Feedback Inhibition of Aspartate Transcarbamylase in Liver and in Hepatoma*

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SUMMARY

Aspartate transcarbamylase was partially purified from rat liver, Morris hepatoma 5123-A or D, Hepatoma 7800, and Novikoff hepatoma and was shown to be inhibited by pyrimidine deoxyribonucleosides and deoxyribonucleotides. The pyrimidine analogs, 5-bromo-2'-deoxyuridine, 5-bromouridine, 5-bromo-2'-deoxycytidine, 5-fluoro-2'-deoxyuridine, 5-fluorouridine, 5-fluoro-2'-deoxycytidine, 5-iodo-2'-deoxyuridine, also inhibited enzymatic activity. The enzyme, however, was most effectively inhibited by 2'-deoxyadenosine-5'-monophosphate (or 2'-deoxyguanosine-5'-monophosphate [d-DMP]) and by 2'-deoxyadenosine or 2'-deoxyguanosine.

Aspartate transcarbamylase was shown to be identical in liver and in the hepatomas by pH optimum, $K_m$ values for carbamyl phosphate and aspartate, $V_{max}$, degree of inhibition by Cu++, by the pyrimidines, and by the inhibition constants for BCDR, FUDR, thymidine, and d-AMP (or d-GMP).

It was concluded that negative feedback inhibition of pyrimidine biosynthesis occurs in hepatomas as well as in liver, and to the same extent. Furthermore, the control of aspartate transcarbamylase by d-AMP (or d-GMP) may play a significant role in vivo.

The original deletion hypothesis for carcinogenesis proposed by Potter (17) in 1944, which stated that neoplasia may be characterized by an absence or reduction in concentration of one or more enzymes, has been modified recently (18) to include the mechanisms responsible for the control of enzyme activity or synthesis. These mechanisms include negative feedback inhibition operative at the substrate level and repression operative at the genome. Aspartate transcarbamylase, an enzyme responsible for the irreversible carbamylation of L-aspartic acid by carbamyl phosphate (I) is subject to both negative feedback inhibition (24, 27) and to repression (28) in Escherichia coli.

$\text{COOH} \quad \text{O}$
$\text{CHNH}_2 + \text{NH}_2 \text{-CO-PO}_3\text{H}_2 \rightarrow \text{CH-} \text{NH-} \text{C-NH}_2 + \text{H}_3\text{PO}_4$

$I$

Negative feedback inhibition by pyrimidines of this enzyme is also manifested in mammalian tissues—i.e., in Ehrlich ascites cells (4) and in liver preparations (2). No repression of this enzyme has been observed in liver by pyrimidines or pyrimidine derivatives to date.1

In the absence of repression in mammalian tissues, the role of feedback inhibition of pyrimidine biosynthesis as a mechanism of enzymatic control would assume added importance. Therefore, an investigation has been undertaken to ascertain the relative importance of such regulatory control in the development of normal liver and of hepatomas. Toward this end, aspartate transcarbamylase has been partially purified from rat liver and from various rat hepatomas, and the effects of various pyrimidine derivatives upon this enzyme have been E. Bresnick, unpublished experiments.

Received for publication July 11, 1962.

* This investigation was supported by an American Cancer Society Institutional Research grant and by a grant from the Anna Fuller Fund.

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investigated. The results of these studies are reported in this manuscript.

MATERIALS AND METHODS

Chemicals.—All pyrimidine and purine nucleotides were obtained from commercial sources. The author is indebted to the Hoffmann-LaRoche Co., Nutley, N.J., for FUDR, FUR, and FCDR and to Dr. Samuel Bieber, Burroughs Wellcome & Co., Inc., Tuckahoe, N.Y., for gifts of BCDR, BUDR, and IUDR. Commercial dilithium carbamyl phosphate (90 per cent pure) was further purified by the method suggested by Gerhart and Pardee (5) and stored over calcium chloride in a desiccator at −5°C.

