Metabolism of Bile Components during Hepatocarcinogenesis

I. Conjugation of Cholic Acid in Vitro in Experimental Liver Carcinogenesis*

CURTIS L. SONGSTER,† SYDNEY S. SCHOCKET, JR.,† AND EMMANUEL FARBER‡

(Departments of Pathology and Biochemistry, Tulane University School of Medicine, New Orleans, Louisiana)

SUMMARY

The conjugation of cholic acid with taurine was measured in liver microsomes and supernatant from male rats (a) fed diets containing ethionine, 3'-methyl-dimethylaminoazobenzene (3'-Me-DAB), or 2-acetylaminofluorene (2-AAF) or (b) subjected to ligation of the common bile duct. A fairly rapid decrease in in vitro conjugation was found in the livers of the animals fed ethionine, beginning 2 days after initiation of the diet and reaching a minimum value of less than 1 per cent of the control level within 10–14 days. The defect in conjugation persisted for as long as the ethionine was fed (34 days). The level of conjugation returned to the control value within 10 days after the ethionine was removed from the diet. A similar defect was not found in the livers of rats fed 3'-Me-DAB or 2-AAF under conditions which lead to the development of liver cancer. Rats subjected to bile duct ligation also showed no defect in taurocholate synthesis.

Since ethionine, 3'-Me-DAB, 2-AAF, and bile duct ligation all induce a similar proliferation of bile duct epithelial cells, it must be concluded that the defect in conjugation of cholic acid with taurine cannot be of significant importance as a biochemical basis for the ductular proliferation. If there is truly a relationship between alterations in the metabolism of bile components or in bile secretion and the development of ductular proliferation, the metabolic correlate must be found in reactions other than the conjugation of cholic acid with taurine.

One of the most interesting early morphologic carcinogens is by no means limited to such reactions of the liver to the administration of agents. The two most notable exceptions are ligation or obstruction of the biliary system (5) and the administration of the noncarcinogen, a-naphthyl-isothiocyanate (19). Even though differences have been observed with both light and electron microscopy, the morphologic appearance and behavior of the hyperplastic ductular cells is sufficiently similar under these various conditions to suggest some common pathogenetic mechanism.

The present study was undertaken with the hope of elucidating the biochemical aberrations underlying or associated with the initiation of ductular proliferation. The experimental approach was based upon the hypothesis that ductular hyperplasia might be triggered by alterations in the metabolism of free or conjugated cholic acid carcinogens, it is by no means limited to such agents. The two most notable exceptions are ligation or obstruction of the biliary system (5) and the administration of the noncarcinogen, a-naphthyl-isothiocyanate (19). Even though differences have been observed with both light and electron microscopy, the morphologic appearance and behavior of the hyperplastic ductular cells is sufficiently similar under these various conditions to suggest some common pathogenetic mechanism.

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and other bile acids. This thesis is based on the following observations: (a) ductular proliferation follows obstruction of biliary flow, as well as the administration of many hepatotoxic agents, (b) biliary obstruction characteristically results in an elevation in serum cholesterol, probably owing to some alteration in the synthesis or metabolism of cholesterol in the liver (23), (c) cholic acid and chenodeoxycholic acid are the major metabolic end-products of cholesterol metabolism in the liver (1) and (d) bile acids are potent pharmacodynamic agents and, if altered, might possibly stimulate bile duct epithelial hyperplasia.

The initial phase of this study, the results of which are reported in this communication, is concerned with the capacity of liver fractions to conjugate cholic acid with taurine at different times after the feeding of diets containing ethionine, 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) or 2-acetylamino-6-fluorene (2-AAF) or after bile duct ligation.

