

Functional and Structural Alterations of Liver Ergastoplasmic Membranes during DL-Ethionine Hepatocarcinogenesis¹

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SUMMARY

Different functional and structural properties of rat liver microsomes were studied during hepatocarcinogenesis induced by 0.25% DL-ethionine. During the first to fourth months of ethionine feeding, great decreases of cytochrome P-450 content, reduced nicotinamide adenine dinucleotide phosphate-dependent lipid peroxidation, and aminopyrine demethylase activity occurred. No changes in the reduced nicotinamide adenine dinucleotide phosphate-cytochrome *c* reductase activity were observed. These functional alterations were paralleled by an increase in membrane-free ribosomes and by changes in the relative proportions of phospholipid fatty acids in microsomes.

After the end of ethionine feeding, when hyperplastic nodules and/or hepatomas were present, the above functional and structural parameters were studied in the latter tissues, as well as in surrounding nonnodular liver. Decreases in cytochrome P-450 content, lipid peroxidation, and aminopyrine demethylase activity were documented in hyperplastic nodules and hepatomas. In hepatomas, the alterations were more marked and decrease of reduced nicotinamide adenine dinucleotide phosphate-cytochrome *c* reductase activity was also found. All these functional parameters were quite normal in surrounding nonnodular liver. Similarly, alterations in phospholipid fatty acid composition disappeared in surrounding nonnodular liver, but they partially persisted in both hyperplastic nodules and hepatomas. In contrast, the increase in membrane-free ribosomes also occurred in surrounding nonnodular liver, although to a lower extent than in hyperplastic nodules and hepatomas.

These data are discussed in relation to the problem of the cellular precursors of hepatomas.

INTRODUCTION

There exist many observations indicating that the oncogenic process consists of multiple steps (8, 13, 43). According to a recent evaluation of the molecular mechanisms of carcinogenesis (13), the carcinogenic stimulus induces the appearance of altered but nonneoplastic cells. The prolifera-

tion of these cells, associated with different cytological and biochemical alterations, ultimately produces neoplastic cells. During this process, some "key precursors" of tumor cells could appear (13). In the course of hepatocarcinogenesis an apparently constant liver lesion is the hyperplastic nodule, which has been considered a precursor of hepatoma (14). If key precursors do exist, some characteristics of tumors could appear before neoplastic transformation takes place. It has indeed been shown that during chemical carcinogenesis the synthesis of new antigens occurs in premalignant mammary tissue (48) as well as in skin papillomas (28, 29) preceding the carcinoma development. The later reappearance of α -fetoprotein has been documented during hepatocarcinogenesis, coincident with the development of hyperplastic nodules (27, 54). Recently, the presence of α -fetoprotein has been demonstrated within hyperplastic nodules by immunofluorescence (39). The loss of feedback control of hepatic cholesterol synthesis has also been documented in precancerous liver (25, 27).

In recent years different morphological alterations have been observed in endoplasmic reticulum of rapidly growing tumors. Studies in several laboratories (7, 30, 31) have given morphological evidence of smooth endoplasmic reticulum hypertrophy. Alterations of the polyribosomal patterns and a decrease in the relative amounts of membrane-bound polyribosomes have been demonstrated in hepatomas (9, 55, 56). Inhibition of several enzymatic activities (38) and alterations of protein (5) and lipid (2) composition of tumor microsomes have also been described.

With the aim of determining when these alterations appear in the course of hepatocarcinogenesis, we investigated the following aspects of precancerous tissues during DL-ethionine feeding: (a) different steps of microsomal electron transport chain; (b) the degree of aggregation of ribosomes and ribosome-membrane interactions; and (c) the lipid composition of microsomes. Preliminary reports of some of the results in this paper have been published previously (17, 20).

MATERIALS AND METHODS

Animals and Diet. Male and female Wistar rats weighing 120 to 150 g at the beginning of the experiment were used. Experimental animals were fed a basal diet (11) supple-

¹ Supported by a grant from the Consiglio Nazionale delle Ricerche of Italy.

