The Prevention of Ethionine-induced Carcinoma of the Liver in Rats by Methionine*

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It was previously reported that rats fed diets containing ethionine, the ethyl analog of methionine, developed unequivocal carcinoma of the liver (10). Since methionine administration prevents most of the other known effects of ethionine (3, 4, 7, 13, 14, 17, 22, 23, 25, 27), it became important to know whether methionine would also protect rats against the carcinogenic effect of ethionine. Also, since the feeding of low methionine-choline-deficient diets leads to carcinoma of the liver in rats (5, 19, 20), chickens (19, 20), and mice (1, 31) and since ethionine decreases the availability of the methyl groups of methionine for choline synthesis (8, 24), it was considered possible that ethionine might induce liver cancer by producing a choline deficiency. For these reasons, the influence of adding methionine or choline at different levels to ethionine-containing diets upon the incidence of liver cancer in rats was observed. Betaine was also tested for its possible effect upon ethionine carcinogenesis, because of the known metabolic relationships between betaine, choline, and methionine (18, 29, 30).

Of the compounds tested, only methionine was found to give complete protection against the carcinogenic effect of ethionine. Betaine was almost as effective as methionine, while choline was much less so.

MATERIALS AND METHODS

White Wistar rats (Carworth Farms) of both sexes, weighing from 150 to 260 gm., were used. The animals, five to a cage, were housed in screen-bottomed cages in an air-conditioned room and were weighed once weekly. In all experiments, the basal diet contained 16 per cent casein, sucrose, corn oil, salt mixture, vitamins, and 0.25 per cent DL-ethionine (1.54 millimoles/100 gm diet) (California Research Foundation), as previously described (9). This diet contained 0.3 gm. choline chloride and 0.1 mg. vitamin B_{12}/kg. The compounds added to this diet replaced a corresponding amount of sucrose.

In the first experiment, 30 animals were divided into three groups of ten each, five of each sex. One group was fed the basal ethionine-containing diet; a second group, a similar diet containing in addition 0.3 per cent DL-methionine (2 millimoles/100 gm diet) (Mann Research Chemicals); and a third group, 0.3 per cent choline chloride (2.1 millimoles/100 gm diet) (Nutritional Biochemicals). These diets were fed for 8 months, after which time the animals of all groups were fed the normal stock diet (Purina Laboratory Chow) for 2 months. The liver and other abdominal viscera of each animal were examined by laparotomy at the end of 8 months before the animals were placed on the stock diet. All surviving animals were sacrificed 10 months after the beginning of the experiment.

In the second experiment, 40 male animals were divided into four groups of ten each. One group was fed the basal ethionine-containing diet; a second group, a similar diet containing, in addition, 0.2 per cent DL-methionine (1.35 millimoles/100 gm diet); a third group, 0.6 per cent DL-methionine (4 millimoles/100 gm diet); and a fourth group, 0.8 per cent DL-methionine (5.4 millimoles/100 gm diet). By error, the concentration of all vitamins in the diets in this experiment was 2.5 times greater than that used in previous studies (9) and in the other experiments. The animals were fed the experimental diets for 4.5 months, followed by the normal stock diet for 7.5 months. The livers of the animals in the first three groups were examined by laparotomy at 10 months. The surviving animals in Groups 1, 2, and 3 and half of those in Group 4 were sacrificed at 10 months. The remainder of the animals in Group 4 were sacrificed at 16 months.

In the third experiment, 60 male animals were divided into six groups of ten each. The first group was fed the basal ethionine-containing diet; the second group was fed a similar diet containing, in addition, 0.6 per cent DL-methionine; the third group, 0.6 per cent choline chloride (4.2 millimoles/100 gm diet); the fourth group, 0.8 per cent DL-methionine; the fifth group, 0.8 per cent choline chloride (5.7 millimoles/100 gm diet); and the sixth group, 0.8 per cent betaine chloride (5.2 millimoles/100 gm diet). The animals were fed the diets for 8 months, followed by the normal stock diet for 4 months. All surviving animals were sacrificed at 12 months.

In each experiment, samples of all lobes of the liver and portions of the pancreas, testis, kidney, and occasionally other organs were fixed in Bouin's solution. Paraffin sections were stained with hematoxylin and eosin.