The in vitro conjugation of cholic acid with taurine in rat liver homogenates and cell fractions was reported in 1955 by Bremer (3), Bremer and Gloor (4), and by Siperstein (29). On fractionating liver cells it was found that the microsome fraction and the supernatant were both essential. Further work by Bremer (3) and Siperstein and Murray (30) with rats and Elliott (7-9) with guinea pigs established the following formulation:

1. Cholic acid + coenzyme A \( \rightarrow \) choly-CoA;
   \[ \text{ATP, Mg}^{++} \]
   \[ \text{microsomes} \]

2. Cholly-CoA + taurine \( \rightarrow \) tauro-conjugate cholic acid + coenzyme A.

These two reactions were the basis for the measurement of the conjugating ability of the liver in the present study.

**MATERIALS AND METHODS**

White Wistar (Carworth Farms) and Holtzman rats of both sexes were used. The animals were housed four to six per cage in an air-conditioned room and maintained on a commercial Purina chow diet until used or until special diets were begun. In the acute experiments with ethionine, five male and five female Holtzman rats, weighing 180-280 gm., were given injections intraperitoneally of a 2.5 per cent aqueous solution of dl-ethionine in a total dose of 1 mg/gm body weight in two equal doses administered 2 hours apart. Equal numbers of control animals were given injections in the same fashion with normal saline. All animals were sacrificed 5 hours after the first injection, and cholic acid conjugation was measured in suitably prepared cell fractions of the liver, as will be described subsequently.

In the first ethionine feeding experiment, 30 male Holtzman rats, weighing from 170 to 220 gm., were divided into six equal groups. Three experimental groups were fed a 16 per cent casein diet containing 0.25 per cent dl-ethionine, as previously described (11), and the other three control groups were fed the same diet without the ethionine. One experimental and one control group were sacrificed at weekly intervals. The second ethionine feeding experiment was essentially a repetition of the first one, with the use of 40 male Holtzman animals weighing from 214 to 240 gm. In this experiment, two experimental and two control rats were sacrificed at daily intervals for the 1st week and subsequently at weekly intervals for 4 weeks. In the third experiment, 98 male Carworth Farms animals, weighing 116-178 gm., were divided into two groups. Thirty-six were fed the control diet, and the remaining 42 were fed the same diet containing ethionine. Two experimental and two control animals were sacrificed twice weekly for 5 weeks. At the end of this period, the remaining animals in the experimental group were put on the control diet without ethionine, and the control animals were continued on the same diet. Two control and two experimental animals were sacrificed twice weekly for 5 weeks and at weekly intervals thereafter until all had been sacrificed.

In the 3'-Me-DAB experiment, twenty Carworth Farms male rats weighing from 189 to 337 gm. were divided into four equal groups. One group was fed the azo dye basal diet containing 16 per cent casein (16) without addition of the azo dye, and the remaining three groups were fed the same diet containing 0.06 per cent 3'-Me-DAB. One control and two experimental animals were sacrificed at weekly intervals for 5 weeks. In the 2-AAF experiment eighteen male Carworth Farms rats, weighing from 128 to 150 gm., were divided into three equal groups. One group was fed the basal 2-AAF grain diet of Miller et al. (21) without the carcinogen, and the other two groups were fed the same diet containing 0.04 per cent 2-AAF. One control and two experimental rats were sacrificed at weekly intervals for 1 month. In the experiment on common bile duct ligation, nine male Carworth Farms animals weighing from 160 to 200 gm. were subjected to ligation of the common bile duct under ether anesthesia. Nine animals of the same strain and weight received a sham operation of comparable duration. Three control and experi-
mental animals each were sacrificed at 2, 5, and 7 days after the operation. In the experiments with the hepatomas, explants of Novikoff hepatoma were transplanted subcutaneously in three male Carworth Farms rats, and Dunning hepatoma was similarly transplanted into three Fischer male rats. One rat bearing a primary hepatocellular carcinoma induced in this laboratory by the feeding of ethionine (10) was also used. This animal had been fed the stock diet without ethionine for 10 weeks before sacrifice. After the rats were sacrificed, all the hepatomas were dissected free of necrotic tissue before homogenization. In each instance, one liver aliquot was taken for measurement of cholic acid conjugation, and others were fixed in Bouin's solution. Sections for microscopic study were stained with hematoxylin and eosin.

