final volume of 3 ml. Reduction was achieved by adding a few mg of Na₂S₂O₄ to one of the cuvettes. For the arbitrary estimates of the cytochrome *b*₅ content, the difference between the maximum and minimum, 424 and 410 m μ , was used (10).

CO-BINDING PIGMENT (Cyt. P-450): The pigment was measured in the same system and on the same samples as cytochrome *b*₅. After determination of the latter, the second cuvette was likewise reduced by the addition of Na₂S₂O₄. Carbon monoxide was

then passed into one of the cuvettes for 3 min. The difference between the 450-m μ maximum and the 490-m μ minimum was taken as an arbitrary estimate of the CO-binding pigment (11).

PHOSPHATASES: The assay system in a final volume of 1 ml contained 0.02 M glucose-6-phosphate and 0.1 M Tris-maleate buffer, pH 6.6, for glucose-6-phosphatase (12); 0.01 M ATP, 0.01 M MgCl₂, and 0.05 M Tris buffer, pH 7.5, for nucleoside triphosphatase (13); 0.01 M IDP, 0.01 M MgCl₂, 0.1% NaDOC and 0.05 M Tris buffer, pH 7.5, for nucleoside diphosphatase (14). After incubation for 20 min at 30°, the reaction was stopped by the addition of 1 ml of 1 N perchloric acid, and the inorganic orthophosphate liberated was determined by the Fiske-Subbarow method (15).

Extraction of Microsomal Lipids

Extraction of microsomes with acetone-water was performed as described by Lester and Fleischer (16) for mitochondria. 8 ml of microsome suspension (1 g tissue equivalent/ml in 0.15 M KCl-0.01 M Tris buffer, pH 7.5) were added dropwise to 392 ml of acetone-H₂O mixture (360 ml acetone + 32 ml H₂O). The final mixture was allowed to stand for 10 min, with occasional swirling, and then transferred into 4 large tubes (100 ml) and centrifuged in an International Centrifuge at 2,000 RPM for 6 min. The supernates were carefully and completely poured off, the tubes drained, the residues homogenized in 44 ml of KCl-Tris and recentrifuged at 105,000 *g* for 15 min in 4 centrifuge tubes (No. 40 rotor, Spinco, model L centrifuge). The pellets were rehomogenized in KCl-Tris, and the washing procedure was repeated. The final pellet was resuspended by homogenization in 4 ml of KCl-Tris (2 g tissue equivalent/ml). The whole procedure was performed in the cold room at ~4°.

Preparation of Lipid Micelles

Asolectin micelles were prepared by 3 successive sonication runs, each of 5 min, in 0.02 M Tris buffer, pH 7.5, (40 mg/ml) under cooling. Micelles of microsomal lipids were prepared by taking pooled pellets of microsomes, isolated from either 8-day-old or adult rats, and homogenizing them in twenty times their volume of chloroform:methanol (2:1). The homogenate was passed through a fine grade sintered glass filter and the filtrate evaporated to dryness in a flash evaporator. The residue was dissolved in butanol-DOC and dialyzed as described by Fleischer and Klouwen (17). The dialyzed solutions (about 10 mg phospholipid micelles per ml) were clarified by high speed centrifugation (105,000 *g*, 30 min), and stored in a dark bottle under nitrogen at ~4°. Micelles of mitochondrial lipids and purified beef heart phosphatides were kindly supplied by Dr.

S. Fleischer, Department of Biochemistry, Vanderbilt University, Nashville, Tennessee.

RESULTS

A. Pattern of Enzymic Differentiation

1. ELECTRON TRANSPORT ENZYMES

The specific activities of electron transport enzymes in hepatic microsomes of fetal rats are very low by comparison with the corresponding values in the adult (Fig. 1). NADPH-cytochrome *c* reductase and NADPH-diaphorase (measured with ferricyanide as electron acceptor) are present already in the fetus, but the major increase in the activities of both enzymes occurs just after birth (see also Table I and Fig. 5), reaching the adult level at +1 day.² This is not the case with the NADPH-NT reductase activity, which does increase after birth but does not exceed 50% of the adult level even at +8 days. The activities of the NADH-cytochrome *c* and NADH-NT reductases increase slowly and reach only about 25% of the adult value at +8 days. On the other hand, NADH-diaphorase activity, assayed with ferricyanide as electron acceptor, rises to the adult level at a higher rate than those of the NADH-cytochrome *c* and the NADH-NT reductases.³ It is difficult to measure the two microsomal cytochromes, *b*₅ and P-450, before +1 day: the heme concentrations are low and the high concentrations of microsomes needed interfere with accurate difference spectroscopy. But after birth the concentration of cytochromes, while low, is measurable; it increases to 75 to 80% of the adult level at +8 days. The oxidative demethylation activity, using aminopyrine as substrate, is very low in the fetus and increases slowly, reaching only 30% of the adult value at +8 days.

The NADPH-dependent enzymic lipid peroxidation (20) could not be assayed by using

² The days before birth are designated from -3 to -1, and after birth from +1 to +8.

³ The literature is in agreement concerning the presence of a NADPH-cytochrome *c* reductase in microsomes, but the evidence concerning the microsomal NADH-NT reductase is contradictory (see 18, 19). This discrepancy, in our experience, is caused by impurities in some commercial NT's used. Certain NT's¹, tested in our system, inhibit the reaction completely in the presence of NADH but only partially with NADPH present.

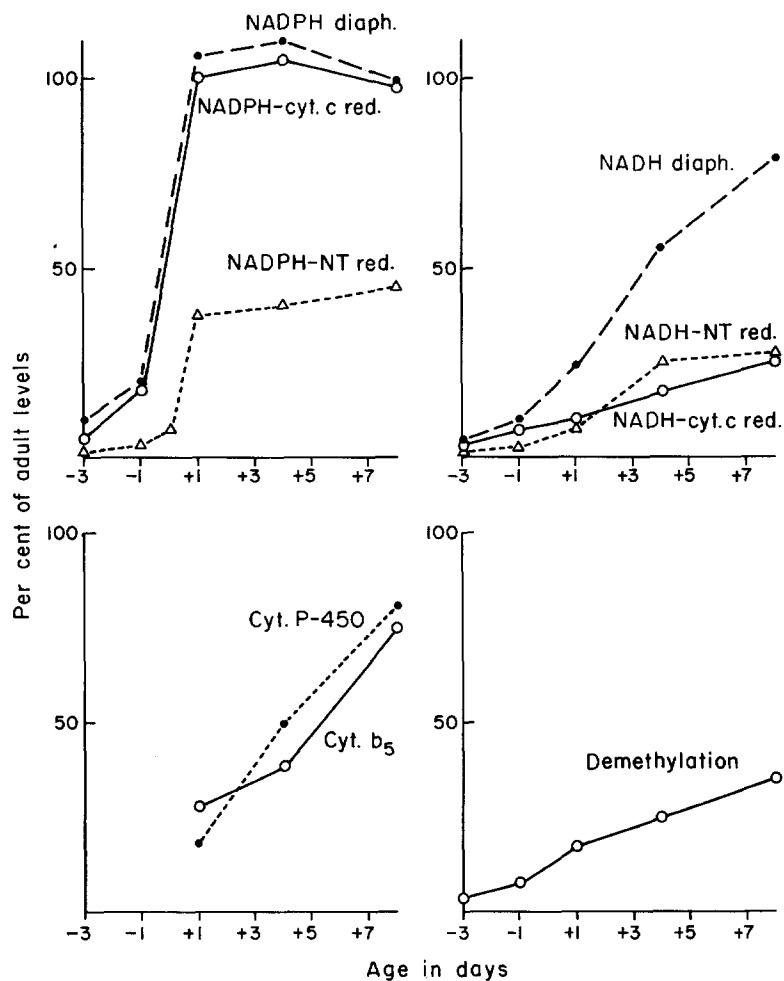


FIGURE 1 Electron transport enzymes in hepatic microsomes as a function of age. Enzymatic activities performed as described in text. Specific enzyme activities or enzyme amounts, calculated on protein basis, and expressed as per cent of adult level, are plotted on the ordinate. Each value represents an average of at least 3 experiments. The average adult enzyme activities and enzyme amounts, which were the bases for calculating the relative activities, were: 0.069, NADPH diaphorase; 0.031, NADPH-cytochrome *c* reductase; 0.23, NADPH-NT reductase; 3.77, NADH diaphorase; 1.17, NADH-cytochrome *c* reductase; 0.28, NADH-NT reductase; all in moles NADPH or NADH oxidized/min/mg protein. For demethylation, the value was 5.01 moles formaldehyde/min/mg protein; for cytochrome *b*₅, the value was 0.049 $\Delta E_{424-410}$ m μ /mg protein; for CO-binding pigment, the value was 0.022 $\Delta E_{450-490}$ m μ /mg protein.

thiobarbituric acid to determine the amount of malonaldehyde produced in the reaction, since fetal liver apparently contains a compound(s) which reacts with thiobarbituric acid. Adrenocortical microsomes have been reported to contain an additional CO-binding pigment with a peak at 420 m μ (21, 22). Nonwashed hepatic microsomes also exhibit a large peak at 420 m μ in the presence of CO. This peak, however, disappears completely after removal of absorbed hemoglobin.

