Biochemical Changes during Experimental Carcinogenesis

II. Glutamine and Glutamic Acid Metabolism in Perfused Livers of Azo Dye-fed Rats*

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In a recent publication from this laboratory (7) data were presented which showed that, during the process of azo dye carcinogenesis in rat livers, several striking changes occurred in the quantitative metabolism of amino acids and protein.

The experiments were carried out by the technic for rat liver perfusion described by Miller et al. (25, 27). These experiments indicated that perfused livers from rats which had been fed the hepatic carcinogen 3'-methyl-4-dimethylaminoazobenzene for 2-3 months showed a considerable impairment of their capacity to oxidatively catabolize a mixture of amino acids added to the blood perfusate as compared with normal rat livers. On the other hand, these livers from azo dye-fed rats, as compared with livers from normal rats, demonstrated an enhanced capacity to incorporate L-lysine-6-C14 into both liver and plasma protein.

From these facts the conclusion was drawn that the liver undergoing carcinogenesis had considerably higher protein synthetic activity than did the normal liver. The data were further interpreted to indicate that there was a biochemical defect or a metabolic block operative in these precancerous livers on the catabolic pathway from amino acid to urea and CO2. The enhanced incorporation of the isotopically labeled lysine into liver and plasma proteins during carcinogenesis is, of course, only presumptive evidence of protein synthesis. It may indicate only an increased rate of protein turnover in preneoplastic tissue.

In the present paper data are presented on the effects of glutamine or of glutamic acid added to the blood perfusate on the processes of C14O2 production, urea formation, and labeled amino acid incorporation into protein by perfused livers from 3'-Me-DAB-fed rats, as compared with normal rat livers.

MATERIALS AND METHODS

All the rats used in these investigations were adult, 225-325-gm. males, either of a strain of Wistar rat which has been maintained at this laboratory over a period of years or Sprague-Dawley rats. No differences were found attributable to the type of rat used.

The normal rats were fed a commercial diet (Purina Checkers). A series of liver perfusions was run on the livers of rats maintained on the synthetic diet (7) without added carcinogen (designated as CD in Chart 1). No difference could be demonstrated between these two dietary groups, as noted below.

Experimental hepatic carcinogenesis was initiated in similar rats maintained on the diet previously described (7) containing .06 per cent of 3'-Me-DAB for a period of 8-9 months. All the rats were fasted 18-20 hours prior to liver perfusion but given free access to water during this period.

DL-Lysine-6-C14 was prepared by the method described by Rothstein and Claus (31); 3'-Me-DAB was prepared by standard methods. The details of the technic used for the preparation of the rat livers for perfusion and for the perfusion itself have been described elsewhere (7, 25, 27).

The supplements to the blood perfusate were as follows: L-Glutamic acid—441.0 mg. (3.0 millimoles) added in Ringer's solution. This supplement contained 42.0 mg. of L-amino nitrogen.

L-Glutamine—219.0 mg. (1.5 millimoles) added to Ringer's solution. This supplement contained 42.0 mg. of total nitrogen.

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† Obtained from the Holtzman Rat Company, Badger Lane, Madison 4, Wis.
The analytical methods used were the same as described previously (7), with the exception of the analysis for blood ammonia, which was determined by the Conway microdiffusion technic (10), and of blood glutamine, which was determined by the method of Boulanger and Osteux (6).

Body surface area of the rats was estimated by a linear formula with extensive data from actual surface area measurements made by Lee (17). The formula used was:

\[ \text{Body Surface Area (sq. cm.)} = 0.78 \times [\text{body weight (gm.)}] + 148. \]

This formula was derived by applying the method of linear regression to the data given by Lee. It is valid for the range 500–400 gm. body weight.

All livers were perfused with approximately 190 ml. of normal rat blood freshly drawn by cardiac puncture, heparinized, filtered through gauze, and then diluted with one-third its volume of Ringer's solution. In addition to either of the above amino acid supplements, 500 mg. of d-glucose and 0.62 mg. (10 µc.) of DL-lysine-6-C\(^{14}\) were added. This dose was the average value of the added amino acid substrate could be interpreted without being complicated by the endogenous metabolism of the rat liver, a series of perfusions were run with no added amino acid substrate. The determination of the endogenous urea nitrogen production in those perfusions containing an amino acid supplement can be understood by references to the series of curves shown in Chart 2 and the data and method of calculation as outlined in Table 1.