All animals were fasted overnight before being sacrificed by a blow on the head. The livers were rapidly removed, cooled, and weighed. A 4-gm. aliquot was homogenized for 45 seconds in a glass homogenizer in 8 ml. of cold, freshly prepared 0.76 M sucrose medium containing 0.004 M MgCl₂, 0.025 M KCl, and 0.035 M KHCO₃. The homogenate was fractionated in the refrigerated Spinco Model L preparative centrifuge with a number 40 rotor. The initial centrifugation was performed at 15,000 r.p.m. for 15 minutes. The supernatant from this run was carefully removed and recentrifuged at 40,000 r.p.m. for 60 minutes to separate the microsomes from the nonparticulate supernatant. The supernatant was used directly. The microsome pellet was washed 3 times with 1-ml. quantities of 0.1 M phosphate buffer, pH 7.6, and was then suspended with gentle homogenization in the same buffer, with approximately 0.5 ml. buffer for every gm. of original liver weight. It is noteworthy that repeated attempts to demonstrate taurocholate formation by conjugation of cholic acid and taurine, with the use of the whole homogenate instead of the mixture of microsomes and supernatant, were uniformly negative.

Conjugation was carried out by incubation for 1 hour at 37° C. in a Dubnoff metabolic shaker. The components of the incubation medium were added to screw-capped tubes in the following order: 0.05 ml. of 0.55 M MgCl₂, 0.1 ml. of 0.1 M potassium cholate, 0.2 ml. of 0.01 M coenzyme A (Sigma Chemicals), 0.1 ml. of 0.1 M taurine, 0.1 ml. of 0.5 M KF, 0.1 ml. of 1 N KOH, 0.5 ml. of 0.1 M disodium adenosine triphosphate (ATP), 0.3 ml. liver supernatant, and 0.6 ml. of the microsome suspension. The addition of versene or cysteine (7) had no effect upon the degree of conjugation of the system. After incubation, 1 ml. of a 3 per cent NaHCO₃ solution was added, and the tubes were placed in a boiling water bath for 10 minutes to coagulate the protein. At the end of this period, 0.4 ml. glacial acetic acid was added to further facilitate the protein precipitation. The tubes were then cooled and centrifuged at 2000 r.p.m. in a small clinical centrifuge for 30 minutes. The clear supernatant solution contained both the unreacted cholic and the product of the reaction, taurocholic acid. Since each reacts equally with the modified Pettenkofer reagent as used in this study, they were first separated by ether extraction. Various reported separation procedures (cf. 30) were tested with uniformly unsatisfactory results. However, by extracting 2 ml. of the supernatant solution twice with 15-ml. samples of anhydrous ethyl ether, free of peroxide, virtually complete separation of the ether-soluble unconjugated cholic acid from the water-soluble taurocholic acid was achieved (Table 1).

The concentration of taurocholic acid in the aqueous phase was measured by a modification of the original Pettenkofer reaction. Gregory and Pascoe (15) first described a modification by which a stable blue color is produced instead of a pink color after addition of the furfural and sulfuric acid as in the original procedure. This was modified again by Reinhold and Wilson (26) and finally by Irvin et al. (17), whose procedure was used in this study. After ether extraction, 0.5 ml. of the aqueous phase was added to 13 ml. of 16 N sulfuric acid. To this was added 1 ml. of a 1:100 dilution of triple-distilled furfural, and the mixture was heated in a water bath at 65° C. ± 0.2° for 15 minutes. The reaction was stopped, and the color was stabilized by cooling in ice for 3 minutes and
then by the addition of 5 ml. glacial acetic acid. The resulting blue color was stable for several hours and was measured in a Beckman DU spectrophotometer at a wave length of 680 m. u. Beer's law was obeyed over the range of concentrations used (up to 5 μmoles of cholic or taurocholic acid). Since the yield of microsomes varied somewhat from animal to animal, all results are expressed as μmoles taurocholate formed per 100 mg. microsomal protein. Protein was determined in the microsome suspension by the method of Lowry et al. (20) after precipitation and washing of the protein with 5 per cent tricholoroacetic acid (TCA) and extraction of the lipides with alcohol and alcohol-ether mixture [3:0]. Control incubation tubes, containing all the components except CoA and ATP, were always run.