PCMB inhibits tetrazolium reductases (23), possibly by reacting with an intermediate (see Fig. 7) in the microsomal electron transport chains. To test whether the PCMB inhibition titer is different in microsomes derived from postnatal and adult livers, the reductases (in microsomes from 15 mg liver/1.5 ml incubation medium) were titrated with PCMB without preincubation. Small concentrations of PCMB stimulate the reactions; larger concentrations

TABLE I
Effect of Actinomycin D and Puromycin on Some Enzymatic Activities of Rat Liver Microsomes during Development

Actinomycin D was injected intraperitoneally either once at 2 hr or twice at 2 and 5 hr after birth. Puromycin was given by intraperitoneal injection three times at 2, 6, and 10 hr after birth. Microsomes were prepared from 10 to 14 pooled livers.

Experiment No.	Conditions	G-6-Pase	ATPase	NADPH-NT reductase	NADPH-cyt. <i>c</i> reductase
		$\mu\text{moles P}_i/20 \text{ min/mg protein}$		$\mu\text{moles NADPH ox/min/mg protein}$	
1	2-hr-old, none	0.80	0.98	0.105	0.0088
	15-hr-old, none	3.76	1.06	0.270	0.0270
	15-hr-old, Actinomycin D				
	1 \times 0.8 mg/kg	1.71	1.19	0.191	0.0202
	2 \times 0.8 mg/kg	1.06	1.15	0.186	0.0185
	2 \times 1 mg/kg	0.72	1.15	0.152	0.0107
2	2-hr-old, none	0.89	1.10		
	15-hr-old, none	2.34	1.20		
	15-hr-old, puromycin				
	3 \times 10 mg/kg	1.44	1.06		
	3 \times 20 mg/kg	1.12	1.08		
	3 \times 30 mg/kg	1.01	1.10		

inhibit both NADPH and NADH-NT reductase activities (50% inhibition at $\sim 8 \times 10^{-5} \text{ M}$ and $\sim 2 \times 10^{-4} \text{ M}$, respectively). However, no difference in the PCMB sensitivity of the two reductase activities appears when the hepatic microsomes of 5-day-old and adult rats are compared.

2. PHOSPHATASES

The three phosphatases assayed (Fig. 2) exhibit three different patterns. Nucleoside triphosphatase (with ATP as substrate) is the only enzyme followed in these experiments whose specific activity remained constant during hepatocyte differentiation. Nucleoside diphosphatase activity, measured with IDP as substrate, is hardly detectable before birth, but increases slowly in the postnatal period. G-6-Pase activity (see also Table I, Table II, Fig. 5, Fig. 6) shows shortly after birth a large increase which exceeds the level of activity found in adult liver microsomes. The overshoot is always present, but its timing and extent show considerable variation from one experiment to another: peak specific activity was reached on the 1st, 2nd, or 3rd day at 200 to 450% of the adult level.

B. Effect of Inhibitors of Protein Synthesis

1. MORPHOLOGY

The postnatal increase in enzyme activities may be due to either net protein synthesis or activation of enzymes already present at birth. To distinguish between these possibilities, Actinomycin D or puromycin was administered to newborn rats. Actinomycin D, which inhibits DNA-dependent RNA synthesis (24, 25), was injected intraperitoneally either once at 2 hr, or twice at 2 and 5 hr after birth. Puromycin, which is known to inhibit protein synthesis by competing with aminoacyl-S-RNA for ribosomal sites (26, 27), was given intraperitoneally three times, at the age of 2, 6, and 10 hr.

The cytoplasm of hepatocytes in actinomycin-treated ($2 \times 0.8 \text{ mg/kg}$) sucklings (Fig. 3) showed extensive but variable alterations of the ER consisting of: moderate to pronounced dilation of the cisternal spaces, loss of cisternal content, partial loss of parallelism of rough-surfaced elements, and decrease in frequency of attached ribosomes. Those ribosomes still present, however, appeared normal and were disposed in

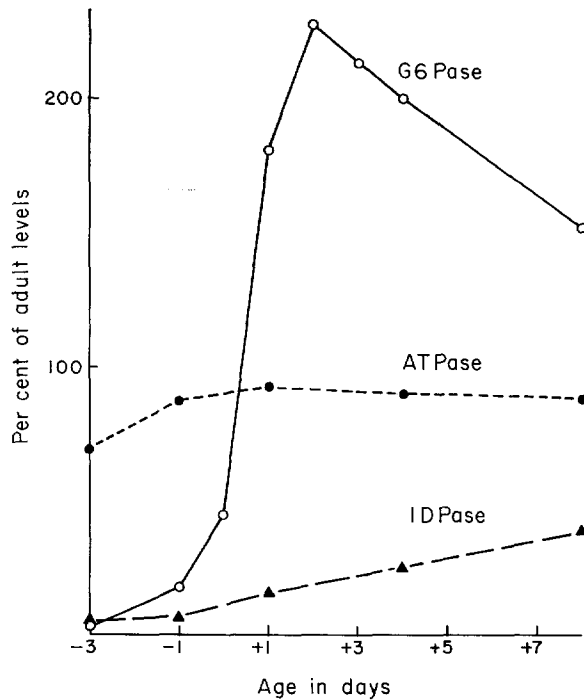


FIGURE 2 Phosphatases in hepatic microsomes as a function of age. Enzymatic activities performed as described in text. Specific enzyme activities, calculated on protein basis, and expressed as per cent of adult level, are plotted on the ordinate. Each value represents an average of at least 3 experiments, except for the G-6-Pase values which represent single experiments. The average adult enzyme activities, which were the bases for calculating the relative activities, were: 1.18, ATPase; 11.1 IDPase; 2.34, G-6-Pase; all in $\mu\text{moles P}_i/20 \text{ min/mg protein}$.

usual patterns (rosettes, spirals, and double rows), a finding which is in agreement with observations recently published by Revel et al. (28). The smooth-surfaced elements were few in number and their typical relationship with particulate glycogen was only occasionally encountered in small areas. The Golgi complex appeared normal and its vacuoles already contained the dense $\sim 300\text{-\AA}$ granules characteristic of postnatal hepatocytes. The cytoplasmic matrix contained free ribosomes—some of them in clusters but many scattered as individual particles.

There was a very drastic reduction in particulate glycogen and most of that still present was found as β particles in autolytic vacuoles, together with various membranous residues. The cytoplasmic matrix contained relatively frequent lipid droplets. The mitochondria were enlarged and of irregular shape. The number of microbodies appeared markedly reduced.

The nuclei showed nucleolar alterations of a type already described (29), i.e., separation of fibrillar and particulate components and reduction in the amount of the latter. Notwithstanding these various changes, there was no indication of nuclear pycnosis, or general uncontrolled cytolysis at the time points examined (+15 hr).

TABLE II
Distribution of G-6-Pase among Some Cell Fractions and Subfractions in Rat Liver during Development

The fractionation procedure was described earlier (1). The activities are given as $\mu\text{moles P}_i/\text{g liver}/20 \text{ min}$.