In the lower curves of Chart 2 the extent of endogenous urea nitrogen production per 500 sq. cm. body surface is shown. These data represent the urea nitrogen production from the blood perfusate and liver alone. No amino acid supplement was added. That a depression of endogenous urea nitrogen production existed in the livers from 3'-Me-DAB-fed rats (without nitrogen supplementation of the blood perfusate) is evident.

RESULTS

The condition of the livers from the carcinogen-fed rats was the same as that seen in previously reported experiments (7). Grossly, the livers were tan in color and slightly larger than normal in size, with an average weight of about 12 gm. Their capsular surfaces were granular and occasionally pseudo-lobulated. Microscopic examination of liver sections revealed some areas of parenchymal and many areas of biliary duct hyperplasia. There was no evidence of hepatoma formation. We believe them to be similar to the precancerous livers described by Cantero (8).

Since the carcinogenic diet containing the azo dye is also somewhat low in protein and deficient in riboflavin, we wished to determine if our use of rats on Purina Checkers as our control animals had any effect upon the results obtained. In Chart 1 are plotted the data for urea nitrogen production in a series of four perfusions of livers from rats on the deficient diet without added 3'-Me-DAB, a series of four perfusions of livers from rats on the Checkers diet, and a series of six perfusions of livers from rats on the deficient diet including 3'-Me-DAB. In all cases the blood perfusate was supplemented with a complete mixture of amino acids (7). Obviously, the differences in diet alone do not account for the decrease in urea-producing capacity of the livers from rats on the deficient diet containing carcinogen.

We have found that a useful method of normalizing data from a series of liver perfusions is to express the data on the basis of the body surface area of the liver donor rat (20). So that the effects of the added amino acid substrate could be interpreted without being complicated by the endogenous metabolism of the rat liver, a series of perfusions were run with no added amino acid substrate. The determination of the endogenous urea nitrogen production from this amino acid mixture described previously (7). In the upper portion of Chart 2 excess urea nitrogen production from this amino acid mixture is plotted after correcting for the endogenous urea production. This correction is made by subtracting from the total amount of urea produced in a particular perfusion the amount of urea which would be produced by a liver from a rat of the body surface area of that used in that specific perfusion if the blood perfusate had been unsupplemented with amino acids. The average values of such calculations for a series of perfusions are plotted.

The data in Chart 2 thus show that the unsupplemented perfused livers of normal rats were capable of producing in a 5-hour period about 12.0

![Chart 1](image-url)

**Chart 1.**—Urea nitrogen production—amino acid mixture added. Vertical bars indicate ranges. Numbers in parentheses refer to number of experiments in each case. "Checkers" refers to stock diet of Purina Checkers. CD refers to control diet as described under methods.
mg. of urea nitrogen/300 sq. cm body surface and that the livers of rats which had been on the azo dye diet were capable of producing only about 7.5 mg/300 sq cm body surface area of urea nitrogen. When an amino acid mixture was added to the blood perfusate, both types of liver were capable of producing more urea, as shown in the center portion of Chart 2. That is, as the top curves demonstrate, the normal liver could produce about 11.0 mg. of excess urea nitrogen from the added 42.0 mg. of amino acid nitrogen, and the liver from an azo dye-fed rat could produce only about 5.1 mg. in excess of the calculated endogenous urea production. It should be pointed out that the added amino acid mixture contained (from L-amino acids) 4.7 mg. of glutamic acid a-amino nitrogen, 23.7 mg. of other a-amino nitrogen, 8.6 mg. of non-a-amino acid nitrogen (from proline, hydroxyproline, histidine, lysine, arginine, and tryptophan), 4.7 mg. of n-nitrogen from u-amino acids, and no glutamine.

