Metabolic Profiles during Carcinogenesis and Related Conditions in Hepatic Tissue

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SUMMARY

An extensive series of rat liver perfusions has been carried out using livers from normal rats, livers from rats treated with carcinogens or carbon tetrachloride, or from rats following partial hepatectomy or bile duct ligation. The metabolic fate, in terms of urea and carbon dioxide production and liver and plasma protein synthesis, of 15 amino acids was determined for each liver condition. Although the results in general are similar, in that enhanced incorporation of amino acids into liver and plasma proteins and decreased amino acid catabolism are features common to most of the liver types, when the metabolism of specific amino acids was investigated it was found that each liver type was associated with a characteristic biochemical defect or metabolic deviation from normal. The data provide evidence that different hepatic carcinogens may have similar over-all effects but the basic metabolic lesions in each case are related to the carcinogen used. The data also provide an indication of the cell of origin of the tumor on the basis of the associated metabolic changes.

Our studies have involved an examination of the process of experimental carcinogenesis in the rat liver with special references to protein and amino acid metabolism.

We have attempted to design experiments, the results of which would provide data relevant to the following questions: (a) Do a number of liver carcinogens have the same over-all metabolic effect? For example, are they all associated with an increase in protein synthesis or a decrease in urea production? (b) Do a number of liver carcinogens have the same specific metabolic effect? (c) Do other liver conditions such as regeneration, bile duct cell proliferation or parenchymal cell necrosis demonstrate changes in metabolic activities which are identical with hepatic metabolism during carcinogenesis?

Our previous investigations of amino acid metabolism in perfused livers have provided data which suggest that the increase in amino acid incorporation found in both precancerous and in regenerating livers is associated with, and possibly maintained by, biochemical lesions involving the metabolic degradation of amino acids to urea and carbon dioxide (1, 3–6). These earlier data demonstrate that the depression of amino acid catabolism is associated with quantitative changes in the activity in vivo of certain metabolic pathways. The metabolic activity involves quantitative and qualitative differences for both carcinogens studied previously and also for the regenerating liver. The present investigations further substantiate and extend this finding.

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MATERIALS AND METHODS

Experimental procedure.—We have used the isolated, perfused, rat liver for these investigations. Tumorigenesis has been induced in this organ by a variety of carcinogens including an azo dye, TAA,2 AAF, ethionine, and DMN. In addition to the carcinogen treated animals we have studied regenerating livers, livers treated with carbon tetrachloride and livers from rats whose bile ducts had been ligated previously. Each of these conditions has biologic features in common with the neoplastic process, viz., proliferation of all hepatic cell types during regeneration, parenchymal cell necrosis and growth of connective tissue following carbon tetrachloride treatment and the extensive intrahepatic bile duct cell proliferation which occurs following bile ligation (14).

Livers from each type of rat were perfused for a 4-hr period. Appropriate normal control livers were also perfused. In each type of liver the metabolic fate of 15 different L-amino acids was studied. The amino acids in amounts of 3.0 mmoles of nitrogen were dissolved in Ringer's solution, neutralized, and added to the perfusate. Measurements were made of urea nitrogen production, 14CO2 expiration, and liver plasma protein incorporation of amino acid14C. For each liver type with each amino acid supplement 4–5 liver perfusions were run.

The technic of isolated rat liver perfusion has been described previously in detail (3, 22, 23). The bile duct,

2 The abbreviations used are : AAF, 2-acetylaminofluorene; MeDAB, 3'-methyldimethylaminoazobenzene; TAA, thioacetamide; and DMN, dimethylnitrosamine.
hepatic portal vein and thoracic vena cava were cannulated with the rat under light ether anesthesia. The perfusate consisted of 90 ml of normal rat blood and 5000 units of heparin, 100 mg of D-glucose, and 5 µc of the appropriate "C-labeled amino acid in 10 ml of Ringer's solution.

