Biochemical Changes during Experimental Carcinogenesis

III. The Production of Urea from Aspartic Acid, Arginine, or Ammonia in the Perfused Rat Liver*†

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In the earlier papers in this series (3, 4) data have been presented which indicate that the isolated perfused liver from a rat fed 8'-methyl-dimethylaminoazobenzene* has a decreased ability to oxidatively catabolize amino acids. Such livers, however, show an increased capacity for incorporation of C14-labeled amino acid into both liver and plasma proteins.

A biochemical block or impairment in the chemical reaction sequence from amino acid nitrogen to urea nitrogen was thought to develop during the process of azo dye carcinogenesis.

To attempt to demonstrate definitively the locus of this metabolic impairment, we have studied the ability of the perfused livers from 3'-Me-DAB-fed rats to produce urea from ammonia, aspartic acid, or arginine added to the blood perfusate.

MATERIALS AND METHODS

The rats used in these studies were adult, 225–325-gm. males of the Rochester strain of Wistar rats. Some of the experiments were repeated with Sprague-Dawley rats and with rats from Carworth Farms; no difference was noted.

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‡ Postdoctoral Fellow of the National Cancer Institute, United States Public Health Service, at the University of Rochester, 1953–1956, where most of these investigations were carried out.

* Hereafter 3'-methyl-dimethylaminoazobenzene will be referred to as 3'-Me-DAB.

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The technic of isolated rat liver perfusion is the same as that described previously (9, 11).

Livers were taken from rats which had been on a diet containing .06 per cent 3'-Me-DAB for 2–3 months. We have referred to these livers as precancerous because they do not show appreciable gross or histological deviation from the normal rat liver (3). These results were controlled by studies of rats fed the experimental diet without carcinogen and studies of rats fed the commercially available food known as Checkers. The results in both series were the same (4).

Most of the chemical and analytical procedures and the experimental rat diets were described in the earlier papers (3, 4).

In the presence of added ammonia the blood ammonia level was determined by the Conway micro-diffusion method without the addition of urease (6). Blood urea-nitrogen levels were determined in these experiments by difference, using the Conway method to determine diffusible nitrogen with and without added urease. The supplements to the blood perfusate were as follows: ammonium carbonate, 144 mg. (3 mmoles nitrogen); L-arginine-HCl, 158 mg. (.75 mmoles arginine), 316 mg. (1.5 mmoles arginine), or 632 mg. (3 mmoles arginine); L-aspartic acid, 300 mg. (1.5 mmoles). All supplements were added to the blood in a small volume (less than 5 ml.) of Ringer's solution, neutralized to pH 7.2 when necessary with small volumes of 2.0 N sodium hydroxide.

RESULTS

Some of the results are expressed as urea nitrogen production in excess of the endogenous urea production from the free amino acids in the blood of the perfusate. The method for estimating this endogenous or basal urea production was described in an earlier paper (10).

Chart 1 shows the total urea nitrogen production from an added supplement of 3 mmoles of ammonia in the form of ammonium carbonate.
in a series of perfusions of normal rat livers as compared with livers undergoing carcinogenesis. No impairment of the urea synthetic mechanism was shown by the livers from the azo dye-fed rats.

In Chart 2 the excess urea nitrogen production from the added ammonia is presented, as well as the disappearance of ammonia, in experiments with both normal livers and with livers from 3'-Me-DAB-fed rats. The livers from animals fed the carcinogen appeared fully as capable of producing urea from ammonia as did normal livers. Note, in addition, that both types of livers showed a production of roughly 30 mg. of urea nitrogen paralleled by the disappearance of about 35 mg. of ammonia nitrogen. This indicates that both types of livers were capable of completely converting ammonia into urea with equal efficiency. These results are in marked contrast to those obtained when the perfusate was supplemented with either glutamic acid or a complete mixture of amino acids. In such experiments the livers of 3'-Me-DAB-fed rats showed a decrease of 50 per cent or greater in their urea-producing capacity (4).

In Chart 3 are shown the curves for urea production (in excess of the calculated endogenous urea production of comparable livers) from three different concentrations of L-arginine. The amounts of urea nitrogen produced in the course of 5 hours by the normal livers from 3.0, 1.5, and .75 mmoles of arginine were 4.2, 3.0 and 1.30 mmoles, respectively, an almost quantitative synthesis of 1 mole of urea from each mole of arginine supplement. These values indicate strikingly the close approximation to normal physiological function, at least insofar as urea production is concerned, of the isolated perfused rat liver. We have noted previously the capacity of the perfused liver, with unsupplemented blood perfusate, to maintain a level of endogenous urea production which is comparable to the 24-hour urea production in the intact rat on a nonprotein diet (10).

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**Chart 1.**—Total urea nitrogen production; 3 mmoles ammonia nitrogen added. Vertical bars indicate ranges of final samples. Numbers in parentheses represent the number of experiments.