Animals.—Livers from male Holtzman rats weighing from 125 to 150 gm. were employed as the source of the enzyme. The author is also grateful to Dr. Harold P. Morris of the National Cancer Institute, Bethesda, Md., who provided the Morris Hepatoma 5123, Hepatoma 7800, Hepatoma H-55, and the Hepatoma 7316-A, as well as the information about these hepatomas. Buffalo rats bearing the Morris Hepatoma 5123-A which had been inoculated subcutaneously and bilaterally were sacrificed when the tumor was 54 days old. A Buffalo rat bearing the Hepatoma 5123-D inoculated intramuscularly and subcutaneously on the right side was sacrificed when the tumor was 87 days old. The Hepatoma 7800 was obtained from a Buffalo rat given inoculations intramuscularly and subcutaneously and was used when 100 days old. The Hepatoma 7800 is a well differentiated hepatocellular carcinoma induced in a male rat by N-(2-fluorenyl)phthalamic acid (13), the same compound used to induce the Morris Hepatoma 5123. Additionally, aspartate transcarbamylase activity was determined in a 169-day-old Hepatoma 7316-A obtained from a Buffalo rat which had been given inoculations bilaterally and intramuscularly, and in the Hepatoma H-55, obtained from the A × C rat previously given inoculations intramuscularly and subcutaneously. The Hepatoma 7316-A is a slowly growing tumor induced originally by 2,4,6-trimethylamline in the Buffalo rat, whereas the Hepatoma H-55 was originally induced by Reuber (23) in the gray A × C rat. The initial Novikoff hepatooma was kindly supplied by Dr. N. B. Fur long of the M. D. Anderson Hospital and Tumor Institute, Houston, Texas. The tumor was maintained by weekly transfers, intraperitoneally, into Holtzman rats. The ascitic hepatoma cells were collected by centrifugation, washed twice with 0.9 per cent saline, weighed, and resuspended to a concentration of 10 per cent with distilled water.

Enzyme preparation.—Homogenates of 10 per cent in 0.25 mM sucrose were prepared from the liver and hepatomas (except the Novikoff hepatoma, which was suspended in water) and were centrifuged at 105,000 × g for 45 minutes at 4°C. All the steps in the purification procedure were conducted at this temperature. The purification procedure has been described in detail elsewhere (3) and will only be presented briefly here.

To the supernatant preparation obtained after centrifugation was added sufficient (NH₄)₂SO₄ to give a 30 per cent saturation. The resultant precipitate was dialyzed against 0.1 mM imidazole buffer, pH 7.0, containing 0.002 M mercaptoethanol, for 1 hour in a rotating dialyzer apparatus, then calcium phosphate gel (6) was added (2 mg/mg protein). The enzyme was eluted from the gel with 0.5 mM phosphate buffer, pH 7.4, and was then dialyzed against the imidazole-mercaptopethanol buffer for 1 hour in the dialyzer. The enzyme was further fractionated on DEAE-cellulose columns (1.5 × 30 cm.) by discontinuous elution with the imidazole-mercaptopethanol buffer, containing 0–0.8 M potassium chloride. The total enzymatic activity was eluted with 0.8 M KCl. This preparation could be maintained for 5 days at 4°C. Without loss in activity but could not be stored below freezing.

Protein was assayed by the colorimetric procedure of Lowry et al. (10) or the spectrophotometric method based upon the absorbance at 280 µm (8).

Enzyme assay.—Enzymatic activity was assayed by the production of carbamylaspartate in a system containing the following: carbamyl phosphate, 10 µmoles; L-aspartic acid, 3.75 µmoles; enzyme; pyrimidines or purines; 0.2 M Tris buffer, pH 9.2, to make a total volume of 1.5 ml. The incubation was conducted at 37°C for 30 minutes, after which enzymatic activity was stopped by the addition of 0.5 ml. of 4 n perchloric acid. The carbamylaspartate concentration was determined in a 0.5-ml aliquot by the recent modification (5) of the method of Koritz and Cohen (7). The amount of carbamylaspartate produced nonenzymatically was simultaneously determined.

RESULTS

A summary of the scheme for the purification of aspartate transcarbamylase from liver, the No-
vikoff hepatoma, Hepatomas 5123-A or D, and 7800 is presented in Table 1. An over-all purification of 56-fold was achieved from rat liver, whereas the purification from the three hepatomas varied from 30- to 54-fold. Only the Novikoff hepatoma was significantly different from liver when the aspartate transcarbamylase activity was expressed on a wet weight basis. The values for liver, Morris Hepatoma 5123-A, 5123-D, Hepatoma 7800, Hepatoma H-35, Hepatoma 7316-A, and Novikoff hepatoma were 20.0, 21.1, 26.4, 27.8, 34.8, 20.1, and 96.9 units per gram wet weight, respectively.

**Table 1**

Partial Purification of Aspartate Transcarbamylase from Liver and Hepatomas

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Hepatoma 5123-A</th>
<th>Hepatoma 7800</th>
<th>Novikoff Hepatoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Homogenate</td>
<td>10%</td>
<td>0.040</td>
<td>0.080</td>
<td>0.084</td>
</tr>
<tr>
<td>10X Supernate</td>
<td>(NH_4)_2SO_4, 80%</td>
<td>0.14</td>
<td>0.090</td>
<td>0.100</td>
</tr>
<tr>
<td>Gel eluate</td>
<td>1.24</td>
<td>0.70</td>
<td>0.90</td>
<td>4.91</td>
</tr>
<tr>
<td>DEAE fraction</td>
<td>2.28</td>
<td>1.39</td>
<td>1.84</td>
<td>10.6</td>
</tr>
<tr>
<td>Purification, x-fold</td>
<td>56</td>
<td>43</td>
<td>54</td>
<td>30</td>
</tr>
</tbody>
</table>

* Similar purification was obtained with the Morris hepatoma 5123-D.
† One unit is defined as the number of μmoles of product (carbamylaspartate) produced under the standard conditions of the assay.