Received April 23, 1975; accepted July 15, 1975.

mented with 0.25% DL-ethionine (Nutritional Biochemicals Corporation, Cleveland, Ohio) for a maximum of 5.5 months, followed by 1 to 4 months of basal diet. The controls were fed the basal diet alone. The animals were housed no more than 3/cage and were given tap water *ad libitum*. They were subjected to a 14-hr day-10-hr night regimen with light from 7 a.m. to 9 p.m. The animals were fasted 16 to 18 hr before decapitation.

Morphological Control. Careful histological study was performed on all tissues used for biochemical determinations. The tissues were fixed in a solution of 10% formaldehyde and 0.1 M sodium phosphate buffer (pH 7.0). Paraffin sections were routinely stained with hematoxylin and eosin; some sections were stained with van Gieson's stain. For determination of the percentages of various cell types, differential cell counts were performed according to the method of Trowell and Westgarth (52).

Preparation of Microsomes. Livers were removed and immersed in ice-cold 0.154 M sodium phosphate buffer (pH 7.4). When hyperplastic nodules and/or hepatomas were present, livers were placed on ice-cooled dishes and carefully dissected. A rim of hyperplastic or neoplastic tissue was routinely left to ensure complete separation of nodular or tumoral tissues from surrounding nonnodular liver. Hyperplastic nodules, hepatomas, and surrounding nonnodular liver were immersed in the above medium. The minced tissues were washed several times by decanting; then they were suspended in a volume of medium equivalent to 3 times their weight and homogenized in a Potter-Elvehjem homogenizer with 5 strokes of a tight pestle running at 1500 rpm. Microsomes were then isolated as in a previous work (16).

Assays. NADPH-cytochrome *c* reductase was determined by the spectrophotometric procedure of Phillips and Langdon (41). Aminopyrine demethylase was determined according to the method of Schoene *et al.* (45), and cytochrome P-450 was determined according to the method of Sato and Hagihara (44). The reaction system of May and Reed (32) was used to study the NADPH-dependent lipid peroxidation.

Preparation and Fractionation of Ribosomes. Male rats were used for these experiments. Postmitochondrial supernatants were prepared by a method patterned on the procedure of Adelman *et al.* (1). In this way a more complete recovery of rough endoplasmic reticulum than is possible with traditional procedures was obtained. Livers, hyperplastic nodules, and hepatomas were suspended in twice their weight of 1 M sucrose and were homogenized in a Potter-Elvehjem homogenizer by 8 strokes of a tight pestle running at 1300 rpm. The homogenates were filtered through a single layer of nylon cloth, and the filtrates were diluted by equal volumes of 2.5 M sucrose. They were then centrifuged for 1 hr at 27,000 rpm in an SW-27.1 rotor in a Spinco-Beckman L-65 centrifuge. Supernatants were mixed with one-half their volumes of water, rehomogenized, and centrifuged for 20 min at 18,000 $\times g$. The final supernatants were diluted with equal volumes of a solution containing 0.04 M Tris-HCl buffer (pH 7.4), 0.2 M KCl, and 0.01 M Mg(CH₃COO)₂. In order to ob-

tain total ribosomes, diluted supernatants were treated with one-tenth their volume of a solution containing 10% sodium deoxycholate and 10% Triton X-100. To obtain free ribosomes, water was substituted for detergents in some samples. Aliquots of these preparations were layered over a solution containing 1.8 M sucrose, 0.02 M Tris-HCl buffer (pH 7.4), 0.1 M KCl, and 0.005 M Mg(CH₃COO)₂ and were centrifuged for 12 hr at 166,000 $\times g$. RNA content of ribosomal pellets was determined according to the method of Fleck and Munro (18). Size distribution patterns of ribosomes were studied as previously described (22).