The rats used were 180–200 gm males obtained from the Holtzman Rat Company. Experimental liver carcinogenesis was initiated by feeding rats a basal diet (ground Purina Checkers) supplemented with one of the following carcinogens (a) 0.06 % MeDAB for 10–12 weeks; (b) 0.25 % "C-ethionine for 14–15 weeks (10, 11, 12); (c) 0.4 % AAF for 6 months, (31); (d) 0.03 % TAA for 5 months (12, 16), and (e) 0.005 % DMN for 30 weeks (20, 21).

Neoplastic growths represented by multifocal nodules were induced in all cases. Further data about the nature of the tumors induced by each type of carcinogen can be obtained from the references cited.

Regenerating livers were removed for perfusions 24 or 48 hr following subtotal heptectomy performed on 350–400 gm rats. The larger rats were chosen to provide a regenerating liver comparable in size to the other livers studied.

Carbon tetrachloride (0.5 ml in mineral oil) was administered to intact rats by stomach tube 48 and 24 hr before isolation and perfusion of the liver.

Normal, control rats were maintained on the same diet without added carcinogen for 4–5 months.

Analytical methods.—Blood glucose was determined by a glucose oxidase method (Glucostat Worthington Biochemical Co.). Liver and plasma proteins were assayed by the Lowry method using a bovine serum albumin standard (19). Free amino acid concentrations in plasma and liver homogenates were determined by the method of Moore and Stein (24) using a leucine standard. Urea nitrogen was determined by the Conway microdiffusion method as previously described (3, 7). In all experiments the specific activity of the added "C-labeled amino acid was adjusted to 5 µc/3.0 mmoles.

Expired carbon dioxide was trapped in 40 % potassium hydroxide. Aliquots of this solution were plated on filter paper discs, dried, placed in vials with toluene-scintillator solution and counted in a Packard Tri-Carb liquid scintillation counter.

Liver and plasma proteins were collected by precipitation with 6 % trichloroacetic acid from liver homogenates or perfusate plasma. The precipitates were suspended in water and aliquots of these suspensions plated and counted.

RESULTS AND DISCUSSION

Table 1 summarizes the data on the metabolic fate of each of 15 amino acids in terms of the production of urea nitrogen, the expiration of "CO2 and the incorporation of amino acid "C into liver and into plasma proteins respectively during 4-hr perfusions of normal rat livers. In Charts 1 through 9 the data for the experimental livers are presented as percentages of these normal values. Each chart presents the data relative to a particular type of liver. The data for each parameter measured for each amino acid are presented as profiles for each liver type studied.

The results in Chart 1 indicate that livers treated with carbon tetrachloride have a normal or slightly reduced functional activity in terms of incorporation of amino acid "C into plasma protein but a considerably enhanced incorporation into liver proteins. The latter may be indicative of a post necrotic reparative process. In these livers, although the conversion of amino acid nitrogen to urea nitrogen is essentially normal, the oxidative catabolism of amino acids is depressed as indicated by a decreased expiration of "CO2. This depression may be responsible.

### Table 1

<table>
<thead>
<tr>
<th>Added amino acid</th>
<th>Urea-nitrogen produced</th>
<th>% added 14C-activity incorporated in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Valine</td>
<td>15</td>
<td>57</td>
</tr>
<tr>
<td>Leucine</td>
<td>15</td>
<td>27</td>
</tr>
<tr>
<td>Methionine</td>
<td>17</td>
<td>49</td>
</tr>
<tr>
<td>Alanine</td>
<td>22</td>
<td>29</td>
</tr>
<tr>
<td>Lysine</td>
<td>38</td>
<td>53</td>
</tr>
<tr>
<td>Glycine</td>
<td>43</td>
<td>20</td>
</tr>
<tr>
<td>Histidine</td>
<td>50</td>
<td>36</td>
</tr>
<tr>
<td>Glutamate</td>
<td>12</td>
<td>52</td>
</tr>
<tr>
<td>Citrulline</td>
<td>31</td>
<td>—</td>
</tr>
<tr>
<td>Aspartate</td>
<td>21</td>
<td>57</td>
</tr>
<tr>
<td>Aspartate + citrulline</td>
<td>38</td>
<td>—</td>
</tr>
<tr>
<td>Arginine</td>
<td>53</td>
<td>39</td>
</tr>
<tr>
<td>Glutamine</td>
<td>55</td>
<td>47</td>
</tr>
<tr>
<td>Asparagine</td>
<td>25</td>
<td>—</td>
</tr>
</tbody>
</table>

- Values given are averages for 4–5 perfusions. Ranges did not exceed ±5% in any series.
- Milligrams/10 gm liver/4 hr, corrected for endogenous urea nitrogen production from amino acids present in blood perfusate (4).