The effect of pyrimidine and purine nucleosides and nucleotides upon the partially purified aspartate transcarbamylase from liver and from the hepatomas was determined (Table 2). The deoxyribonucleosides were very effective inhibitors of this enzyme from all sources investigated, and thymidine appeared to possess the greatest inhibitory efficacy of the pyrimidine derivatives; the deoxyribonucleoside of uracil was as effective as the corresponding cytosine compounds. The nucleotides, however, of uracil, cytosine, or thymine were not as inhibitory as the nucleosides. The degree of inhibition of the enzyme from the hepatomas was similar to that observed with the liver enzyme. The deoxyribonucleosides and deoxyribonucleotides of adenine and guanine were much more effective than thymidine (by approximately four-fold), and the inhibition produced by the nucleoside was equal to that observed with the nucleotide. The corresponding ribose derivative, at comparable concentrations, exerted no inhibition upon this enzyme. Comparable inhibition of the Novikoff and 5123-D hepatomas by the purine derivatives was also observed.

Pyrimidine analogs proved to be effective inhibitors of the partially purified enzyme of liver as well as of the hepatomas (Table 3). The deoxyribonucleosides of bromouracil and fluorouracil were slightly more active as inhibitors than the corresponding ribonucleoside. The most potent inhibitor of the pyrimidine analogs was BCDR, which at 50 μmoles resulted in a reduction of liver aspartate transcarbamylase by 69.6 per cent. Once again, the pyrimidine analogs exerted comparable inhibitions upon the enzymes isolated from the Novikoff or 5123-D hepatomas.

In Chart 1 the inhibitions of the liver aspartate

**Table 2**

Inhibition of Aspartate Transcarbamylase from Liver and Hepatomas by Nucleosides and Nucleotides

<table>
<thead>
<tr>
<th>Source of Enzyme</th>
<th>Liver</th>
<th>Novikoff</th>
<th>5123-D</th>
<th>7800</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-UR</td>
<td>20</td>
<td>31.5</td>
<td>49.9</td>
<td>47.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>54.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-UMP</td>
<td>10</td>
<td>11.5</td>
<td>17.4</td>
<td>17.1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20.0</td>
<td>30.1</td>
<td>47.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>41.3</td>
<td>30.1</td>
<td>15.2</td>
</tr>
<tr>
<td>UMP</td>
<td>20</td>
<td>20.0</td>
<td>29.0</td>
<td>17.2</td>
</tr>
<tr>
<td>d-CR</td>
<td>20</td>
<td>41.1</td>
<td>43.6</td>
<td>34.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>50.1</td>
<td>49.6</td>
<td>35.9</td>
</tr>
<tr>
<td>d-CMP</td>
<td>20</td>
<td>21.6</td>
<td>30.1</td>
<td>15.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>43.0</td>
<td>27.2</td>
<td>39.7</td>
</tr>
<tr>
<td>CMP</td>
<td>20</td>
<td>10.0</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>TdR</td>
<td>10</td>
<td>20.0</td>
<td>15.1</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20.0</td>
<td>43.6</td>
<td>34.1</td>
</tr>
<tr>
<td>TMP</td>
<td>10</td>
<td>11.5</td>
<td>18.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>41.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-AMP (or d-GMP)</td>
<td>1</td>
<td>1.0</td>
<td>22.2</td>
<td>29.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>11.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>30.0</td>
<td>52.5</td>
<td>63.2</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>65.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-AdR (or d-GdR)</td>
<td>20</td>
<td>61.2</td>
<td>70.4</td>
<td>78.9</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>88.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results are reported as an average of two experiments. The amounts of carbamylaspartate produced by the enzyme from the livers, Novikoff hepatoma, Hepatoma 5123-D, and Hepatoma 7800 in Experiment 1 was 0.415, 0.427, 0.375, and 0.398 μmoles; in Experiment 2, 0.397, 0.443, 0.397, and 0.396 μmoles.
transcarbamylase by TdR, FUDR, BCDR, d-AMP (or d-GMP) have been plotted according to the method of Lineweaver and Burk (9). The data clearly define the inhibition by these compounds as competitive. The inhibition constants are presented in Table 4 and will be discussed subsequently. Similar Lineweaver and Burk plots have been obtained with the hepatoma enzymes.