Analytical Determinations. Total lipids were isolated according to the method of Folch *et al.* (19). Neutral and polar lipids were separated by silicic acid columns (Unisil; Clarkson Chemical Co., Inc., Williamsport, Pa.). Phospholipid phosphorus was determined according to the method of Shin (46). Cholesterol was isolated by thin-layer chromatography using as a solvent *n*-heptane:isopropyl ether:formic acid (60:40:2) and was determined according to the method of Bowman and Wolf (3). Fatty acid methyl esters were prepared from the phospholipid fraction according to the method of Morgan *et al.* (37) and were separated by gas-liquid chromatography (Fractovap G. V.; Carlo Erba, Milano, Italy). Proteins were determined by a biuret method (21).

RESULTS

Liver Changes. The microscopic findings in livers of ethionine-treated rats are shown in Table 1. The table includes the changes observed during ethionine feeding as well as those observed at the termination of the feeding. Histological changes were classified according to the system of Farber (12). It appears that a few oval cells were present from the beginning of the experiment. Although they increased during the ethionine feeding, they rarely exceeded 30% of total liver cell population at any phase of the proceedings. No evident increase in mesenchymal cells was observed at any stage of induction. Similarly, no evident fibrosis was found in livers. Intensified nuclear basophilia and occasional increase in nuclear diameter were observed. The nuclear irregularities became evident at the 4th month

Table 1
Histological changes induced in rat liver by ethionine

Time (mos.)	Oval cells	Nuclear irregularity	Fibrosis	Nodular hyperplasia	Cancer
1	± ^a	-	-	-	-
2	+	-	-	-	-
3	+	±	-	-	-
4	±	++	-	±	-
6	+	++	-	+	±
7-8	+	+++	-	+++	++

^a The number of + signs refers to the intensity of the alterations, *i.e.*, number of oval cells, as assessed by differential cell counts, degree of nuclear irregularities, number and volume of hyperplastic nodules and hepatoma nodules. ±, very small changes; -, no changes.

of ethionine feeding and they increased in nodules and hepatomas, where occasional double nuclei and mitotic figures were present. Hyperplastic areas were observed at the 4th to 6th month. Sometimes small nodules of 1 to 3 mm in diameter were grossly evident at this time. Hyperplastic nodules of 7 to 10 mm in diameter and/or hepatoma nodules were present at the 7th to 8th month. At this time almost 70% of the livers contained hepatomas and about 90% contained nodules. Hepatomas were present in all rats that survived 10 months from the beginning of the experiment. The microscopic features of hyperplastic nodules and hepatomas were nearly identical to those described by Farber (12, 14).

Modifications of Drug-metabolizing System. In order to study the activities of the drug-metabolizing system in microsomes of precancerous tissues and hepatomas, the NADPH-cytochrome *c* reductase activity, cytochrome P-450 content, NADPH-dependent lipid peroxidation, and aminopyrine demethylase activity were determined (Chart 1). It appears that NADPH-cytochrome *c* reductase activity did not change in any tissue during or after the ethionine feeding except in the hepatoma, in which about 75% decrease occurred. By contrast, the cytochrome P-450 content, NADPH-dependent lipid peroxidation, and aminopyrine demethylase activity greatly decreased during ethionine feeding. Once the feeding was stopped, cytochrome P-450 content and the peroxidative and demethylase activities were quite normal in liver between nodules, but they were lower than the control values in nodules and hepatoma. Data reported in Chart 1 refer to female rats. Experiments performed with male rats gave practically identical results.