In Chart 3 are presented curves for total urea-nitrogen production by both normal livers and by livers from the carcinogen-fed rats in the presence of added glutamine and of added glutamic acid (both without the added amino acid mixture). In all cases, the amino acid supplement to the blood perfusate contained 42.0 mg. of total added nitrogen. As can be seen, the glutamine served as a much more effective source of urea nitrogen than either the amino acid mixture or the added glutamic acid in the case of both the normal liver and the liver from the 3'-Me-DAB-fed rat. In Chart 4 this difference is shown to be even more striking when the excess urea production is plotted. Both the normal and the precancerous liver exhibited an ability to synthesize urea from glutamine to an extent that was far in excess of that from the amino acid mixture and several times that produced from glutamic acid.

On the basis of determinations of the a-amino nitrogen level in the perfusing blood during the course of 5-hour perfusions of glutamine or glutamic acid, the disappearance of only 4-8 mg. of a-amino nitrogen in all cases could be shown. From these data it may be concluded that only a few mg. of blood a-amino nitrogen were taken up by the precancerous livers, and not much more than this amount by the normal livers, or that a-amino nitrogen was being released continuously by the liver even in the presence of a high degree of urea synthesis. Analyses showed that this nitrogen was not due to ammonia or glutamine.

The conversion of L-lysine-6-C\textsuperscript{14} into C\textsuperscript{14}O\textsubscript{2} is shown in Chart 5. Surprisingly, the presence of glutamine was associated with decreased oxidation of lysine-6-C\textsuperscript{14} to C\textsuperscript{14}O\textsubscript{2} in both the normal and the precancerous livers. The preneoplastic livers in the glutamine perfusions showed an even greater depression than the normal. The metabolism of supplementary glutamine was associated with a depression of the oxidative catabolism of the isotope labeled lysine. In this connection no significant differences were evident when glutamic acid was added to the perfusate, as compared with the unsupplemented blood.

In Chart 6 are plotted the data on the incorporation of L-lysine-6-C\textsuperscript{14} into combined liver and plasma protein. The glutamic acid supplement resulted in the same pattern which was found for the amino acid mixture—i.e., an increased incor-
poration in the livers from carcinogen-fed rats as compared with the normal livers.

In the presence of glutamine, incorporation of the isotope into protein was the same for normal livers as for precancerous livers, and in both cases was considerably greater than in the presence of glutamic acid. The preneoplastic livers with no amino acids added showed an incorporation as high as the livers supplemented with glutamine.

**DISCUSSION**

Three facts which require explanation are: (a) increased urea nitrogen production by the normal livers in the glutamine-supplemented perfusions and the apparent absence of any biochemical disturbance of urea synthesis from glutamine in the precancerous livers in this type of experiment; (b) the maintenance of the initially high level of blood a-amino nitrogen which, at the end of the experiments, and after the production of large amounts of urea, is not due to glutamine or to ammonia; (c) enhanced protein synthesis in both normal livers and in livers from rats on the carcinogen diet in the presence of added glutamine, as measured by incorporation of lysine-6-C\(^{14}\).

The fact that glutamic acid demonstrates only a very slight capacity for promoting urea production may be a result of the slow penetration of glutamic acid into cells postulated by some investigators (12, 16, 32, 33), although the work of Hers and Rittenberg (15) is at variance with these results. They interpret their data as indicating that glutamic acid can penetrate the rat liver cell to a significant level. Our data showing a persistence of a-amino nitrogen in the perfusate might be considered to add support to the contention that glutamic acid is slow to penetrate the liver cell. However, we have recently published the results of perfusions in which L-glutamic acid uniformly labeled with C\(^{14}\) was added to the perfusate (27).