**Chart 1**—Metabolic profiles of perfused livers from carbon tetrachloride treated rats. The following abbreviations are used: LLEU, I leucine; VAL, valine; L EU, leucine; MET, methionine; ALA, alanine; LYS, lysine; GLY, glycine; HST, histidine; GLA, glutamate; CIT, citrulline; ASA, aspartate; ARG, arginine; GLN, glutamine; and ASN, asparagine.
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After 48 hr the regenerating liver has returned to an approximately normal metabolic profile with the exception of the conversion of amino acid carbons to carbon dioxide. Interpretation of this profile can be only speculative.

Ligation of the bile duct is known to be followed by extensive proliferation of bile duct cells and an extension of biliary tubules (or pseudo tubules) throughout the hepatic parenchymal areas (14, 30). The morphologic changes following duct ligation resemble some of the cellular changes associated with azo-dye feeding. However, the azo-dye has the additional capacity to induce a proliferative or regenerative response of the hepatic cells. This latter reaction does not form a part of the response pattern to bile duct ligation. Whether ligation would ultimately result in precancerous changes or tumor formation seems doubtful but cannot be definitely determined as the animals usually die within about 40 days. Up to that time tumors do not develop.

The data in Chart 4 indicate that bile duct ligation is accompanied by a considerable generalized depression of amino acid metabolic activity. This decrease may be a reflection of the marked increase in biliary cells and the concomitant decrease in proportion of parenchymal cells rather than a functional impairment or inhibition. It provides an indication that the cellular localization of many enzymes (both catabolic and anabolic) of amino acid metabolism may be within the parenchymal cells.

With regard to the metabolic effects of the 5 carcinogens investigated the data presented in Charts 5 through 9 indicate some interesting individual variations in addition to a common tendency toward decreased amino acid catabolism and increased incorporation of amino acids for a potentiation of protein synthesis rather than merely a reflection of increased \(^\text{14C}\)-amino acid incorporation into protein.

The measured metabolic activities of regenerating livers (Charts 2, 3) are dependent on the time between partial hepatectomy and liver isolation for perfusion. After 24 hr the livers manifest a decreased amino acid degradation and a substantially enhanced incorporation of amino acids into liver proteins up to those experiments which involve glutamate and the other amino acids to the right of glutamate on the chart. Our interpretation of these results is that the process of hepatic regeneration involves a deletion or inhibition of a metabolic reaction which participates in the transfer of amino acid nitrogen to urea nitrogen. The amino acids, whose nitrogen is converted to urea nitrogen to an extent comparable to the activity in the normal liver, are all located at points beyond glutamate on the pathway of urea synthesis. Greenbaum et al. (15) have reported a decrease in the activity of glutamic dehydrogenase and glutamic-aspartic transaminase following partial hepatectomy. Our results may be related to such decreases. It is at least teleologically satisfying to note the existence of a depression of urea synthesis which is concomitant with and may be the impetus for enhanced protein synthesis.

Chart 2.—Metabolic profiles of perfused, regenerating livers from rats 24 hr following partial hepatectomy. For explanation of abbreviations, see Chart 1.

Chart 3.—Metabolic profiles of perfused, regenerating livers from rats 48 hr following partial hepatectomy. For explanation of abbreviations, see Chart 1.

Chart 4.—Metabolic profiles of perfused livers from rats with ligated bile ducts. For explanation of abbreviations, see Chart 1.
into protein. At this point note should be made of the fact that the livers from carcinogen treated animals have retained a number of activities characteristic of normal liver function. Of special interest among these is the capacity to maintain reasonably constant levels of blood glucose and to produce and excrete bile. In all of these liver perfusions the bile ducts were cannulated and bile collected throughout the experiment. In all cases 2–3 ml of the bile were produced during the 4-hr period. In each case the tissue which is perfused is a heterogeneous complex of normal liver tissue, tissue affected by changes ancillary to carcinogenesis, e.g., fibrosis and necrosis, and neoplastic tissue at various stages of tumor progression.