Preparation	Age of rats		
	+5 hr	+15 hr	+90 days
Homogenate	55.9	159.0	85.6
Final supernatant	6.2	5.9	6.4
Total microsomes	25.3	43.0	27.6
Submicrosomal fractions:			
“Soluble” in 0.26% DOC	2.0	3.9	2.7
“Soluble” in 0.50% DOC	7.9	17.2	7.9
Ribosomes	0.2	0.5	0.3

The cytoplasm of hepatocytes in puromycin-treated ($3 \times 20 \text{ mg/kg}$) sucklings (Fig. 4) showed similar but less pronounced changes in the organization of the ER. The number of attached ribosomes was closer to that found in normal animals of the same age, and the frequency of “polysomes,” free as well as attached, was high. A small number of smooth-surfaced elements was

present, and in a few small areas they formed networks amidst small deposits of particulate glycogen. Such associations were more advanced and more frequent than in actinomycin-treated sucklings. Glycogen depletion, however, was as advanced as in the latter, but glycogen-containing autolytic vacuoles were absent. The cytoplasmic matrix was heavily loaded with lipid droplets. As in the case of actinomycin-treated animals, there were no signs of pycnosis or extensive, uncontrolled cytolysis at the time point examined (+15 hr).

2. ENZYME ACTIVITIES

Liver microsomes were prepared from 15-hr-old animals which had been previously treated with either Actinomycin D or puromycin as described earlier. Depending on the amount injected, both Actinomycin D and puromycin inhibited strongly (Table I) the increase in G-6-Pase activity and partially the increase in NADPH-cytochrome *c* and NADPH-NT reductase activities. ATPase activity was not affected. These results are taken to indicate that the postnatal increases in enzyme activity are due to synthesis of new enzymes.

C. Enzyme Distribution in Microsomal Subfractions

In adult rat liver, all the enzymes we have studied are about equally concentrated in the rough and smooth microsomes (19). Assays carried out on rough and smooth microsomes showed that this is not the case in the developing liver. At birth, for instance, the concentration of G-6-Pase is considerably higher in the rough than in the smooth microsomes (Fig. 5). After birth, enzyme concentration increases in the smooth microsomes, but during the first 3 days it remains far below that found in the rough microsomes. The same situation appears in the case of the NADPH-cytochrome *c* reductase. For ATPase, however, the findings are entirely different: the enzyme concentration changes little with age in the two subfractions. The specific ATPase activity is constantly higher in the smooth microsomes, a finding at variance with the situation in the adult, in which the rough and smooth microsomes have equal ATPase specific activities (19); no explanation can be given at present for this difference.

More detailed information concerning the distribution and development of G-6-Pase activity is given by the experiment in Fig. 6. The curves describing the concentration of enzyme activity

in rough and smooth microsomes, as a function of age during the first 28 hr after birth, are not parallel. The enzyme appears later and its concentration increases at a somewhat slower rate in smooth microsomes. The data suggest a gradual transfer of G-6-Pase from the rough to the smooth microsomes, but the curves do not demonstrate a clear precursor-product relationship. It should be pointed out that one of the fractions compared (smooth microsomes) consists almost entirely of "new" membranes while the other (rough microsomes) is a mixture of "old" and "new" membranes. For this reason, the specific enzyme activities in the smooth membrane fraction are expected to be higher than they would be in the classical precursor-product relationship, and a higher specific activity is actually found, as the slope of the curves shows.

D. G-6-Pase in Liver Fractions and Submicrosomal Fractions

The suggested transfer of G-6-Pase from rough to smooth ER membranes could be effected along various pathways, among them through the cytoplasmic matrix or through the cisternal space (ER content). To test the former possibility, distribution of G-6-Pase between the final supernate, which corresponds to the cytoplasmic matrix, and the microsomes was studied as a function of age (Table II). Only a small percentage of the total activity is recovered in the final supernate, usually less than 10%, and this percentage is not higher at the expected time of rapid transfer (+15 hr). Hence G-6-Pase transfer through the cytoplasmic matrix appears unlikely. To test the possibility of G-6-Pase transfer through the ER content, total microsomes were subfractionated by DOC (1) into: (a) ribosomes, (b) 0.26% DOC-soluble fraction (content and certain membrane constituents), (c) 0.5% DOC-soluble fraction (membranes). Table II shows that little activity is detected in the ribosomes, and this is probably due to contamination by membranes, while the bulk of the activity is recovered in the membrane fraction and little activity, with no significant variation with age, appears in the microsomal content. However, these results should be interpreted with caution since the DOC concentrations used inhibit G-6-Pase activity (30, 31) and since total recovery was less than 50%.

E. Effect of Fat Diet on Synthesis of Enzymes in Developing Liver

The previous experiments suggest that the increase in enzymic activity is due to synthesis of new enzymes, rather than activation of preexisting proteins. Yet our previous investigations (1) showed that PLP-fatty acids vary extensively with age. Hence it is conceivable that changes in enzyme activity during differentiation are partly due to changes in certain membrane lipid constituents. In other words, emerging enzyme activities may reflect both synthesis and activation by special PLP's, since the activity of some microsomal enzymes is known to depend on their association with lipids (32-34).

To investigate the role of PLP-fatty acids in the development of microsomal enzyme patterns, female rats at the 7th day of pregnancy were put on a standard diet supplemented with either 20% corn oil or 20% lard. Corn oil and lard contain a large amount of 18:2 and 18:1 fatty acids, respectively; and both lack long chain (above 20) fatty acids. The control rats were given only the standard diet which contains 3.8% fat. The mothers were kept on the respective diet until 5 days after delivery, when the livers of the sucklings were removed for cell fractionation. The micro-

somal lipids were extracted and the FA of their phospholipids determined by gas chromatography. The results (Table III) show that, by comparison with the standard diet controls, there is an increase in 16:0 and especially in 18:2 FA concomitant with a marked decrease in 22:6 FA in the hepatic microsomal phospholipids of sucklings whose mothers were kept on a diet supplemented with corn oil. The lard-supplemented diet of the mothers gave an increase in 16:0, 18:0, and especially 18:1 FA, and a sharp decrease in 22:6 FA in the microsomal phospholipids of the sucklings. But in spite of these differences, the enzymic pattern was not significantly influenced (variations did not exceed 10 to 15%). Enzymes and enzyme systems which have low activities at +5 days, such as NADH-cytochrome *c* reductase, oxidative demethylation, and IDPase, remain low; while enzymes which reach or have the adult level at +5 days, such as NADPH-cytochrome *c* reductase and ATPase, exhibit the same activities, irrespective of the diet-induced changes in FA distribution. Thus, changes in the FA composition of the ER membrane during hepatocyte differentiation do not seem to influence the pattern of enzymic differentiation on the same membrane.

FIGURE 3 a* Representative field in a hepatocyte of a 15-hr-old rat suckling treated with Actinomycin D (2×0.8 mg/kg).

The cisternae of the rough-surfaced ER (*rs*) are irregularly dilated, appear "empty," and have lost their usual arrangement in parallel arrays. The number of attached ribosomes (arrows) is decreased, but those present are still found disposed in characteristic patterns, i.e. double rows (1), rosettes (2), spiral (3), loops (4), wherever the plane of the section is parallel to the cisternal membranes.