RESULTS

Ethionine alone.—The animals on the basal ethionine-containing diet showed a high incidence

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of liver cancer in all three experiments (Table 1). Out of a total of 27 animals, 26 had obvious carcinomas, many with metastases. Increasing the vitamin level by 2.5 times, as was accidentally done in Experiment 2, did not influence the cancer incidence. The tumors were all liver-cell cancers, and no bile duct carcinomas were found. The appearance of the tumors as well as of the pancreas, testis, and kidney was the same as previously described (9, 10). The non-neoplastic portions of the liver showed changes similar to those already reported in rats fed ethionine (9, 10). These included varying degrees of oval-cell (bile duct epithelial cell) proliferation, with differentiation to bile ducts and to cholangiofibrosis, dissociation of the normal liver architecture with some pseudolobule formation, and nodular hyperplasia.

Methionine plus ethionine.—The administration of 0.6 or 0.8 per cent methionine completely protected the animals against liver cancer induced by ethionine (Table 1). These levels of methionine were effective when the experimental diets were fed for either 4.5 or 8 months. Smaller amounts of methionine showed less complete protection, which varied with the length of time the experimental diets were fed. With 0.3 per cent methionine added to the ethionine-containing diet, the tumors occurred in five out of ten animals when the diet was fed for 8 months (Table 1). With a lower level of methionine (0.2 per cent), the cancer incidence was less (two out of eight animals) when the diet was fed for only 4.5 months. Apparently, the longer feeding period of experimental diets offers a more rigorous test of the efficacy of compounds in counteracting the carcinogenic action of ethionine. Although the experimental conditions were not exactly the same in these two experiments because of the larger quantity of vitamins in the diets of the second experiment, the tumor incidence in the groups without added methionine was about equal in both groups, suggesting that the different levels of vitamins had no serious effect upon the carcinogenic process.

With 0.6 or 0.8 per cent methionine, the liver was normal on both gross and microscopic examination. Oval-cell proliferation, cholangiofibrosis, or nodular liver cell hyperplasia was not seen in any of the livers of the animals on diets containing these larger amounts of methionine. With the smaller levels of methionine, minimal oval-cell proliferation and, rarely, small areas of cholangiofibrosis were seen in some livers, especially those with cancer.

The effects of the different amounts of methionine upon the morphologic changes in other organs were interesting. The degree of protection afforded by methionine against ethionine damage to the pancreas and testis did not correspond closely with the protection against liver damage. For example, with 0.6 per cent methionine, the livers of animals on this diet were normal, but the pancreas showed minimal to moderate acinar atrophy and the testis a moderate amount of tubular cell atrophy. However, at the highest level of methionine administration (0.8 per cent), the pancreas and testis of almost every animal were normal.

Choline.—In the first experiment, choline supplementation at a level of 0.3 per cent in the diet was about as effective as an approximately equimolar amount of methionine in protecting the animals against ethionine-induced liver cancer (Table 1). However, with higher levels of dietary choline, a striking difference was obtained between the effects of choline and of methionine. The addition of 0.6 or 0.8 per cent choline to the basal ethionine-containing diet produced no greater protection against liver cancer than did the lower level of choline (Table 1), in contrast to the complete protection afforded by 0.6 or 0.8 per cent extra methionine.

The livers of the animals fed supplementary choline showed interesting differences in the degree of tissue reaction to ethionine. In the non-neoplastic portions of liver with cancer, a moderate oval-cell proliferation, cholangiofibrosis, and nodular hyperplasia were seen, regardless of the level of dietary choline. In contrast, in the livers without cancer, only slight degrees of these changes were found, again regardless of the size of the choline supplement.

The pancreas and testis of the animals on the choline-ethionine diets showed varying degrees of acinar and tubular cell atrophy. In general, the pancreatic lesions were less severe with the larger amounts of choline. However, even with the largest dose of choline, no complete protection was found against the ethionine-induced pancreatic damage. The testicular changes were pronounced, regardless of the level of choline in the diet. This contrasted with the results with methionine in which virtually complete protection against the testicular changes was observed with the largest amount of methionine used.

Betaine.—Addition of 0.8 per cent betaine chloride to an ethionine-containing diet also caused a striking reduction in the incidence of liver cancer, as is shown in Table 1. In this experiment, betaine was much more effective than choline in protecting the liver against cancer induction and was only slightly less effective than methio-

nine. In another experiment, not reported here, 0.5 per cent betaine was added to an ethionine-containing diet, and this ration was fed to five rats for 5 months, followed by 3 months of stock diet. All the animals on this regimen had normal livers, while those on ethionine alone or ethionine plus homocystine or thymine had liver cancer.