In Chart 5 it can be seen that livers from AAF treated rats show a moderate increase in plasma protein and a considerable increase in liver protein incorporation of amino acid-\(^{14}\)C.

The capacity of these livers to produce urea nitrogen is decreased for most of the amino acids tested. However, the contribution to urea production from the nitrogen of aspartate is only slightly lower than normal. A perfusate supplement containing an equimolar mixture of citrulline and aspartate or an arginine supplement is equivalent in regard to urea synthesis in these livers to the capacity of the normal liver with these supplements. The metabolic defect in AAF treated livers may be related to the capacity of these livers to convert glutamate to aspartate. The expiration of \(^{14}\)CO\(_2\) from aspartate as compared with glutamate is compatible with this conclusion. In addition the low synthesis of urea from citrulline alone may be explained by a decreased availability of aspartate.

The data in Chart 6 indicate that, although the effects of MeDAB resemble superficially those of AAF (including enhancement of liver and plasma protein incorporation of amino acid-\(^{14}\)C), the resulting metabolic profiles show that the underlying biochemical alteration involves a different reaction in the pathway of urea synthesis in each case.

The effects of TAA present another type of profile as can be seen in Chart 7. In the case of this carcinogen there is a striking enhancement of liver protein \(^{14}\)C-activity and a variable increase of amino acid-\(^{14}\)C incorporation into plasma proteins. The latter depends on the nature of the amino acid supplement. The 300 % increase in the incorporation of amino acid-\(^{14}\)C into liver protein from lysine-\(^{14}\)C represents the most notable feature of the protein pattern. The production of urea nitrogen from the various amino acids is normal with an exception in the case of lysine. The expiration of \(^{14}\)CO\(_2\) is greatly reduced from all amino acids.

In livers from rats treated with a large dose of TAA a decrease in the oxidation of malate, pyruvate, octanoate, and glutamate has been reported by Gallagher et al. (13). These investigators ascribed these findings as due to an
inhibition of enzyme systems by an increased intracellular accumulation of calcium due to increased membrane permeability brought about by TAA.

These data contribute to a metabolic profile which may be representative of the synthesis of a liver protein much higher than normal in its content of lysine. In this regard we have previously reported an increased synthesis of acid-soluble proteins in perfused livers from carcinogen treated rats (2). The suggestion that the protein may represent a nuclear histone provides special relevance when the extensive work of Davis and Busch (8) and Starbuck and Busch (29) on acid-soluble protein synthesis in a variety of rat tumors is considered.

Chart 8 outlines the results with livers from DMN-treated animals. The generalized depression of amino acid metabolism found with DMN is comparable to the profile obtained after bile duct ligation. A decrease in the incorporation of the amino acids valine and leucine into liver protein following within 3 hr the administration of DMN has been found by Hultin et al. (17) using an in vitro system and by Magee (21) in intact animals. The present work confirms and extends these findings to include the other amino acids studied. Thus, this pattern of hepatic activity may be regarded as indicating a loss of normal parenchymal function as a result of either hepatic necrosis or a tendency toward bile duct cell proliferation and cholangioma formation. Preliminary histologic examination of routine hematoxyline and eosin stained sections of these and all of the other liver types indicate a tendency of all the carcinogens studied to induce both hepatoma and bile duct cell type tumors and also to be associated with some degree of fibrosis, necrotic degeneration and occasional fatty infiltration. The latter pathologic changes may be the result of the nutritional status of the animals. We have concluded, however, that cholangioma formation is more usual and extensive in DMN-treated rats and that ethionine produces hepatic parenchymal cells tumor more frequently. Data on the histologic effects of the various treatments will be published separately.