Polyribosomes Association. Chart 2 shows the size distribution patterns of ribosomes from normal liver, surrounding nonnodular liver, hyperplastic nodules, and ethionine-induced hepatoma. For purposes of comparison, the patterns relative to the transplanted hepatomas, Yoshida ascites AH-130 and Morris 5123, were included. It appears that no alterations in the size distribution patterns of ribosomes occurred in the surrounding nonnodular liver and in hyperplastic nodules. Similarly, no changes occurred in ethionine-induced hepatoma, which in this respect, differs from hepatomas AH-130 and Morris 5123. In the latter hepatomas, as in various other rapidly growing hepatomas (55, 56), there was a relative increase in the amount of monomeric and dimeric ribosomes. The polyribosomal patterns were also studied in liver cells during ethionine feeding. No evidence of changes was obtained at any time during the feeding.

Membrane-Ribosome Association. Total and membrane-free ribosomes in liver, hyperplastic nodules, surrounding nonnodular liver, and hepatomas are reported in Table 2. No variations in the total amount of ribosomes took place in any tissues studied except the hepatoma, in which a small but significant increase occurred. However, the increase could be a consequence of a lower content of blood and connective tissue in the tumor. The relative amounts of membrane-free ribosomes progressively increased in liver during the ethionine feeding, so that 3 months after the beginning of the experiment membrane-free ribosomes were

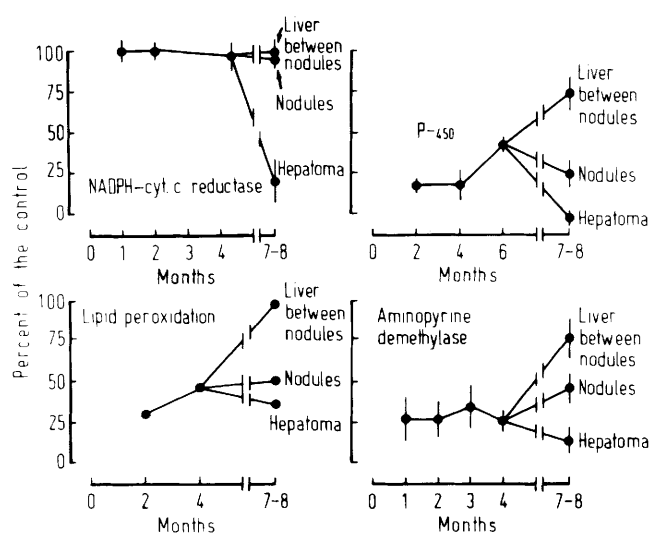


Chart 1. NADPH-cytochrome *c* (cyt. *c*) reductase cytochrome P-450, NADPH-dependent lipid peroxidation, and aminopyrine demethylase in microsomes from livers of ethionine-fed rats, as well as from hyperplastic nodules, surrounding nonnodular liver and hepatoma. Samples of different tissues were taken at the times indicated. A basal diet substituted for ethionine diet after 5.5 months of treatment. Data are expressed as percentage of the control values. The mean values \pm S.D. of NADPH-cytochrome *c* reductase and aminopyrine demethylase activities of control microsomes are 103.2 ± 8.6 and 6.91 ± 0.12 nmoles of substrates transformed per min and per mg of protein, respectively. Lipid peroxidation was determined by measuring the absorbance spectra, between 200 and 400 nm, of microsomal suspensions (0.4 to 0.8 mg of protein per ml) in a medium containing 0.1 M Tris-HCl buffer (pH 7.5), 8×10^{-5} M ADP, 2.4×10^{-6} M FeCl_3 , 5×10^{-6} M NADP, 2×10^{-6} M glucose 6-phosphate and 9 units of glucose-6-phosphate dehydrogenase. Data were calculated as $\Delta A_{237-400 \text{ nm}}/90 \text{ min/mg}$ of protein. The mean value of lipid peroxidation in normal liver microsomes is 0.310 ± 0.054 . The content of cytochrome P-450 of control microsomes is 14.05 ± 3.5 nmoles/g equivalent of liver. Each point represents mean of 4 to 8 experiments \pm S.D., with the exception of lipid peroxidation where the values are means of 2 experiments. Liver between nodules, nodules, and hepatomas from 2 to 3 animals were pooled for each experiment.