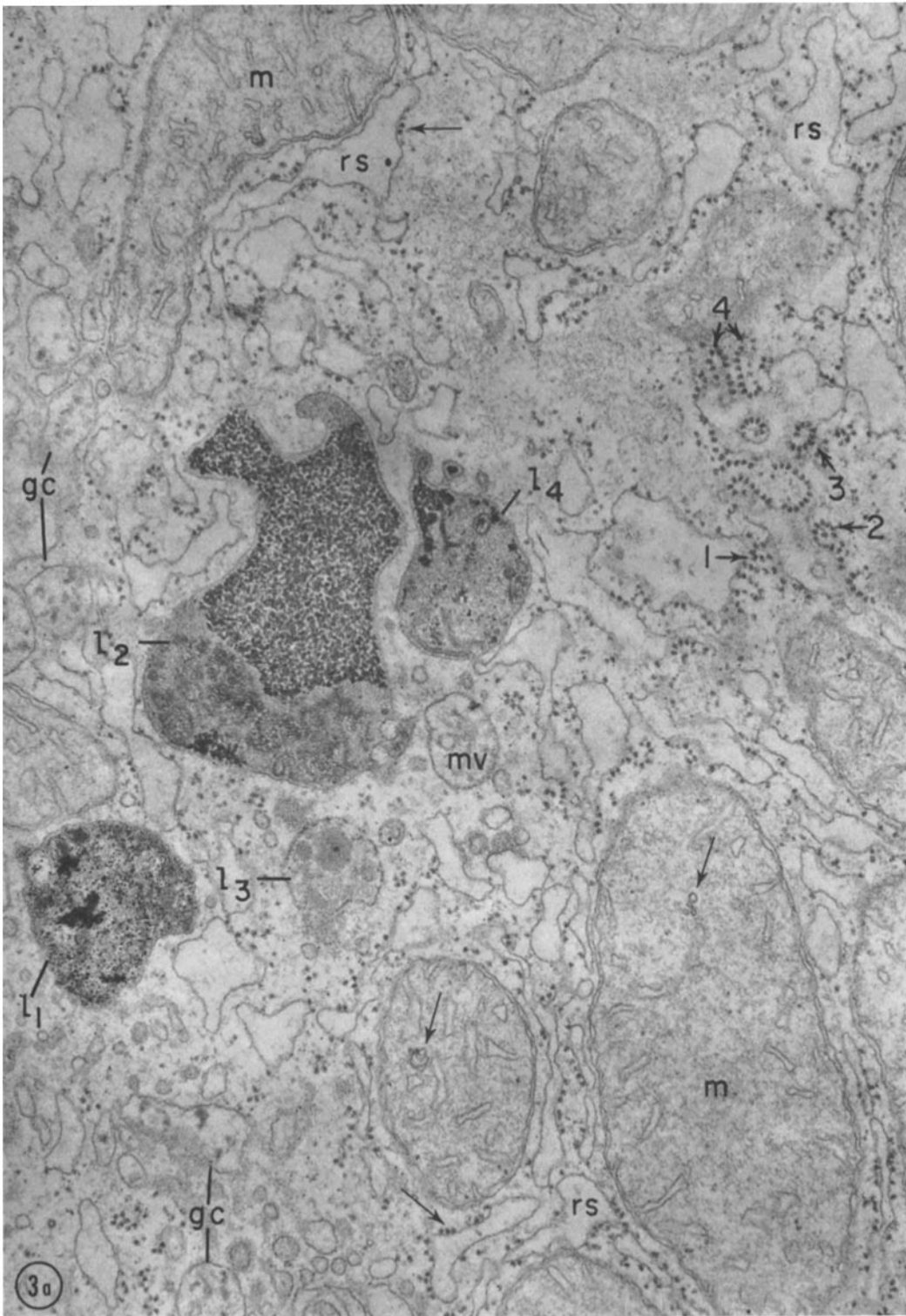
There is little or no development of smooth-surfaced ER.

Golgi complexes appear along the left side of the field and some of their dilated cisternae (*gc*) contain irregular, dense granules reminiscent of, but smaller and less regular than, those found in the hepatocytes of control sucklings.

Four autolytic vacuoles (lysosomes) are marked l_1 to l_4 . Two of them (l_2 and l_4) contain masses of recognizable glycogen particles of β type. Small vesicles are seen along the upper side of this vacuole; they either bring to, or remove glycogen particles from, l_4 . A multivesicular body is marked *mv*.

The mitochondria (*m*) appear normal, except for clusters of small irregular vesicles (arrows) located in the inner compartment. $\times 40,000$.

* All micrographs represent sections of liver tissue fixed for 2 hr at $\sim 0^\circ$ in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4), dehydrated in alcohol and embedded in Epon. The sections were doubly stained with uranyl and lead and examined and micrographed in a Siemens Elmiskop I.



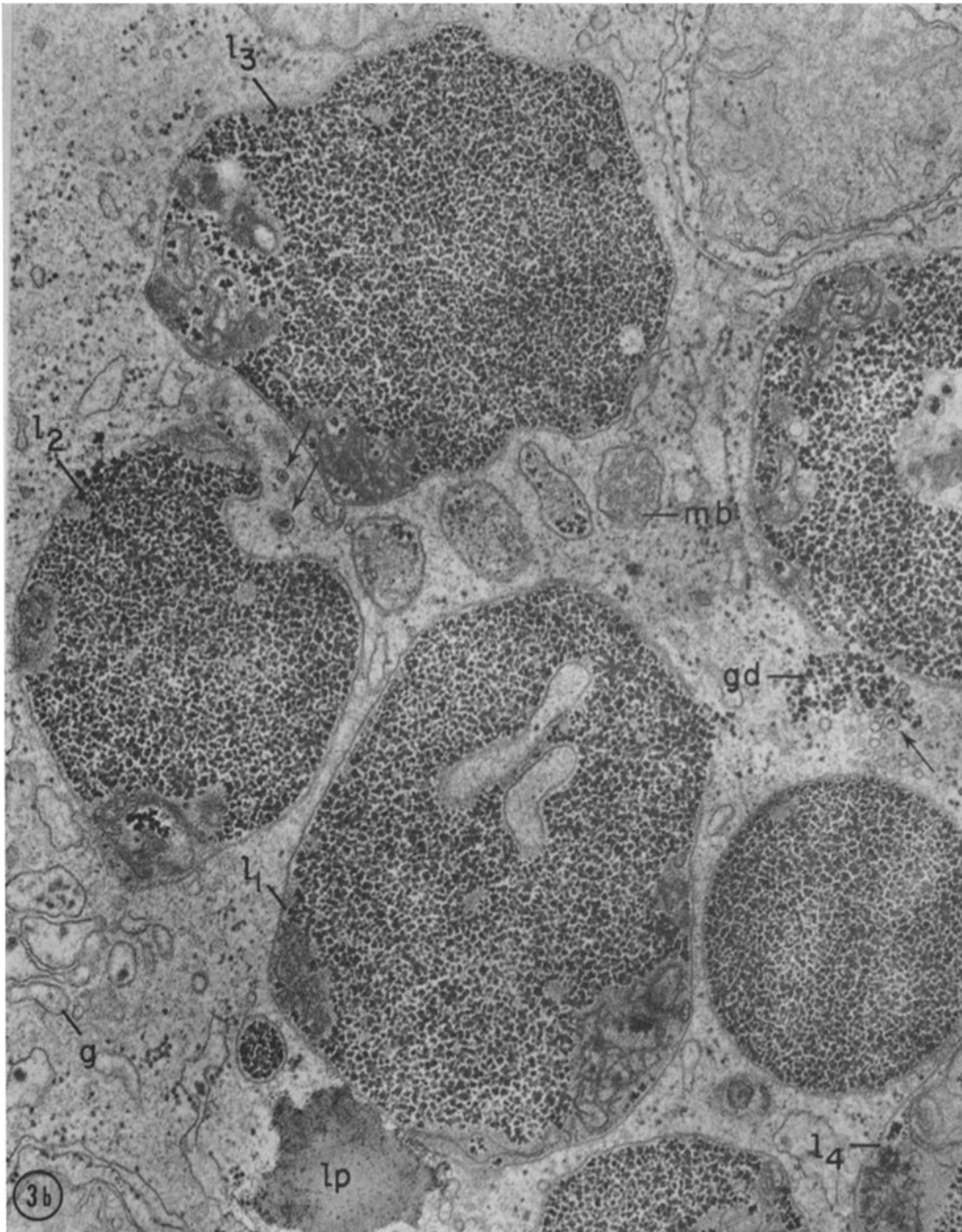


FIGURE 3 *b** Small field in a hepatocyte of a 15-hr-old rat suckling treated with Actinomycin D (2×0.8 mg/kg).

It contains a group of relatively large autolytic vacuoles loaded primarily with glycogen β particles. Some of these vacuoles (l_1 to l_4) contain, in addition, membranous residues of various subcellular components.

A small normal deposit of β glycogen particles appears in the cytoplasmic matrix at *gd*. In its vicinity, and in other parts of the field, glycogen particles are found enclosed individually or in small clusters in small smooth-surfaced vesicles (arrows).

A Golgi complex, whose distended cisternae have the characteristic granular content of postpartum hepatocytes, is seen at *g*. A lipid droplet is marked *lp* and a microbody *mb*. $\times 40,000$.