**TABLE 1**

**METHOD OF CALCULATING THE EXCESS UREA NITROGEN PRODUCTION IN RAT LIVER PERFUSION 216**

<table>
<thead>
<tr>
<th>Time (hours):</th>
<th>1</th>
<th>1.5</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Observed endogenous urea nitrogen production/300 sq cm body surface area*:</td>
<td>2.5</td>
<td>4.1</td>
<td>5.5</td>
<td>6.6</td>
<td>9.1</td>
<td>10.9</td>
</tr>
<tr>
<td>b) Calculated endogenous urea nitrogen production/384 sq cm (surface area of liver donor in RLP 216)†</td>
<td>3.1</td>
<td>5.3</td>
<td>7.1</td>
<td>8.5</td>
<td>11.7</td>
<td>14.0</td>
</tr>
<tr>
<td>c) Observed total urea nitrogen production in RLP 216:</td>
<td>5.8</td>
<td>11.0</td>
<td>15.4</td>
<td>20.3</td>
<td>22.1</td>
<td>24.4</td>
</tr>
<tr>
<td>d) Calculated excess urea nitrogen production in (c-b):</td>
<td>2.7</td>
<td>5.7</td>
<td>8.3</td>
<td>11.8</td>
<td>10.4</td>
<td>10.4</td>
</tr>
</tbody>
</table>

* From the data in Chart 1—perfusions run with no amino acid supplement.
† Normal Wistar rat, weight 304 gm.; body surface area, 384 sq. cm. (calculated on the basis of the formula: body surface area = .78 X body weight + 148); 30.0 mg. of amino acid nitrogen added to perfusion.
Our results show that normal livers are capable of converting 20–50-mg. quantities of added glutamic acid-U-C\(^{14}\) to an extent of 20–40 per cent of the added activity to C\(^{14}\)O\(_2\). This is comparable to the ability of these livers to oxidize other amino acids. Data presented by McHenry and White (21, 35) show that rats with tumors do not produce urea from glutamic acid to the same extent as do comparable controls.

Glutamine may contribute to urea production in the liver by merely providing a means of blood transport and liver cell penetration for amino acid nitrogen of extra-hepatic origin. Inside the liver cell the glutamine could be converted to glutamic acid by transamination reactions of the type observed by Greenstein and Carter (13), and subsequently investigated by Meister (22–24).

Impaired urea formation from amino acid in the precancerous liver thus could be accounted for by postulating that it results from impaired transamination reactions involving the transfer of nitrogen from other amino acids to glutamate. Glutamine transaminase, on the other hand, has been shown by Meister to have a very narrow amino group specificity but to have a broad specificity with respect to the a-keto acid (22). This marks it rather definitely as a separate and distinct enzyme from other transaminases. The nitrogen of this intracellular glutamic acid could be converted to urea by at least two possible pathways: by transamination with oxalacetate to form aspartic acid and subsequent condensation of this amino acid with citrulline to enter the urea cycle (29, 30); or by supplying the nitrogen for the formation of carbamyl phosphate, which would react with ornithine to form citrulline (9).

The classical Krebs urea cycle requires a source of ammonia (in some form) in two reactions: namely, the synthesis of citrulline from ornithine; and the synthesis of arginine from citrulline. In the first reaction the source of nitrogen is most probably through carbamyl phosphate (9, 14), which itself could be generated from glutamine without participation of glutamic acid, as shown at the bottom of this page.

Enzymes catalyzing such reactions have not been described.

The conjecture might be made that the reaction or reactions involved in the synthesis of urea from glutamine nitrogen could occur independently of the reactions concerned with the catabolism of most of the commonly occurring amino acids, including the amino acids used in our mixture. This,
of course, would explain how the synthesis of urea from glutamine could proceed to a great extent in the livers of the azo dye-fed rats in the presence of a biochemical impairment of the usual pathway of urea synthesis from amino acids.

The existence of a series of reactions for the conversion of glutamine amide nitrogen to urea by a pathway independent of the Krebs-Henseleit cycle has been proposed by other workers (1-3, 16, 18, 19). The results obtained in the experiments described here are compatible with this possibility.

We might assume that the glutamine amide nitrogen is the sole source of the urea nitrogen produced. Obviously, the amino group could be involved also, but note might be made of the fact that the amount of urea nitrogen produced in those perfusions to which a glutamine supplement is added is approximately 21.0 mg., which is the amount (1 mmimole) of glutamine amide nitrogen initially present in the perfusate. The speculation that it is the glutamate amide alone which contributes to the urea nitrogen found in these experiments is inviting. In this case the level of α-amino acid nitrogen remaining in the blood perfusate might be explained by a diffusion of glutamic acid or some other amino acid back into the blood after removal of the glutamine amide moiety.