In the animals on betaine, the non-neoplastic portions of the liver showed only minimal oval-cell proliferation without cholangiofibrosis or nodular hyperplasia. The pancreas of many of these animals showed only minor degrees of acinar-cell atrophy. The testis was more severely damaged than in animals fed supplements of 0.6 or 0.8 per cent methionine but showed less damage than in animals on comparable amounts of choline.

Growth curves.—The growth curves of the animals in the third experiment, in which the two largest supplementary levels of methionine or choline and one level of betaine were used, are shown graphically in Chart 1. Since the weights of the animals on the two levels of methionine supplements were very similar, the results were combined, as were those on the two levels of choline. It is evident from the growth curves that methionine and choline, as well as betaine, counteract the depression of growth produced by the addition of ethionine to the diet. Of the three compounds, methionine was somewhat more effective than choline which, in turn, appears to be slightly better than betaine in reversing the growth inhibition. Although choline appeared to be slightly more effective than betaine in counteracting the inhibition of somatic growth induced by ethionine, it was less effective in protecting against the induction of liver cancer.

DISCUSSION

The results of this study clearly show that adequate levels of methionine prevent all the chronic morphologic changes in the liver, including cancer formation, induced in rats by ethionine. In two different experiments none out of a total of 38 animals fed supplementary methionine at levels of 0.6 or 0.8 per cent developed cancer, as compared with seventeen out of seventeen fed the basal ethionine diet without added methionine. In contrast, the addition of extra amounts of choline at approximately the same levels is only partially effective. Betaine is more effective than choline but is not as active as methionine. Me-

TABLE 1

THE INFLUENCE OF METHIONINE, CHOLINE, OR BETAINE UPON THE INDUCTION OF RAT LIVER CANCER BY ETHIONINE

Experiment no.	Compound added to ethionine-containing diet (Gm./100 gm diet)	Period experimental diet fed (months)	No. animals surviving for 8 mo.	No. animals with liver cancer*	Cancer incidence (per cent)
1	None	8	10	9	90
	Methionine, 0.3	8	10	5	50
	Choline chloride, 0.3	8	9	5	56
2	None	4.5	9	9	100
	Methionine, 0.2	4.5	8	2	25
	" 0.6	4.5	10	0	0
	" 0.8	4.5	10	0	0
3	None	8	8	8	100
	Methionine, 0.6	8	9	0	0
	" 0.8	8	9	0	0
	Choline chloride, 0.6	8	10	6	60
	" " 0.8	8	10	7	70
	Betaine chloride, 0.8	8	10	1	10

* The time of termination of each experiment was different and is given in the text.

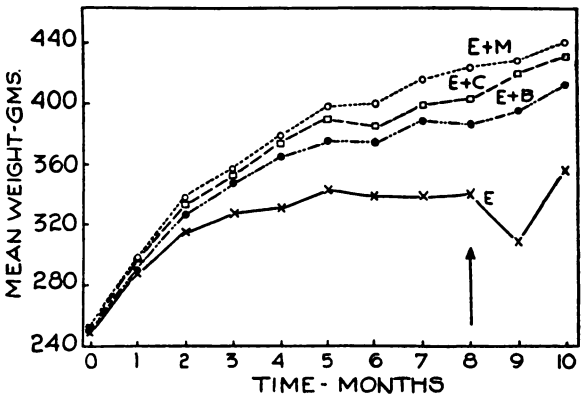

CHART 1.—Growth curves of rats fed diets containing 0.25 per cent ethionine (EX————X), 0.25 per cent ethionine plus 0.6 or 0.8 per cent methionine (E + MO-------O), 0.25 per cent ethionine plus 0.6 or 0.8 per cent choline chloride (E + C□-----□), or 0.25 per cent ethionine plus 0.8 per cent betaine chloride (E + B●——-——●). For E + M and E + C, each point is the mean of from eighteen to twenty rats. For E and E + B, each point is the mean of from eight to ten rats. The arrow at 8 months marks the time of change from the experimental to the stock diet.

thionine and choline also differ in another respect in regard to their effects on ethionine-induced carcinogenesis. With methionine, the degree of protection increases with increasing levels, while with added choline the higher levels are no more effective than the lowest one used in this study.