F. Effects of Lipid Extraction and Lipid Supplementation upon Microsomal Enzyme Activities

The possibility that specific phosphatides influence the development of the microsomal enzymic pattern was put to further test by extracting the lipids of total microsomes and assaying the effect of lipid micelles from various sources on the reactivation of the NADH-cytochrome *c* reductase activity of such lipid-depleted preparations. Extraction by acetone-water (10:1) removes 95% of the microsomal phospholipid, causes a 15-fold decrease in microsomal PLP/protein ratio (Table IV), and inactivates NADH-cytochrome *c* reductase to the extent of 80 to 85%. The acetone-water extraction, however, results in heavy protein losses (55 to 60%) (Table IV) which may represent mostly nonmembrane protein (i.e., adsorbed protein or protein of the microsomal content) although some losses of membrane protein by extraction or incomplete sedimentation are not excluded. The results are the same irrespective of the age of the animal: 8-day suckling or adult. The lipid preparations used in the reactivation experiments included asolectin, and phospholipid micelles of total lipid extracts, prepared from (a) hepatic microsomes (adult and 8-day-old animals) and (b) beef heart mitochondria, and purified individual phospholipids prepared from beef heart mitochondria. Some of these lipids proved inhibitory for the NADH-cytochrome *c* reductase activity of whole (unextracted) microsomes, but others were not (Table V). All lipids tested partially reactivated the enzyme, especially when the extracted microsomes were incubated with the lipid (4°, 10 min) prior to enzyme assay (Tables VI and VII). The extent of reactivation was the same for microsomes isolated from adult (Table VI) and 8-day-old (Table VII) animals. It did not increase with the amount of lipid added, and its extent proved to be little influenced by the nature of the lipid: asolectin was just as effective as microsomal lipid (~50% reactivation); microsomal lipid from adult animals was not better than microsomal lipid from 8-day-old sucklings; and the best results (60% reactivation) were obtained with cardiolipin, a mitochondrial rather than microsomal phosphatide.

The results are taken to confirm the influence of lipids on the activity of a typical membrane enzyme system (NADH-cytochrome *c* reductase),

and to indicate at the same time that no specific lipids are required for this activity. Furthermore, the findings suggest that no specific activating lipids are present in fully differentiated hepatic microsomal membranes which are absent from the corresponding membranes of the newborn rat.

DISCUSSION

In the interpretation of our data, we assume that the enzymatic activities found in our microsomal fractions represent the enzymes of the ER membrane in situ. Some contamination by plasma membrane fragments and enzymes (*see* 35) is most probably present, but we expect it to be negligible because the amount of intracellular membranes (ER) far exceeds that of plasma membranes in hepatocytes.

Some microsomal enzymes have already been reported to undergo changes in the early postnatal period. For instance, a sudden and large increase of G-6-Pase activity soon after birth has been described in liver homogenates and microsomes of several species (36-42). Certain electron transport enzymes, which are partly or completely microsomal, such as NADH- and NADPH-cytochrome *c* reductases and NADH-ferricyanide reductase, have a low activity in the whole chicken embryo (43), in the liver microsomes of newly hatched chicks (44), in microsomes of new born rabbit (45), and in liver homogenates of newborn rats (46). Cytochrome *b₅*, however, is apparently present in the hepatic microsomes of chick embryos (47). A low drug detoxication (48-50) and ascorbic acid synthesizing (51) ability have also been reported in liver microsomes of newborn rabbits and rats. The formation of bilirubin glucuronides by microsomes of human, rat, and guinea pig livers in the early postnatal period is also deficient (45, 52, 53). Synthesis of plasma proteins, one of the important functions of liver rough ER, was found to be partially lacking in the newborn (54, 55), but the rate of lipid synthesis (56) and the rate of incorporation of labeled amino acids (57) into proteins were shown to be much higher in the liver of the newborn than in that of the adult. In this connection it should be mentioned that, during early fetal development, some of the antigens found in adult liver microsomes are missing (58). Thus, at birth the only constitutive enzymes known to be present in the microsomal membrane are phospholipid-synthesizing enzymes and ATPase.

Actinomycin and puromycin inhibit the post-

natal increase of the activities of the enzymes studied. The effect of these metabolic inhibitors is a clear indication that RNA and protein syntheses are involved in the enzymic differentiation of the ER, and that simple activation of enzymes pre-existing in the newborn is improbable. The possibility that these antibiotics are generally cytotoxic, in addition to their interference with protein synthesis, has been discussed in the literature (59, 60). In our experiments, the electron micrographs (Figs. 3, 4) showed rather extensive alterations in the organization of hepatocytes, e.g., vacuolization of the ER, distention of the cisternal space, and decrease in frequency of attached ribosomes, but no definite signs of cytolysis. The unchanged specific activity of ATPase is also an argument against acute cellular injury.

Although these findings indicate that the pattern of enzymic differentiation of the ER is dependent on protein synthesis, we felt that further experiments were needed to test the possible role of the lipid components of the ER membrane, because it is already known that many microsomal enzymes are active only when part of an organized membrane, and that their catalytic properties are greatly modified by changes in membrane structure (61, 62). Since phospholipids are essential membrane components, their role in the differentiation process was investigated by dietary experiments that caused alterations in their FA composition and by extraction-reactivation experiments. The results indicate that:

1. The FA composition of the total microsomal phospholipids is dependent, at least in part, on

the FA content of the diet. This is in agreement with other investigations on total homogenates (63-65) and on subcellular particles (66-69).

2. Enzymic activity and the pattern of enzymic differentiation of the ER are neither dependent on, nor influenced by, the FA composition of the membrane phospholipids.

3. Phospholipids are certainly required for at least some of the enzymatic activities of liver microsomes. Similar phospholipid requirements have been described, among others, for beef mitochondrial enzymes (70), muscle microsomal enzymes (34), isolated cytochrome *c* (71), and G-6-Pase (32).

4. ER phospholipids are not responsible for the low activity of NADH oxidase in newborn rats. Phospholipids from 8-day-old and adult rats are equally effective in the reactivation of the newborn's NADH oxidase system in lipid-extracted microsomes. It has been proposed, in relation to other systems, that the key function of membrane lipids is to maintain hydrophobic conditions for catalytic processes, and to bridge the gap between hydrophilic and hydrophobic functional groups (72). This could explain the finding that the reactivation of microsomal NADH oxidase after acetone extraction is nonspecific and occurs even in the presence of a nonmicrosomal lipid, such as cardiolipin.

Although it appears that enzyme synthesis and insertion into the ER membrane does not require a strictly determined phosphatide composition, it should be pointed out that, in fact, there are consistent and substantial differences (*see* 73) in