**Chart 2.**—Excess urea nitrogen production and ammonia nitrogen disappearance; 8 mmoles ammonia added. Vertical bars indicate ranges of final samples. Numbers in parentheses represent the number of experiments.
In contrast to the urea-forming capacity of the normal rat livers, decreases of up to 60 per cent of the normal values could be seen when the same amounts of arginine were added to the precancerous livers. Although the impairment of the capacity to produce urea from arginine of these livers was not complete, they obviously had only a small fraction of the capacity of normal, control livers.

These results might be explained on the basis of low permeability of the liver cell to arginine.

In a series of perfusions of normal livers we added 2 mg. (5 μc.) of arginine randomly labeled with C¹⁴. In these experiments the perfused normal livers converted an average of 33 per cent of this tracer dose of arginine to C¹⁴O₂ in a period of 5 hours. In livers from rats fed 3'-Me-DAB, however, only 6.3 per cent of the C¹⁴ from labeled arginine appeared as C¹⁴O₂ in the same period. Thus, arginine appears capable of entering the normal hepatic cell. Differential and lowered permeability of the hepatic cells from livers undergoing carcinogenesis seems unlikely. A lowered arginase activity in hepatoma induced by 4-dimethylaminoazobenzene as compared with normal livers has been reported by Greenstein and Leuthardt (see Greenstein, 1954).

The data in Chart 4 indicate the relative urea-forming capacity of perfused normal livers as compared with livers from rats fed azo dye in the presence of 1½ mmoles of aspartic acid supplementing the blood perfusate. Although the difference between the two types of livers appears substantial, note must be made of the fact that the data here plotted represent total urea nitrogen production values. When these are converted to values for excess urea nitrogen produced from the added aspartic acid, both curves are so reduced as to show no appreciable amount of urea formed in either the precancerous or the normal liver. Data presented in our previous paper showed the results obtained with glutamic acid to be similar (4). The possibility that these carboxylic acids do not penetrate the liver cell readily appears likely.

**DISCUSSION**

The isolated perfused rat liver from azo dye-fed rats can synthesize urea from added ammonium carbonate to the same extent as can perfused normal livers, as demonstrated by the data in this paper. The question as to whether this ammonia is converted directly to urea without transfer to glutamine as an obligatory intermediate cannot be answered from these data; however, as shown in our previous publication (4), glutamine amide nitrogen is efficiently and rapidly utilized for urea synthesis in perfused normal livers, and, further, the synthesis of urea from...
glutamine in the precancerous liver is not affected by the disturbance in urea synthesis shown to exist in these livers. In this connection data presented by Duda and Handler (7) indicate that N\(^+$\) from N\(^+$\)-labeled ammonia may be converted first to glutamine before appearing in urea. Bach has also presented evidence for the intermediary formation of glutamine from ammonia and tissue glutamic acid, and for the formation of urea from glutamine (1).

The results obtained with aspartic acid are difficult to interpret because of the lack of more definitive evidence in regard to the permeability of hepatic cells to the aspartic acid molecule. If the aspartic acid does get into the cell the failure of the normal liver to convert at least some of its nitrogen to urea is difficult to understand; however, on the basis of total urea nitrogen production from this amino acid, the existence of a biochemical disturbance in urea synthesis in livers of rats fed 3\(^{-}\)-Me-DAB is further substantiated.

An examination of the data so far presented leads to the conclusion that the only materials which the precancerous liver is capable of readily utilizing as a source of urea nitrogen are glutamine and ammonia (with the possibility that the latter is first converted to the former). The proposal which has been made by several investigators (1) for a chemical reaction sequence from glutamine to urea unique and different from the pathway followed by the nitrogen from other amino acids gains support from these results.

The considerable decrease of urea synthesis from arginine in the livers of the carcinogen-fed rats may indicate a biochemical defect involving a marked decrease in arginase activity. One may speculate that this is due to some form of enzyme inhibition by a mechanism such as Potter has postulated for enzyme inactivation during carcinogenesis (12, 13). On the other hand, the decrease in arginase activity may be the result of a process of enzyme loss in accord with the enzyme deletion hypothesis of tumor formation as formulated by Rusch (14). Whatever the explanation, the data presented here show, at least, that the normal liver is capable of producing urea and carbon dioxide from arginine, whereas this capacity is diminished significantly in the precancerous liver.

A further possibility is that those amino acids which show a decreased ability to act as urea precursors during the process of hepatoma formation may be preferentially utilized for other metabolic purposes, such as protein synthesis and cell proliferation. From the standpoint of what one might expect to occur during carcinogenesis, this is a satisfying, if not fully substantiated, conclusion.

**SUMMARY**

The isolated perfused livers from rats fed 3\(^{-}\)-Me-DAB synthesized urea from arginine and aspartic acid nitrogen to an extent of 50 per cent or less than did normal control livers. In contrast, ammonium carbonate added to the blood perfusate was utilized efficiently for urea synthesis by both types of liver. In accord with data on the metabolism of a mixture of amino acids, of glutamic acid, or of glutamine, which we have published previously, we conclude that urea synthesis from glutamine or ammonia may proceed by a pathway other than the classical Krebs-Henseleit urea cycle. Further, a loss either of arginine or of arginase activity may be associated with a preferential utilization of arginine for other metabolic purposes, such as liver and plasma protein synthesis, during the process of azo dye carcinogenesis.

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