It is well established that cancer of the liver frequently occurs in rats fed choline-deficient low-methionine diets (5, 19, 20). It is also known that ethionine administration to rats decreases the transmethylation from methionine to choline precursors (8, 24). Therefore, it might be assumed that chronic choline deficiency is an important factor in the mechanism of liver carcinogenesis by ethionine. However, the following available evidence is not in accord with this hypothesis: (*a*) the addition of as much as 0.8 per cent choline to the ethionine-containing diet was only partially effective in preventing liver cancer, while even 0.2 per cent was completely effective in rats on choline-deficient diets (19); (*b*) animals fed carcinogenic levels of ethionine did not develop the severe fatty liver with fatty cirrhosis seen in chronic choline deficiency in rats (15, 16); and (*c*) methionine and betaine were considerably more effective than choline in preventing the chronic tissue changes, including liver cancer, that develop in rats fed ethionine. In rats on choline-deficient diets, choline was about 3 times as effective as either methionine or betaine in preventing the fatty liver (32).

One is therefore forced to conclude that ethionine does not cause liver cancer simply by inducing a chronic choline deficiency and that the partial protection afforded by choline is probably a result of the "sparing" effect of choline on the available methionine. Apparently, this action of choline becomes saturated at the lowest level of added choline used in this study, since this level was as effective as the two higher levels in protecting against liver cancer.

On the basis of available evidence, a more attractive hypothesis is that the carcinogenic action of ethionine is in some way related to an interference with one or more of the roles of methionine in cellular metabolism other than that concerned with the synthesis of choline. The capacity of methionine, but not of several other amino acids or of choline, to prevent almost all of the acute effects of ethionine on the liver and pancreas (13, 14, 25), as well as to prevent the induction of liver cancer, is consistent with this view. Also, the greater effectiveness of betaine as compared with choline in protecting against liver cancer would be expected according to this hypothesis. Both betaine and choline are generally considered to be capable of contributing one methyl group each for the synthesis of methionine from homocysteine (2, 6, 18, 29, 30). The administration of adequate levels of each compound might, therefore, be expected to conserve tissue methionine by assisting in its resynthesis after it loses its methyl group and forms homocysteine. Betaine can apparently act directly as a source of a methyl group for this resynthesis of methionine, while choline must first be oxidized to betaine, probably via choline oxidase and betaine aldehyde dehydrogenase, before it can be so utilized (6, 18). Therefore, betaine should be more effective than choline in counteracting the ethionine effects if these effects are mediated primarily through disturbed methionine metabolism and not indirectly through choline. The inhibition of choline oxidase by ethionine (28) would tend to accentuate this difference in effectiveness between betaine and choline.

The apparent requirement of considerably larger amounts of methionine than of ethionine to counteract the carcinogenic effect of ethionine, as observed in this study, would appear to be in conflict with the suggested antimethionine action of ethionine. However, the high requirement is understandable on the basis of studies on the acute effects of ethionine. These have suggested that methionine is much more rapidly metabolized than is ethionine (13, 14, 21) and that in experiments lasting more than 12 hours periodic administration of methionine is required to counteract the effects of a single dose of ethionine (13, 14).

The nature of the relationship between ethionine and methionine in the production of liver cancer by ethionine is as yet completely unknown. An interference with the formation of cystine or homocystine from methionine does not seem to be directly involved in the carcinogenic action of ethionine, since the addition of neither compound to an ethionine-containing diet proved to be effective in preventing to any degree the induction of liver cancer (11, 12). Also, the adenine-depletion hypothesis of Stekol *et al.* (26) would not appear to be important in the carcinogenic action of ethionine, since the addition of dietary adenine in the form of deoxyribonucleic acid had no effect upon the induction of liver cancer by ethionine (11). The study of the many other known metabolic reactions of methionine in relationship to carcinogenesis by ethionine offers a potentially fruitful biochemical approach to some of the problems of the pathogenesis of experimental liver cancer.

SUMMARY

The effects of adding extra amounts of DL-methionine, choline chloride, or betaine chloride

upon the occurrence of liver cancer in rats fed a 16 per cent casein diet containing 0.25 per cent ethionine was investigated. The addition of 0.6 or 0.8 per cent methionine completely prevented all the chronic morphologic changes in the liver, including liver cancer. Lower levels of methionine were partially effective. The addition of choline supplements to the ethionine-containing diet was of limited effectiveness in reducing the incidence of liver cancer. Three levels of added choline, 0.3, 0.6, and 0.8 per cent, all exerted about an equal degree of protection. Betaine was more effective than choline and somewhat less effective than methionine in preventing liver cancer. On the basis of what is known concerning the possible importance of chronic choline deficiency in the carcinogenic action of ethionine, it is concluded that the induction of a choline deficiency is probably not an important mechanism in ethionine carcinogenesis. The preponderance of evidence favors the view that ethionine exerts its carcinogenic effect by interference with some as yet unidentified metabolic reaction of methionine.

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