A comparison of the aspartate transcarbamylase enzymes partially purified from liver, Morris Hepatoma 5123-A or D, Novikoff hepatoma, and Hepatoma 7800 is presented in Table 4. The pH optimum of the enzymes was 9.2; the $K_m$ for aspartate did not vary significantly and ranged from 1.6 to $2.2 \times 10^{-3}$ M; the $K_m$ with carbamyl phosphate as substrate also did not vary significantly and ranged from $3.0$ to $4.8 \times 10^{-4}$ M; the $V_{max}$ also did not differ and ranged from $1.6$ to $2.5 \times 10^{-4}$ M.

Liver aspartate transcarbamylase was inhibited by heavy metals—i.e., Cu++, Hg++, Cd++, and Ag+ (3); this was relieved upon the addition of EDTA to the medium. An examination of the relative inhibition of the enzymes isolated from liver and from the various hepatomas revealed no essential difference. Furthermore, the inhibition constants for FUDR, BCDR, TdR, d-AMP (or d-GMP) did not significantly differ in hepatomas from that observed with liver. It is interesting to note that d-AMP (or d-GMP) was the most potent of the inhibitors in this series and was bound to the enzyme approximately 10 times better than was aspartic acid. The above data appear to indicate that aspartate transcarbamylase is the same in the liver and in the hepatomas, independent of the growth rate of the hepatoma.

**DISCUSSION**

The group of the slowly growing hepatomas employed in this study, Hepatomas 5123-A or D, 7316-A, 7800 or H-35, possess aspartate transcarbamylase activities equivalent to that observed in adult normal liver. Previously, it had been noted that the Morris Hepatoma 5123 possessed primarily an aerobic energy metabolism, resembling adult liver (1); a carbohydrate enzyme composition close to liver (25), although there did occur a decreased glycogen synthesis from labeled carbon and a depressed phosphoglucomutase (26); a similar composition of enzymes involved in the anabolism and
catabolism of pyrimidines (11, 20). The Novikoff hepatoma, on the other hand, was at the other end of the spectrum. A comparative enzymology of the Novikoff hepatoma and liver indicated differences in the enzymes involved in carbohydrate metabolism (16, 26), in pyrimidine anabolism and catabolism (11, 12, 19, 20), as well as other enzymes (15). The activity of aspartate transcarbamylase may also be employed to illustrate this pattern—e.g., the Morris Hepatoma 5123 more closely resembles liver. It is also interesting that the activity of the next enzyme involved in the biosynthesis of pyrimidines, dihydroorotase, is identical in the Morris Hepatoma 5123 and in the liver.1

The results presented in this report agree essentially with those obtained with crude liver preparations (2) and with Ehrlich ascites cells (4) but do not conform to the results observed in bacterial systems (5, 27). However, the different requirements for feedback inhibition for the two systems are not surprising, since the two enzymes do differ markedly in properties (8, 5).

Gerhart and Pardee (5) initially observed the inhibition of E. coli aspartate transcarbamylase by guanosine triphosphate but found that the latter was not as effective as deoxyctydine triphosphate. In the partially purified liver system, d-AMP (or d-GMP) was much more effective than the pyrimidine. Regulatory control of pyrimidine biosynthesis by purine derivatives has already been recorded. Reichard and co-workers (21, 22) observed the inhibition of the reduction of cytidine diphosphate to the corresponding deoxy-derivative by deoxyguanosine triphosphate and deoxyadenosine triphosphate and, to a lesser extent, by thymidine triphosphate. A similar observation has been made by Morris and Fischer (14), who reported the inhibitory action of thymidine upon the conversion of cytidylic acid to d-CMP in tissue culture. This regulatory mechanism—e.g., the control of both the de novo synthesis of pyrimidines and the reduction to deoxyribonucleotides by the deoxyribonucleotides of thymine and adenine (or guanine)—may play a significant role in the mammalian cell.

The data presented indicate that aspartate transcarbamylase, the locus for negative feedback inhibition in E. coli (24, 27), Ehrlich ascites cells (4), and in liver (2), is not significantly different in either slowly growing hepatomas, a fast-growing hepatoma, or normal liver. This conclusion is based upon the similarity of $K_m$ values for aspartate, carbamyl phosphate, the $V_{max}$, the pH optimum, inhibition by Cu++, and the inhibition constants for FUDR, BCDR, TdR, and d-AMP (or d-GMP). Negative feedback inhibition of aspartate transcarbamylase, therefore, appears to operate equally well in hepatomas or in liver in vitro.

This regulatory mechanism, if operative in vivo, does not appear to play any significant role in the development of hepatoma. An alternative explanation, however, may lie in the amounts of pyrimidine or purine derivatives present within the hepatoma cell.

**REFERENCES**


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