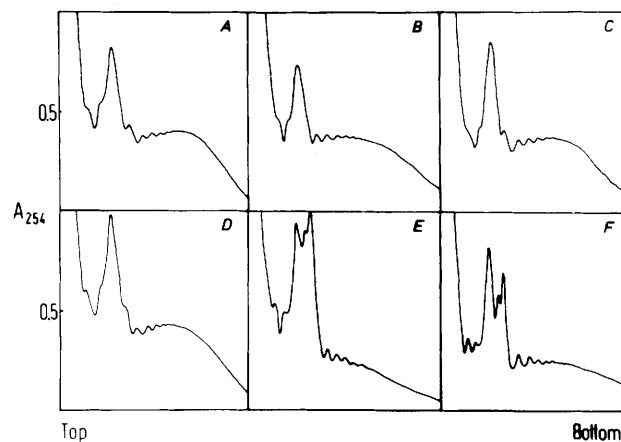


Chart 2. Patterns of ribosome distribution in postmitochondrial supernatants from normal liver (A), surrounding nonnodular liver (B), hyperplastic nodules (C), ethionine-induced hepatoma (D), hepatoma AH-130 (E), and hepatoma 5123 (F).

Table 2

Total and membrane-free ribosomes in liver from normal and ethionine-fed rats, as well as in hyperplastic nodules, in surrounding nonnodular liver and in hepatoma

Samples of the different tissues were taken at the times indicated after the beginning of the ethionine feeding. A basal diet was substituted for the ethionine diet at the 5th month of treatment.

Tissues and treatment	Mos.	No. of experiments	Total ribosomes ^a	Membrane-free ribosomes ^a	$\frac{\text{Free}}{\text{Total}} \times 100$
Liver					
Control	1	4	2.55 ± 0.09 ^b	0.67 ± 0.07	26.3
Ethionine-fed	1	4	2.46 ± 0.12	0.88 ± 0.07	35.8
Liver					
Control	2	3	2.73 ± 0.07	0.74 ± 0.04	27.1
Ethionine-fed	2	5	2.52 ± 0.10	1.05 ± 0.10	41.7
Liver					
Control	3	2	2.61	0.70	26.8
Ethionine-fed	3	3	2.51 ± 0.14	1.29 ± 0.08	51.4
Liver					
Control	6-7	5	2.72 ± 0.05	0.82 ± 0.12	30.1
Nodules ^c	6-7	4	3.04 ± 0.34	2.00 ± 0.08	65.7
Surrounding nonnodular liver ^c	6-7	3	2.48 ± 0.31	1.20 ± 0.16	48.4
Hepatoma ^c	6-7	4	3.30 ± 0.20	2.83 ± 0.11	85.8

^a mg of RNA/g equivalent of tissue.

^b Mean ± S.D.

^c Tissues from 2 to 3 animals were pooled for each experiment.

almost doubled with respect to control livers. At the end of the ethionine feeding, when hyperplastic nodules and/or hepatomas were present, the amount of membrane-free ribosomes in surrounding nonnodular liver was 1.5 times higher than in control liver. In hyperplastic nodules and hepatoma, a great increase of membrane-free ribosomes occurred. In the former tissue there were 2.4 times as many membrane-free ribosomes as in control liver. In hepatoma the increase was 3.5 times as many. The above alterations appear more clearly if the values of membrane-free ribosomes as percentage of total ribosomes are considered. As shown in Table 2, the percentage of membrane-free ribosomes ranged between 26.3 and 30.1% in control livers. There was a progressive increase from 35.8 to 51.4% in livers of ethionine-fed rats between the 1st and the 3rd month of treatment. After termination of the feeding, membrane-free ribosomes increased further in hyperplastic nodules and hepatomas to 65.7 and 85.8%, respectively, whereas they did not change significantly in surrounding nonnodular liver.