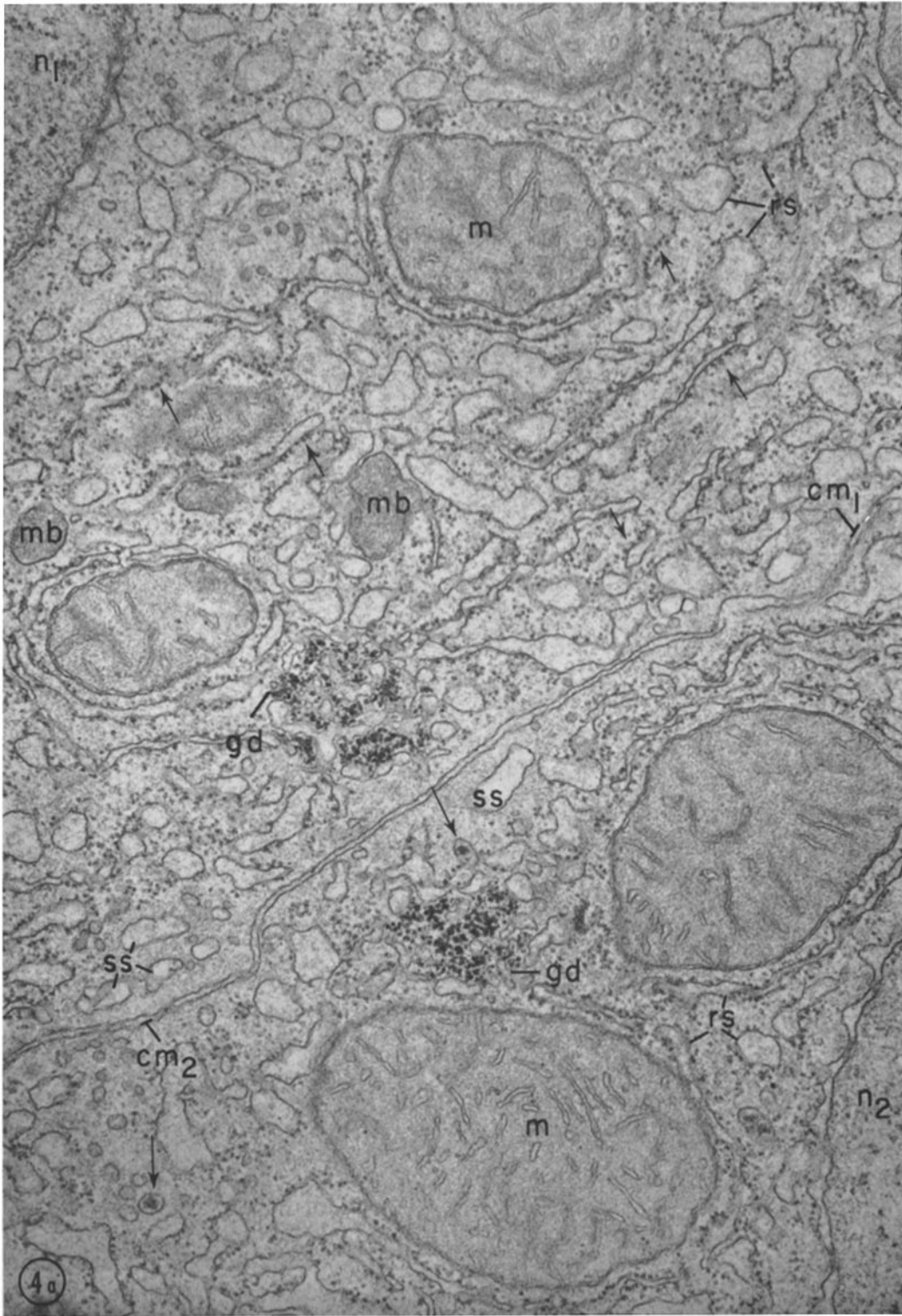
FIGURE 4 *a** Representative field in the liver of a Puromycin-treated (3×20 mg/kg), 15-hr-old rat suckling.

The micrograph shows small fields in two adjacent hepatocytes whose cell membranes are marked *cm*₁ and *cm*₂. The cisternae of the rough-surfaced ER (*rs*) are irregularly dilated, have a content of low density, and have lost in part their usual arrangement in parallel arrays. These changes are similar to, but less pronounced than, those seen in Actinomycin-treated sucklings (Fig. 3 *a*). The frequency of attached ribosomes (short arrows) is higher than in the latter and nearly equal to that found in controls.

There is a moderate production of smooth-surfaced ER (*ss*) along the cell periphery and in the vicinity of glycogen deposits (*gd*). Smooth as well as intermediate elements (part rough and part smooth) penetrate these small glycogen deposits which consist mainly of β particles.

Some smooth-surfaced ER elements contain dense granules (long arrows) of the type usually found in postpartum hepatocytes.

The nuclei are marked *n*₁ and *n*₂; mitochondria, *m*; and microbodies, *mb*. $\times 38,000$.



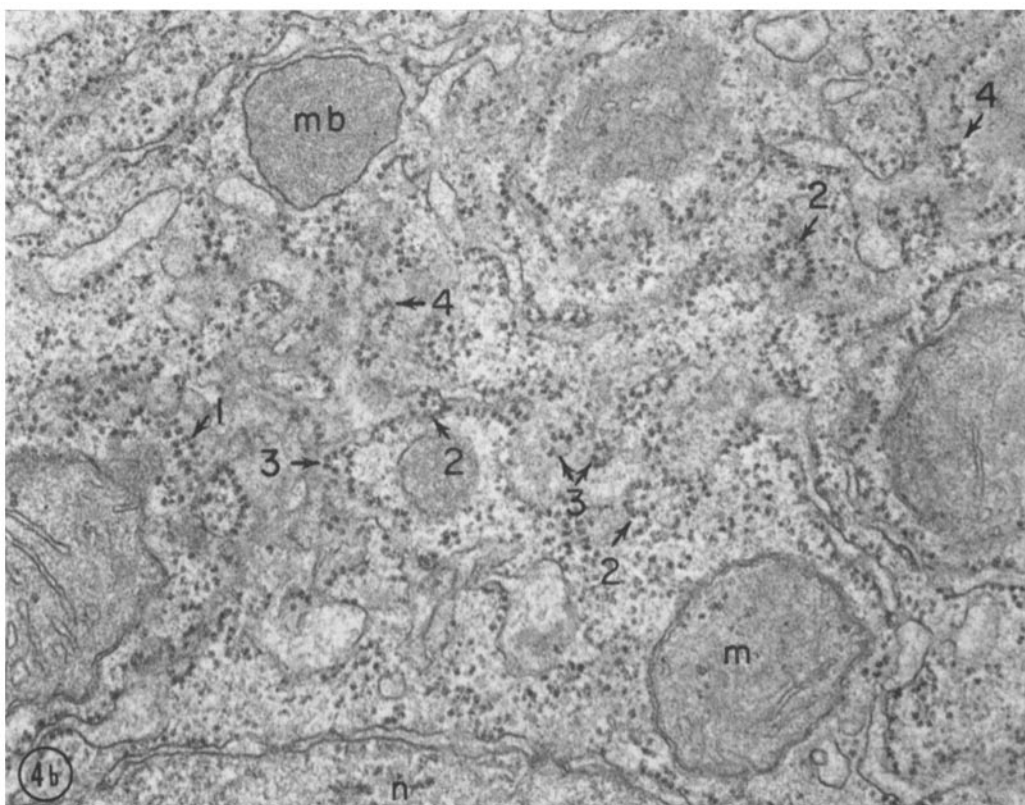


FIGURE 4 *b** Small field in a hepatocyte of a 15-hr-old rat suckling treated with Puromycin (3×20 mg/kg).

The section cuts obliquely through a series of cisternae and reveals the presence usual of ribosome patterns (arrows), i.e. double rows (1), rosettes (2), circles (3), and loops (4), on the surface of the cisternal membranes.

The nucleus is marked *n*; mitochondria, *m*; and microbodies, *mb*. $\times 55,000$.

phospholipid content and composition among various cellular membranes (e.g. microsomal, mitochondrial, plasmalemmal). It could be argued that, in addition to general functions such as partition building and enzyme activation, various mixtures of membrane phospholipids have more subtle structural functions which lead to different membrane conformations, characteristic for each type of cell organelle (cristae in mitochondria, cisternae in the ER, etc.).

Some of our findings bear on the organization of the microsomal electron transport chains whose functional role has been partially elucidated by recent work. The previously isolated NADPH-cytochrome *c* reductase (74, 75) and cytochrome *P*-450 (76, 77) have been identified (78, 23) as components of an electron transport chain (Fig.

7), which oxidizes NADPH and serves as electron donor for the hydroxylating detoxication system. The nature of component X_1 is unknown, but copper (79), quinone (80), a sulfhydryl-group-containing enzyme (75, 81), and recently an iron protein (82, 83) have been proposed as direct electron acceptors of the reduced flavoprotein. This component, which is PCMB sensitive, appears to react with NT. The "detoxication system" in Fig. 7 represents the enzymes (84) assumed to catalyze hydroxylation. However, there are strong indications that the hydroxylation step itself may be a nonenzyme free radical reaction (85). In microsomes from developing rat liver, low detoxication activity cannot be explained by rate limitation at the level of the flavoprotein or cytochrome *P*-450 (see Fig. 1). The low NADPH-NT

reductase activity suggests that component X_1 is lacking. This explanation assumes that the "detoxication system" itself is not limiting in these microsomes and that the electron requirement for NT reduction is not greater than for the whole chain. At present, a satisfactory explanation of the situation cannot be given because the site of PCMB attack is not exactly known and because no difference was found in PCMB inhibition titer between microsomes derived from postnatal and adult livers. The presence of $-SH$ groups in microsomal proteins other than the NT-reductases could mask changes in the inhibition titer of the latter.