RESULTS
In the acute experiments with ethionine, the capacity of the liver to conjugate cholic acid with taurine was the same in preparations from both control and ethionine-treated animals. Under similar conditions it has been shown that ethionine-treated female rats, but not male rats, show a significant inhibition of protein synthesis (13, 25) and a striking decrease in the concentration of hepatic ATP (27). In the first ethionine feeding experiment the in vitro conjugation of cholic acid with taurine was remarkably decreased to less than 20 per cent of the control values within 1 week after starting to feed the ethionine-containing diet. This level dropped further by the end of the 2d week to only 3 per cent of the control value and remained at this level to the end of the 3d week.

The second experiment was run in the hope of delineating more accurately the time sequence of the decrease in hepatic conjugating ability that followed the feeding of ethionine. The results of this experiment are shown in Chart 1. It can be seen that there was a progressive fall in the activity of the taurocholate conjugation system beginning on the 2d day after the animals were placed on the ethionine-containing diet and reaching a minimum sometime between 1 and 2 weeks. The activity remained virtually absent thereafter to the end of the 4th week, when the experiment was terminated. Histologic sections of the livers showed only a slight degree of fatty change with no obvious ductular proliferation for the first 2 weeks. Hyperplasia of the ductular cells began to appear during the 3d week and progressed slowly during the following week.

At this time in the study, we learned through other experiments in the laboratory that Holtzman rats are much less susceptible to the induction of liver cancer by ethionine than are Wistar-Carworth Farms or Fischer rats (12). It appeared important to determine whether the dramatic inhibition of cholic acid conjugation in Holtzman rats could also be demonstrated in a strain more susceptible to ethionine carcinogenesis. In addition, it was of considerable interest to learn whether the liver would or would not recover the capacity to form taurocholate in vitro after ethionine had been removed from the diet. The results of this experiment (Experiment three) are plotted in Chart 2. Here it may be seen that the Carworth Farms-Wistar rats showed essentially the same pattern of response to ethionine as did the Holtzman rats. The capacity to conjugate cholic acid with taurine in the in vitro liver system virtually disappeared sometime between 8 and 12 days after the beginning of the ethionine regimen and did not return as long as ethionine was fed. However, the liver completely recovered from this inhibition within 8–10 days after ethionine was removed from the diet. The shape of the curve for the period from the beginning of loss of the enzyme activity to the time of full recovery was remarkably symmetrical.

Since the taurocholate-conjugating system is composed of two cellular fractions, the microsomes and the nonparticulate supernatant, it was important to know whether the loss in enzyme activity attendant upon the feeding of ethionine involved one or both of the fractions. This was
studied by testing various combinations of microsome and supernatant fractions from control and ethionine-treated animals. The results of one such experiment are presented in Table 2. It may be seen in this table that the defect was located predominantly in the microsomes, although some loss of activity might be attributable to the supernatant fraction as well. The results shown on lines 4 and 5 are especially interesting. From line 4 it would appear that the supernatant from the control liver could partially restore the loss of activity of the microsomes from the ethionine-treated liver. However, if the preparation was centrifuged for 2 hours instead of 1 hour, thus presumably removing some residual microsome fragments, then the capacity of the supernatant fraction to restore lost activity disappeared. The same supernatant retained full activity when combined with normal microsomes. The results with mixtures of microsomes from ethionine-treated and control animals and with different combinations of microsomes and supernatants do not favor the view that the loss of activity in microsomes from ethionine-fed animals is the result of a rapidly acting soluble or diffusible inhibitor but do not rule out the presence of a more slowly acting inhibitory substance.