In Chart 9 the data derived from ethionine treated livers have a strong resemblance to those derived from livers of rats fed the azo-dye. Also livers from ethionine treated rats have free amino acid levels which are half again as high as normal and equivalent to the levels in azo-dye livers (Chart 10). Levy et al. (18) found several amino acids to be increased after acute ethionine intoxication. Farber (10, 11) has noted previously that the early histologic changes induced in the liver by ethionine, AAF and MeDAB are similar, and indeed, he proposed that these carcinogens might have common metabolic effects. This seems to be the case in general but, when the over-all data are considered, each carcinogen has a unique profile. In the case of ethionine the metabolism of glutamine in particular is of interest. In a previous publication we pointed out that nitrogen from this amino acid could be converted to urea nitrogen to a completely normal extent even though urea production from all other amino acids including arginine was reduced to about half that found in normal livers. We have commented previously on the
unique role of glutamine in urea synthesis and the possibility that the nitrogen of this amino acid is transferred to urea by a metabolic pathway other than the Krebs cycle—a possibility, incidentally, which seems less and less likely in view of results from other laboratories (28).

Finally the data presented in Chart 10 indicate that the changes in amino acid metabolism described may not be the result of changes in the level of liver free amino acid pools. With the exception of livers from DMN-treated rats all of the experimental livers have concentrations of amino acids in excess of the normal level.

CONCLUSIONS

The results of the experiments described in this paper are interpreted to indicate that a number of hepatic carcinogens have several similar over-all metabolic effects. The effects of feeding these carcinogens are all related to a decrease in the hepatic synthesis of urea and production of carbon dioxide from amino acids and are usually associated also with an increased incorporation of amino acids into liver and plasma proteins. However, the underlying metabolic defects or alterations are related to the type of carcinogen used. Thus AAF is associated with a defect in aspartate metabolism, MeDAB is related to a decrease in urea formation from all amino acids tested. TAA treatment results in livers with an almost normal capacity for urea synthesis (except from lysine and glutamate) but a generalized depression of 14CO2 production.

Hepatic carcinogenesis initiated by ethionine results in a metabolic profile similar to that related to azo-dye induced carcinogenesis. In contrast DMN treatment, which results in a preponderance of tumor foci of bile cell origin, is associated with a generalized decrease of both protein synthetic activities and activities related to amino acid degradation.

The conclusion that tumors may show a general metabolic similarity is perhaps, a restatement of Grenstein's concept of convergence but with the realization that the specific biochemical effects of different carcinogens may be diverse. In this connection, Davis and Morris (9) have found that the enhanced incorporation of lysine-14C into the proteins of the Walker 256 tumor and into the Morris 5123 hepatoma is related to a decreased rate of lysine degradation beyond piperolic acid. Although the degradation of lysine is decreased considerably in livers from rats fed several of the carcinogens which we have investigated, this depression in liver tumors is unlikely to be related to a block in piperolic acid metabolism since in these livers a depression in the conversion of lysine-nitrogen to urea nitrogen also occurs. The latter step is known to precede the formation of piperolic acid. The general results of Davis and Morris are thus confirmed but the biochemical "lesion" is found to differ.

In addition the quantitative pattern of amino acid incorporation into protein is compatible with the possibility that different proteins are synthesized as a result of the type of carcinogen treatment used.

Other liver conditions are related also to changes in amino acid metabolism. The over-all changes are similar in some cases to the changes during carcinogenesis (for example, those found at 24 hr after partial hepatectomy) but the specific nature of the metabolic change is characteristic of the pathologic or physiologic alteration in the liver and does not, in any specific way, resemble the profiles of livers undergoing carcinogenesis.

Although the investigations reported here did not uncover any significant variations in the metabolic profiles of the livers from animals treated with the same carcinogen such variations might also be common to chemically induced hepatic neoplasms. That such a situation is indeed the case is indicated by studies on several minimal deviation hepatomas all of which were induced by the carcinogen N-(2-fluorenyl) phthalamic acid (25, 27).

We are now extending these studies to include an examination of the nature of nuclear and cytoplasmic protein synthesized in the various types of livers and to include investigations of changes in protein and nucleic acid metabolism occurring soon after the onset of carcinogen feeding.

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