Of interest in this connection is a report by Berenbom and White that greater than 60 per cent of the N15-labeled amide group of glutamine is converted quantitatively to urea after intravenous administration to rats (5). Hers and Rittenberg, however, found very little N15 from the N15-labeled amide group of glutamine to appear in the urea produced in liver slices (15). We have compared the reported (15) endogeneous urea-producing capacity of washed liver slices with that of the isolated perfused liver and found this capacity in the former so slight as to be quantitatively insignificant; however, data reported by Bach (2) on the production of urea from glutamine or ammonium chloride by rat liver slices is, while lower, of the same order of magnitude as the quantity of urea produced from these substrates by the perfused rat liver.

The association of glutamine with increased protein synthesis or turnover, as measured by the incorporation of 1-lysine-6-C14, cannot be explained on the basis of our present knowledge. Increased protein synthesis itself, however, might be expected in tissue undergoing carcinogenesis. In a recent report Rabinowitz, Olson, and Greenberg (28) have interpreted results on the stimulation of incorporation of radioactive leucine, phenylalanine, lysine, and methionine into Ehrlich ascites cell protein in the presence of glutamine in terms of a rapid deamination and slow resynthesis of both added and endogenous glutamine, the glutamine thus acting merely as a limiting and essential amino acid in protein synthesis. We are inclined to agree with their conclusion that "There appears to be no necessity for assigning a role for glutamine in the protein synthetic mechanism other than as an essential constituent amino acid of protein."

Reference should also be made to work of Fisher (11) and of Levintow and Eagle (20). In experiments with a number of normal and malignant mammalian cell lines glutamine has been found to be required for growth and survival in tissue culture. These findings, as well as those of Barry (4), lead to the conclusion that glutamine and glutamic acid are utilized independently in the synthesis of protein.

The review by Waelsch (34) also contains considerable data which indicate that the metabolic activity of glutamine on growth and maintenance of bacterial cells is considerable and further that glutamic acid must be converted to glutamine before comparable effects can be demonstrated. Glutamate could not be used as a substitute for the required glutamine except in very high concentrations. However the facts may be explained ultimately, glutamine obviously occupies an important role in the synthesis of protein in mammalian cells. The results presented here are consistent with our hypothesis that there exists a biochemical disturbance of amino acid catabolic activity and an increase in protein synthesis during the precancerous period in livers from rats fed 3'-Me-DAB. Of further interest is the finding that glutamine participation in the process of urea production by the precancerous liver is unaffected by this apparent metabolic disturbance. The experiments show also that glutamine has the capacity to enhance the incorporation of C14-labeled lysine into protein in both normal and precancerous livers.

**SUMMARY**

The capacity of the isolated perfused rat liver to synthesize urea and to incorporate L-lysine-6-C14 into liver and plasma protein has been compared by using livers from normal rats and livers from rats on a 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) diet.

The addition of glutamine to the blood perfusate was associated with the rapid production of
an amount of urea-nitrogen which was chemically equivalent to the amount of glutamine amide-nitrogen added. This capacity to utilize glutamine nitrogen for the production of urea was found in both the normal rat livers and in the livers from rats maintained on the azo dye diet.

These findings are in contrast to data on the synthesis of urea nitrogen when glutamic acid or a mixture of amino acids was added to the perfusate. In such cases the isolated perfused precancerous livers showed a marked decrease in their capacity to produce urea.

In contrast to glutamic acid, glutamine addition enhanced the capacity of livers from both normal rats and from 3'-Me-DAB fed rats to incorporate L-lysine-6-C14 into both liver and plasma proteins. A corresponding decrease in the production of C14O2 was noted.

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Biochemical Changes during Experimental Carcinogenesis II. Glutamine and Glutamic Acid Metabolism in Perfused Livers of Azo Dye-fed Rats

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