Changes in Lipid Composition. The lipid composition of microsomes from different sources was studied during and after ethionine feeding. At no stage of the experiment were alterations found in microsomal total lipid, triglycerides, free fatty acids, or free and esterified cholesterol in any tissues examined. The phospholipid content was only 0.140 mg/mg of protein in hepatoma microsomes versus 0.257 mg/mg protein in control microsomes. No changes in phospholipid content were found in other tissues, during or after ethionine feeding. Thus, ethionine-induced hepatomas behaved, as concerns the phospholipid content, as a number of transplanted hepatomas (16).

Evident changes were found, during tumor induction, in major fatty acids of microsomal phospholipids. As shown in Table 3, an increase occurred in myristate during the 1st 3 months of treatment with ethionine. Palmitate increased

during the 1st, 2nd, and 4th months, and palmitoleate, oleate, and linoleate increased during the whole treatment. In contrast, stearate and arachidonate sharply decreased during the feeding. At the end of the ethionine administration, no significant changes in the relative proportions of phospholipid fatty acids took place in surrounding nonnodular liver, compared with control liver. On the contrary, evident modifications occurred in both hyperplastic nodules and hepatomas. In hepatoma microsomes there was an increase in myristate, palmitate, palmitoleate, and oleate, whereas stearate, linoleate, and arachidonate decreased. In microsomes from hyperplastic nodules very similar changes were observed, with the exception of a smaller increase in palmitoleate and no changes in myristate and linoleate.

DISCUSSION

The data in this paper indicate that evident alterations of hepatic microsomal membranes take place during ethionine feeding. Impairment of some steps of the drug-metabolizing system, hypertrophy of smooth endoplasmic reticulum and modifications of the relative proportions of fatty acids in phospholipids, appear early in liver cells and persist during ethionine feeding (see also Ref. 14).

Alterations of the drug-metabolizing system in liver microsomes of ethionine-fed rats include lowering of cytochrome P-450 content, NADPH-dependent lipid peroxidation, and aminopyrine demethylase activity. Inhibition of some microsomal enzymes has already been shown during acute treatment with ethionine (24). Phenobarbital-induced increase in microsomal strychnine metabolism was prevented in the rat liver following a 6-day treatment with low doses of ethionine (33). Inhibition of *p*-dimethylaminoazobenzene *N*-demethylation was observed by

Table 3

Fatty acid composition of phospholipids in microsomes from normal and ethionine-fed rats, as well as from hyperplastic nodules, surrounding nonnodular liver and hepatomas