The composition of the NADH chain (Fig. 7) is less well understood than that of its NADPH counterpart, particularly since there are no data

about the final electron acceptor, *in vivo*. The isolated flavoprotein reacts with cytochrome b_5 , but not with cytochrome c (86, 87). The PCMB-sensitive NADH-NT reductase (19), like its NADPH counterpart, probably contains an additional component (X_2) besides the flavoprotein, perhaps iron (88). In the newborn, the low NADH-cytochrome c reductase activity is clearly not caused by rate limitation at the level of the flavoprotein or cytochrome b_5 (see Fig. 1). The low NADH-NT reductase activity suggests that the intermediate X_2 is partially missing. The nature of the postulated intermediates, X_1 and X_2 , is not known. They could be low molecular weight components, or proteins. If they are proteins, then it follows that the enzymes of these electron-transport chains are not synthesized at the same time

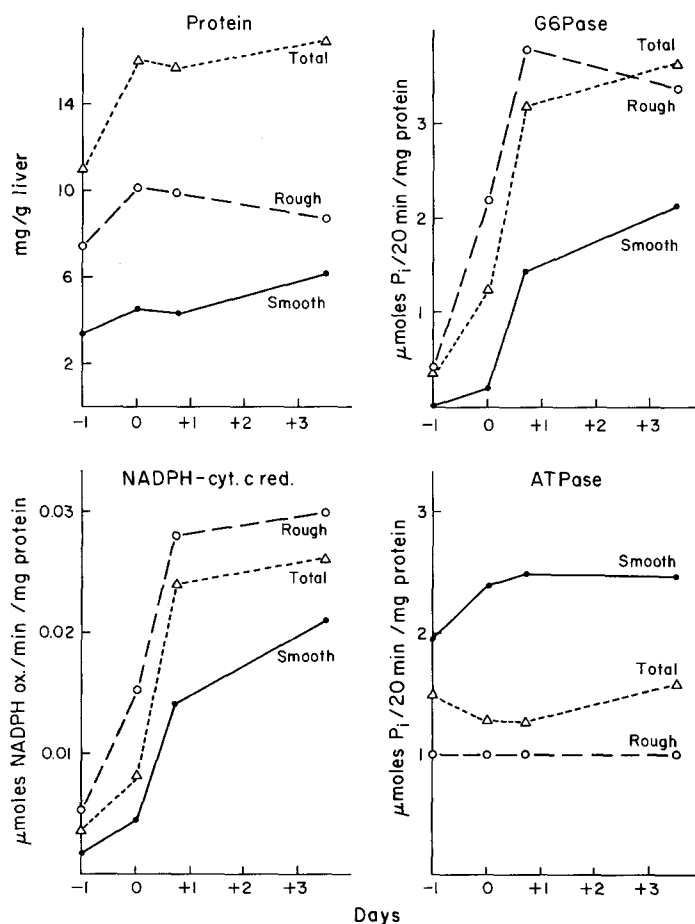


FIGURE 5 Distribution of some enzymes in rough and smooth microsomes of developing rat liver. The method of separation of rough and smooth microsomes is described previously (1).

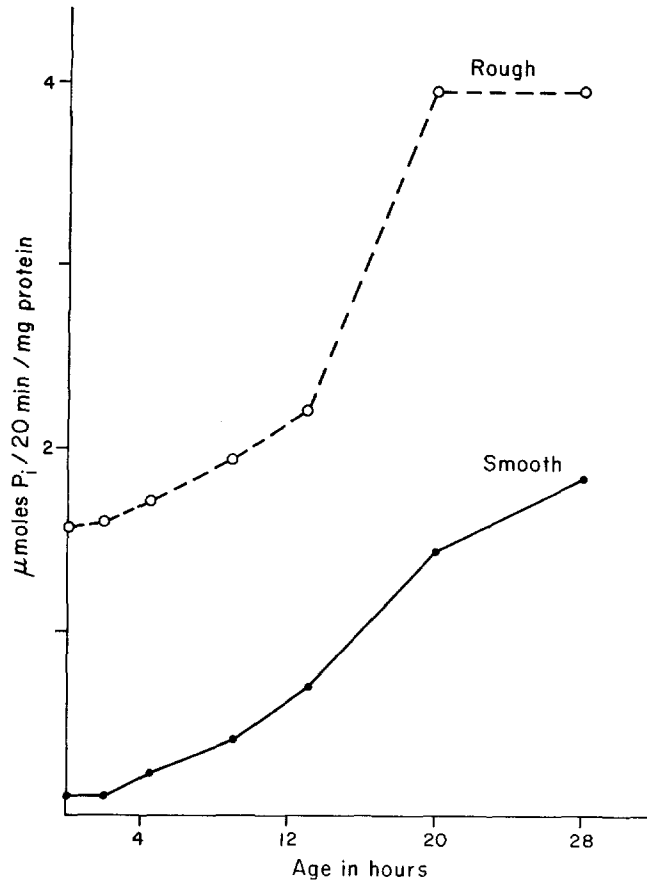


FIGURE 6 Glucose-6-phosphatase of rough and smooth microsomes during the 1st day after birth. Methods are given in the text.

TABLE III

Effect of Diet on Composition of Phospholipid Fatty Acids and on Enzymic Activities of Hepatic Microsomes

Diet:	Corn oil	Lard	Basic	
Age of rats:	5-day-old	5-day-old	5-day-old	Adult
Phospholipid/protein of microsomes:	0.24	0.23	0.24	0.31
Fatty acid composition (% of total fatty acids)				
16:0	34.4	29.9	24.8	10.6
18:0	18.4	22.6	18.9	22.1
18:1	6.6	10.8	5.0	13.9
18:2	12.6	5.7	6.4	23.5
20:4	18.7	21.0	22.9	17.4
22:6	9.3	9.9	21.1	12.1
Specific enzyme activities				
NADPH-cyt. <i>c</i> reductase*	0.023	0.017	0.019	0.023
NADH-cyt. <i>c</i> reductase*	0.099	0.101	0.111	1.11
Demethylation‡	0.84	0.89	0.98	4.37
IDPase§	2.34	2.02	2.04	12.1
ATPase§	0.91	1.04	1.17	1.17

* μmoles NADPH or NADH oxidized/min/mg protein.

‡ μmoles formaldehyde/min/mg protein.

§ μmoles P_i/20 min/mg protein.

TABLE IV

Acetone Extraction of Liver Microsomes

	Protein mg/g liver	PLP mg/g liver	% PLP remaining	PLP Protein
Adult rat				
Microsomes	22.2	6.82	100.0	0.308
Extracted microsomes	9.3	0.20	6.8	0.021
8-day-old rat				
Microsomes	20.4	5.03	100.0	0.247
Extracted microsomes	9.2	0.13	5.7	0.014

TABLE V

Effect of Various Phospholipids on NADH-Cytochrome *c* Reductase Activity of Liver Microsomes from Adult and 8-Day-Old Rats

Microsomes derived from 1 g of rat liver were preincubated for 10 min at 4° before assay with the lipids indicated below. The maximal dilution of the microsomes with micelle solution was 1:6. Further dilutions were made just before the measurements.

Lipid	Lipid added*		μ moles NADH oxid./ min/mg protein	
	During preincub. in assay		Adult	8-day-old
	mg	mg		
None	—	—	1.28	0.200
Asolectin	200	—	1.26	0.208
Asolectin	200	8	1.26	0.209
Adult rat				
Microsomal	10	—	1.30	0.200
Microsomal	10	1	0.89	0.131
Microsomal	10	3	0.70	0.120
8-day-old rat				
Microsomal	7	—	1.25	0.210
Microsomal	7	0.7	0.86	0.122
Microsomal	7	2.1	0.67	0.102

* 0.2 ml microsomal suspensions, containing microsomes from 200-mg liver and containing ~4 mg protein, were preincubated for 10 min at 4°C with 1.0 ml micelle solution containing the indicated amount of lipid. At the end of the preincubation, the microsomes were further diluted with 0.25 M sucrose from 6 to 50 times, depending on the activity of the enzyme. 0.1 ml of this suspension was added to 2.9 ml of the assay medium, which contained the indicated amount of lipid.