Animals fed the ethionine-containing diet always lose weight for the first 2 weeks and then resume a normal pattern of weight gain (10). Conceivably, the loss of weight rather than the ethionine might be responsible for the loss in enzyme activity. This was checked several times during this study by measuring the taurocholate synthetic activity of liver microsome and supernatant fractions prepared from rats with comparable weight loss induced by fasting them for periods up to a week. The levels of conjugation in preparations from these animals were consistently within the same range as the control rats.

The close correlation in time between the loss in the in vitro conjugating ability and the first appearance of ductular proliferation in the liver suggests a possible relationship between these effects and naturally led to experiments with bile duct ligation and with hepatic carcinogens, 3'-Me-DAB and 2-AAF. Male rats of the Carworth Farms-Wistar strain had their common bile duct ligated and were subsequently sacrificed at 2, 5, and 7 days thereafter. Control animals were sham-operated. All the livers of these animals showed varying degrees of bile ductular proliferation. However, the activity of the cholic acid-conjugating system in every animal was within the

<table>
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<tr>
<th>SOURCE OF MICROSOMES</th>
<th>SOURCE OF SUPERNATANT</th>
<th>TAUROCHOLATE SYNTHESIZED (MICROMOLE/100 MG MICROSOMAL PROTEIN)</th>
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<tr>
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<td>Ethionine</td>
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The column in which the "x" is placed indicates in each instance whether the material added to the incubating solution came from a control or an ethionine-fed rat. 

x/2 Indicates that half the total amount of microsomes used in the incubation came from the source shown.

* Supernatant centrifuged for one additional hour.

TABLE 2

PRODUCTION OF TAUROCHOLATE IN VITRO FROM TAURINE AND CHOLIC ACID INCUBATED WITH MICROSES AND SUPERNATANT FROM CONTROL RATS AND RATS FED ETHIONINE FOR 28 DAYS

Chart 2.—Levels of in vitro conjugation of cholic acid with taurine in the livers of Wistar (Carworth Farms) rats fed ethionine-containing (X-X-X) or control (-----) diets. The animals on the ethionine were changed to control diet as indicated by the arrow. Each point is the mean of the value from two animals. The error shown is the standard deviation.
normal range (46–56 μmoles taurocholate formed /100 mg microsomal protein). Similarly, animals fed 3'-Me-DAB or 2-AAF in appropriate diets showed no striking or consistent departure from the normal capacity to form taurocholic acid from cholic acid and taurine in the in vitro microsome-supernatant system prepared from their livers (Table 3). Although some animals showed some decrease from the corresponding control, the pattern was not consistent. The results clearly indicate that the feeding of these potent hepatic carcinogens does not produce the same biochemical defect in bile acid metabolism in the liver as was observed with ethionine.

A few determinations were made on microsome-supernatant preparations from the Dunning hepatoma, the Novikoff hepatoma, and one hepatoma induced by ethionine. These tumor preparations were found to be uniformly without conjugating activity. Unfortunately, it was not determined whether this was the result of an abnormality in the microsomal fraction, in the supernatant, or in both. It is noteworthy that the livers of the animals bearing the subcutaneous hepatoma transplants and the non-neoplastic portion of the liver of the rat bearing the ethionine-induced tumor showed normal activities of the conjugating system. The latter animal had been on a stock diet without ethionine for several weeks.

### DISCUSSION

It is evident from this study that the feeding of ethionine-containing diets under conditions which will ultimately lead to the appearance of liver cancer induced a rapid and striking decrease of an enzyme system in the liver which conjugates cholic acid with taurine. It is also apparent that it is the microsomal component which shows the greatest change on ethionine feeding, even though the effectiveness of the supernatant component also appears to decrease to some degree.