Source of microsomes	Mos.	No. of experiments	Fatty acids (relative %)							
			14:0	16:0	16:1	18:0	18:1	18:2	20:4	
Liver										
Control	1	3	1.40 ± 0.01 ^a	15.94 ± 1.62	1.27 ± 0.00	33.35 ± 4.10	9.17 ± 1.93	7.97 ± 0.84	34.76 ± 3.60	
Ethionine-fed	1	4	10.13 ± 3.73	24.00 ± 4.80	2.65 ± 0.00	20.10 ± 2.47	14.44 ± 2.92	14.13 ± 1.14	16.55 ± 2.55	
Liver										
Control	2	3	3.60 ± 0.60	19.48 ± 3.10	2.54 ± 0.41	31.43 ± 2.54	7.58 ± 0.76	5.90 ± 0.69	32.47 ± 2.30	
Ethionine-fed	2	4	5.57 ± 1.04	22.31 ± 1.60	2.44 ± 0.56	23.47 ± 6.87	12.09 ± 0.76	14.11 ± 1.94	22.01 ± 6.84	
Liver										
Control	3	3	3.79 ± 0.80	17.30 ± 2.80	1.23 ± 0.21	32.66 ± 1.22	7.10 ± 0.38	7.22 ± 0.75	32.70 ± 3.00	
Ethionine-fed	3	5	7.28 ± 2.97	20.15 ± 3.27	2.37 ± 0.10	18.04 ± 2.41	18.04 ± 2.62	13.14 ± 2.42	16.98 ± 3.59	
Liver										
Control	4	3	1.40 ± 0.08	14.44 ± 1.62	1.25 ± 0.23	33.38 ± 3.10	7.63 ± 0.41	8.62 ± 1.27	33.35 ± 1.59	
Ethionine-fed	4	4	1.41 ± 0.67	24.47 ± 1.33	3.04 ± 0.00	22.93 ± 2.54	16.73 ± 0.24	16.31 ± 1.91	21.22 ± 1.87	
Liver										
Control	7-9	6	1.55 ± 0.20	18.66 ± 0.00	0.90 ± 0.00	35.11 ± 5.40	10.08 ± 0.81	5.84 ± 1.01	31.41 ± 2.60	
Surrounding nonnodular liver ^b	7-9	5	1.03 ± 0.10	17.78 ± 4.45	0.18 ± 0.03	30.69 ± 3.82	12.09 ± 1.97	7.99 ± 0.22	33.24 ± 1.11	
Nodules ^b	7-9	4	0.94 ± 0.33	27.78 ± 3.64	2.18 ± 0.00	21.04 ± 4.63	17.66 ± 2.65	9.15 ± 2.74	24.25 ± 2.84	
Hepatoma ^b	7-9	4	2.50 ± 0.00	28.01 ± 2.86	6.20 ± 1.18	21.29 ± 3.00	18.78 ± 4.32	3.94 ± 0.68	22.28 ± 2.70	

^a Mean ± S.D.

^b Tissues from 2 to 3 animals were pooled for each experiment.

Takamiya *et al.* (51) as a consequence of a 6- to 22-week treatment with 0.05% DL-ethionine. Such low doses of the carcinogen did not affect the cytochrome P-450 content. The latter result apparently contrasts with our observation of a 70% decrease in cytochrome P-450 content after 2 to 4 months of ethionine feeding. However, we used a dose of carcinogen 5 times higher than that used by Takamiya *et al.* (51). Evident decrease of cytochrome P-450 content was also found by Oyanagui *et al.* (40) in liver microsomes after a 22-week feeding of 3'-methyl-4-dimethylaminoazobenzene or 4'-methyl-4-dimethylaminoazobenzene. Finally, inhibition of lipid peroxidation as well as of aminopyrine demethylase activity was demonstrated by Meyer and Barber (36) during chronic *p*-dimethylaminoazobenzene feeding. Those authors also documented an inhibition of NADPH-cytochrome *c* reductase, which we did not observe during ethionine feeding.

No satisfactory explanation for the decrease in microsomal functional activities during ethionine feeding can be given at this time. One possibility is that the observed changes depend upon inhibition of protein synthesis in liver. However, no polyribosome disaggregation was observed in liver cells during chronic ethionine administration. Since ethionine-induced inhibition of protein synthesis was shown to be dependent on polyribosome disaggregation (53), the results in our experimental system suggest that no extensive inhibition of protein synthesis would occur. In addition, Farber and Corban (15) found that, during the treatment with high doses of ethionine, inhibition of protein synthesis takes place in female but not in male rats. Our observation of functional impairment in microsomes of both female and male rats could indicate that inhibition of protein synthesis probably does not play a role in the observed phenomena.