TABLE VI

Effect of Various Phospholipids on NADH-Cytochrome *c* Reductase Activity of Lipid-Extracted Microsomes of Adult Rats

Preincubations and dilutions as in Table V, except that twice the concentration of microsomes was used.

Lipid	Lipid added		μ moles NADH oxid./min/ mg protein
	During pre- incub.*	in assay	
	mg	mg	
None	—	—	0.19
Asolectin	110	—	0.36
Asolectin	110	8	0.35
Asolectin	110	16	0.35
Adult rat			
Microsomal	28	—	0.33
Microsomal	28	2	0.36
Microsomal	28	4	0.36
8-day-old rat			
Microsomal	16	—	0.33
Microsomal	16	1.4	0.34
Microsomal	16	2.8	0.34

* Doubling the amount of lipid added during preincubation gave identical results.

from a "polycistronic messenger," and that components X₁ and X₂ are synthesized and inserted later into the corresponding enzyme sequences.

Our salient findings bearing on processes involved in the biogenesis of ER membranes are the following:

(a) Constitutive enzymes are produced at different times and different rates during development; this apparently applies even for enzymes which in the adult are part of a common functional chain.

(b) Proteins, including enzymes, are probably produced in the rough part of the system and subsequently transferred to the smooth.

(c) Lipid components are initially assembled in a membrane in the rough ER; their gross composition is essentially stable throughout development and, within the limits tested, appears to have no influence on the differentiation process.

These findings can be integrated with a number of available data obtained in studies of the synthesis of membranes and membrane-bound enzymes in hepatomas (*see* 89), regenerating liver (*see* 90), and liver of phenobarbital-treated animals. The

TABLE VII

Effect of Various Phospholipids on NADH-Cytochrome c Reductase Activity of Lipid-Extracted Microsomes of 8-Day-Old Rats

Preincubations and dilutions as in Table V, except that twice the concentration of microsomes was used.

Lipid	Lipid added		μ moles NADH oxid./ min/mg protein
	During pre- incub.	In assay	
	mg	mg	
None	—	—	0.042
Asolectin	100	—	0.092
Asolectin	100	8	0.092
Asolectin	100	16	0.093
Adult rat			
Microsomal	25	—	0.089
Microsomal	25	2	0.091
Microsomal	25	4	0.093
8-day-old rat			
Microsomal	18	—	0.079
Microsomal	18	1.4	0.083
Microsomal	18	2.8	0.083
Cardiolipin	24	—	0.062
Cardiolipin	24	1.5	0.126
Cardiolipin	24	3	0.117
Phosphatidyl choline	24	—	0.064
Phosphatidyl choline	24	1.5	0.066
Phosphatidyl choline	24	3	0.066
Mitochondrial	30	—	0.064
Mitochondrial	30	4	0.071
Mitochondrial	30	10	0.084

latter case is characterized by a great proliferation of ER, predominantly of the smooth variety (91-93), and by the preferred synthesis of all enzymes of the NADPH oxidase system, as already described by Ernster and his collaborators (8, 94-96). In many respects, synthesis of membrane and membrane-bound enzymes by drug induction appears to be similar to ER development in the newborn, but is basically a different phenomenon. In the newborn, the possible role of substrates in enzyme induction is suggested by the partial lack of enzymes synthesizing pyridine nucleotides (97), by the levels of NAD and NADP (98-102) and by the disappearance of glycogen (101-103). Moreover, the situation in the newborn is probably more complex, since its hormones and those of the mother, metabolites coming from the digestive tract, and delivery itself (*see* 104, 105) are certainly important influencing factors.

These various elements can be used to formulate some general hypotheses on membrane biogenesis to be used as a base for further work. A priori, membrane could be produced in either a single-step or a multiple-step process. The first hypothesis implies that all constituents, lipids, structural proteins, and enzymes, are simultaneously assembled in a single operation which produces a "standard membrane," i.e. a membrane with characteristic and rigid specifications. At a first approximation, such a process is favored by the finding that protein and lipid are assembled concomitantly in the rough ER, but it is not compatible with data showing that constitutive enzymes are synthesized at different times and different rates and with the results obtained by Ernster et al. (8, 94-96) in their phenobarbital experiments. To account for such findings, the single-step hypothesis must be modified by additional premises, to wit: (a) membranes can be produced in "incomplete form," and all membrane components have a high enough turnover to make completion of incomplete membranes unnecessary; (b) the ER membrane is a mosaic comprised of a number of types of functionally different patches (tesserae), each bearing a characteristic enzyme system or enzyme set. Each tessera is assembled in a single-step operation from structural protein, phosphatides, and corresponding enzymes, but various types of tesserae are produced in different proportions at different times.

The second hypothesis implies that a functional membrane is produced in a multistep operation in which at the first step only the "basic" constituents, i.e. lipids and "structural proteins," are assembled as a framework on which constitutive enzymes will be added as needed in a series of successive steps. This hypothesis is compatible with most data so far obtained. It assumes, however, without any supporting evidence, that ER membranes are long lived, and it introduces additional complications concerning the means by which new enzymes are inserted, supposedly at the right place, into old membranes. This could be effected by inserting either individual enzymes, synthesized elsewhere in the cell, or by bringing in complementary patches of membrane, or finally by attaching to old membrane the ribosomes programmed to synthesize and insert the new enzymes. For the moment, the evidence available renders unlikely the first hypothesis in its unmodified form (one-step assembly of a homogeneous membrane).

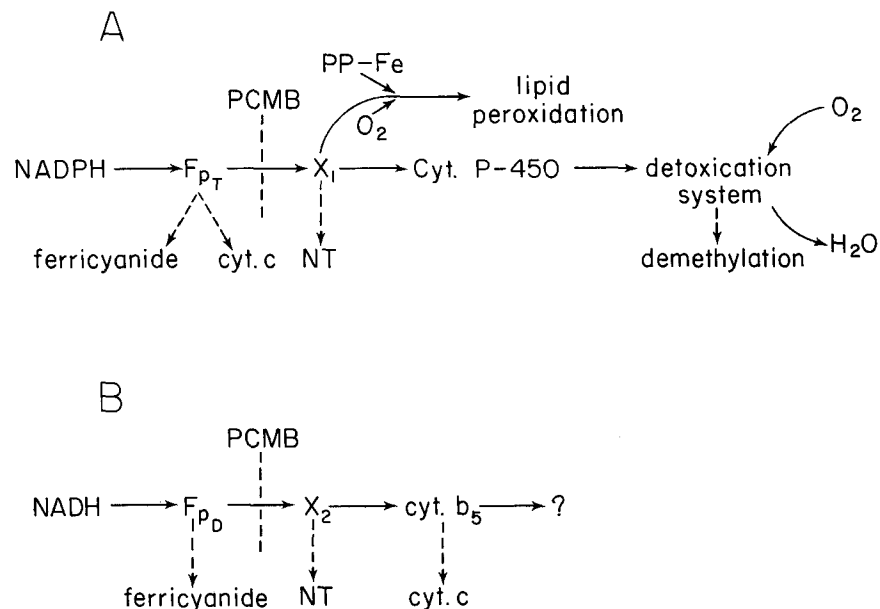


FIGURE 7 Tentative scheme of sequence of components in the two electron transport chains of rat liver microsomal membranes. Abbreviations are given in footnote 1.

A final problem to be considered concerns the relationship of rough to smooth ER. In the first paper of this series, we have already discussed possible means by which membrane synthesized in the rough ER could reach the smooth part of the system. The same means could apply for "basic," "incomplete," as well as functionally complete, membrane. Since detachment and attachment of ribosomes to the ER membranes should also be considered, it follows that, in the case studied, the membranes of two parts of the system should not be considered as permanently rough or smooth, respectively. Extensive exchange and interconversion already appear as probable features of the system.

For the moment, we cannot go beyond this step of formulating hypotheses. Additional data on the

turnover time of various ER membrane proteins, on the relationship of individual proteins in the rough vs. smooth ER, and on the sites of enzyme activity determined in situ during early stages of differentiation are needed before a choice among the various possibilities mentioned becomes possible.

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