It is well known that cholic acid and other bile acids occur in the bile in the conjugated form (1). The two compounds with which cholic acid is combined are taurine and glycine. Taurine is the predominant one in the rat, and glycine is the main one in man. If the upset in hepatic taurocholate formation, as measured in vitro in this study, is truly a reflection of the condition in the intact functioning liver, one would anticipate that the feeding of ethionine-containing diets should be accompanied by alterations in the composition of the bile, at least insofar as the state of conjugation of bile acids is concerned. Popper et al. (24) have found characteristic alterations in the excretion of bile acids under these circumstances. They reported that the volume of bile as well as the quantity of bile solids excreted is increased several-fold during ethionine feeding. Simultaneously, the rate of bile acid secretion decreased, with a reversal of the ratio between trihydroxy and dihydroxy acids. La Rocca et al.2 found that the dominant conjugation form of cholic acid in bile from rats fed ethionine-containing diets is glycocholate rather than taurocholate and that a new bile acid appears which is apparently not present in bile from control rats. No causal relationship has thus far been demonstrated between any of these biochemical alterations and ductular hyperplasia in the liver. Nevertheless, the results are interesting enough in themselves to call for exploration.

The mechanism of the inhibitory effect of dietary ethionine on the conjugation system of cholic acid is not clear. Since the parenteral administration of ethionine induces a decrease in the concentration of hepatic ATP (27) and since this decrease in turn (33, 34) inhibits liver protein synthesis, the possibility was entertained that the loss in the activity of cholic acid-conjugating system might be due to an inhibition of enzyme synthesis. However, recent work by Villa-Trevino and Shull3 failed to show any decrease in ATP levels or in

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*H. S. La Rocca, J. G. Hamilton, and E. Farber, unpublished work.

3S. Villa-Trevino and K. H. Shull, personal communication.
the ribosomal incorporation of amino acids into protein in the livers of rats fed the same ethionine-containing diet for periods up to 5 weeks. The failure to observe any effect of ethionine upon the in vitro conjugation of cholic acid in the acute experiment is consistent with these findings.

Alternatively, the loss of capacity to form taurocholic acid in the ethionine-fed rat could be the result of a direct inhibitory effect of an abnormal metabolite, S-adenosylmethionine. This ethionine derivative is produced from ethionine in the liver by S-activation via the methionine S-activating enzymes (6) and is capable of inhibiting several enzymes in vitro (32). Although our observations as reported in this paper do not suggest that bile acid conjugation was inhibited by a soluble, rapidly acting inhibitor such as S-adenosylmethionine, it is possible that this ethionine metabolite could exert delayed inhibitory effects through a chain of interactions.

It may be concluded from this study that one facet of bile acid metabolism, the conjugation of cholic acid with taurine, is not related to ductular hyperplasia. Ligation of the common bile duct and feeding of diets containing 3'-Me-DAB and 2-AAF failed to reproduce the biochemical lesion that resulted from the feeding of ethionine. It is thus evident that the ductular proliferation that develops under all these conditions is not dependent on the inhibition of taurine conjugation. However, these findings do not rule out the possibility that other disturbances in the metabolism of bile and bile acids could be involved in bringing about the morphologic change. Duct bile duct proliferation is characteristically seen in the early stages of hepatic carcinogenesis regardless of the nature of the carcinogenic agent employed. Striking alterations in the composition of the bile have also been found following the administration of a number of hepatic carcinogens. Spain and Griffin (31) and Mirvish and Gillman (22) have described changes in the bile acid and bile pigment metabolism in rats fed diets containing 4-dimethylaminobenzene or its derivatives. Also, Goldfarb, Singer, and Popper (14) described alterations in bile acid secretion and composition in animals fed α-naphthylisothiocyanate which were similar to those observed in ethionine-fed rats (24). There may be no causal relationship between these chemical changes in the bile and hyperplasia of the bile duct epithelium, but it is still an attractive hypothesis that the many different agents, both carcinogenic and noncarcinogenic, that induce bile duct hyperplasia in the liver could also do so by altering the metabolism of bile acids or other bile components in characteristic and reproducible ways.

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