Our data indicate that ethionine treatment causes a

progressive increase in the relative amounts of membrane-free ribosomes. Morphological evidence of this phenomenon was given by Svoboda and Higginson (49) for experimental conditions similar to those adopted in this study. The question arises whether there is some relationship between these morphological changes and functional impairment of microsomes during the ethionine feeding. Some experimental evidence exists indicating that the rough endoplasmic reticulum is involved in the synthesis of different microsomal constituents such as catalase, glucose-6-phosphatase, and glycoproteins (4, 6, 23, 50). The decrease of cytochrome P-450 content and of aminopyrine demethylase activity, paralleled by an increase in membrane-free ribosomes during the ethionine treatment, might be in line with these observations. NADPH-cytochrome *c* reductase, which does not change during the ethionine feeding, appears to be synthesized by both free and bound polyribosomes (42). However, we also found an increase in membrane-free ribosomes in surrounding nonnodular liver, which exhibits quite normal cytochrome P-450 content and aminopyrine demethylase activity. Thus, it appears that microsomal constituents studied in this paper behave differently from catalase, glucose-6-phosphatase, and glycoproteins.

According to our observations, changes in cellular composition that are as marked as those found by others (26) do not exist during the administration of 0.25% ethionine. Oval cells reach, at maximum, 30% of the liver cell population; there are no evident changes of mesenchymal cells and no extensive necrosis was observed. Thus, the possibility of structural changes causing the functional impairment of liver microsomes of ethionine-fed rats seems unlikely.

After the end of the ethionine feeding, the cytochrome P-450 content and the peroxidative and demethylase activi-

ties are decreased in hyperplastic nodules and in hepatomas, but they are nearly normal in surrounding nonnodular liver. The alterations of the phospholipid fatty acids in microsomes at the end of the ethionine feeding disappear in surrounding nonnodular liver, but they persist in hyperplastic nodules and in hepatomas. This indicates that the functional impairment and the alterations of chemical composition became irreversible in both hyperplastic nodules and hepatomas. These results are in agreement with the histochemical evidence by Epstein *et al.* (10) of a progressive decrease in glucose-6-phosphatase and glycogen phosphorylase activities in hyperplastic nodules, but not in surrounding nonnodular liver.

The observation of a great increase in the relative amounts of membrane-free ribosomes in hyperplastic nodules and hepatomas is in accord with the morphological evidence by Merkow *et al.* (34, 35) of a decrease of rough endoplasmic reticulum in ethionine-induced nodules and hepatomas. The modifications of ribosome-membrane interactions behave differently from functional activities and lipid composition, regarding their reversibility. In fact, increased amounts of free ribosomes are still present after the end of the ethionine feeding, not only in hyperplastic nodules and hepatomas but also in surrounding nonnodular liver. The increase in membrane-free ribosomes, however, is significantly lower in the latter tissue than in nodules and in hepatomas. However, irreversibility of the increase in membrane-free ribosomes in surrounding nonnodular liver is not explained by our data. No evidence of degenerative processes or necrosis, which could explain these features, was obtained by microscopic observations of hyperplastic nodules or surrounding nonnodular liver. In addition, the normal polyribosomal patterns in both tissues would exclude the existence of appreciable necrotic phenomena. It could be tentatively suggested that both hyperplastic nodules and surrounding nonnodular liver possess a certain degree of biochemical heterogeneity concerning the ribosome-membrane interactions. One might hypothesize that the observed differences between these 2 tissues could depend on varying ratios between cellular types, *i.e.*, normal and altered cells, or cells with different degrees of alterations.

Our findings, taken as a whole, seem to support the hypothesis that during the ethionine feeding the smooth endoplasmic reticulum of liver cells becomes hypoactive and hypertrophic (14). When the carcinogen administration is stopped the alterations disappear or decrease in most of the liver cell population, but they persist in hyperplastic nodules. This could indicate that hyperplastic nodules take origin from a new type of liver cells, in which the alterations become irreversible. At a certain time of the ethionine treatment these cells proliferate to form hyperplastic areas (14) and then nodules. On the basis of many parameters considered in this study, hyperplastic nodules show some functional and structural patterns to be similar, although not identical, to those of hepatoma, and clearly different from surrounding nonnodular liver. This behavior seems to be consistent with the hypothesis (14) that some cells of hyperplastic nodules are precursors of cancer cells. Some hepatomas develop within hyperplastic nodules (12, 14).

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