Metabolic Adaptations in Rat Hepatomas

II. Tryptophan Pyrrolase and Tyrosine α-Ketoglutarate Transaminase*

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SUMMARY

The administration of tryptophan or cortisone to rats bearing the Morris 5123 Hepatoma resulted in significant increases in tryptophan pyrrolase activity in the host liver but in no change in the low tryptophan pyrrolase activity of the neoplasm. The repressed level of this enzyme in the neoplasm was not a result of the lack or excess of cofactors or enzymes necessary for the assay procedure employed, nor was it due to inhibitors present in the tissue. By means of induction with C14-labeled tryptophan, the acid-soluble and protein fractions of both the liver and tumor were found to contain essentially the same quantity of the labeled compound at the end of the induction period, showing that the failure of the response of tryptophan pyrrolase of Hepatoma 5123 was not due to an inadequate amount of inducer available to the neoplasm.

The tyrosine α-ketoglutarate transaminase activity of Hepatoma 5123 was very high (derepressed) in tumors carried in intact hosts. Tyrosine or cortisone administration did not change the level of the enzyme in the tumor significantly, although both agents increased the activity of the host liver markedly. The tyrosine α-ketoglutarate transaminase of Hepatoma 5123 in adrenalectomized hosts was much lower than that in tumors in intact animals. Administration of cortisone to adrenalectomized animals markedly stimulated the activity of this enzyme in the hepatoma.

A simple working hypothesis is presented, based on these and related findings, to explain the biologic malignancy expressed by the Morris Hepatoma 5123.

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† Lillian Israel Memorial Fellow in Cancer Research of the American Cancer Society, 1957-1960.

In the first paper in this series (18) we showed that the enzyme tryptophan pyrrolase was repressed1 in the Morris Hepatoma 5123 and that threonine and serine dehydrase were derepressed in this neoplasm under certain physiologic conditions, whereas their activity was repressed in adrenalectomized animals. The levels of these enzymes in the tumor did not change with changes in dietary protein, although the same enzymes in the host liver did increase with an increase in dietary protein level. In view of the failure thus far to find any qualitative enzyme deletion in this neoplasm when compared with that in normal liver, coupled with the observation of an alteration or loss of control of the synthesis of these three enzymes, a study was made of several liver enzymes exhibiting specific control mechanisms in order to see how far the derangement of control extended. The substrate and hormonal control of these enzymes, tryptophan pyrrolase and tyrosine α-ketoglutarate transaminase, was studied in the Morris Hepatoma 5123 and normal liver. The results of this investigation comprise the subject of this paper.

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MATERIALS AND METHODS

Buffalo strain rats raised at the National Cancer Institute or the McArdle Memorial Laboratory were used in all these experiments. Hepatoma 5123 was inoculated via trocar subcutaneously, intramuscularly, or intraperitoneally into these animals. The animals bearing tumors were maintained at the McArdle Laboratory until 2–3 months after inoculation, at which time they were used in the experiments reported here. Both male and female animals and neoplasms of sublines A–D were used in the experiments reported here, no difference being noted with respect to sex or subline of tumor. All the animals were maintained on Purina Laboratory Chow except for some of the animals used for the transaminase assay that were maintained on the 2 and 91 per cent protein diets described in the first paper (18). Some tumor-bearing animals were bilaterally adrenalectomized at the Endocrine Research Laboratories, Madison, Wisconsin. These animals were maintained on 1 per cent NaCl in their drinking water after the operation and were not used for at least 1 week after surgery.

Homogenates were prepared and tissues examined as described in the previous publication (18). Tryptophan pyrrolase activity was measured on the whole homogenate by the procedure of Knox and Auerbach (11). Tyrosine a-ketoglutarate transaminase was measured by a slight modification of the method of Linet et al. (13). The reaction was carried out in 25-ml. Erlenmeyer flasks containing 0.1 or 0.2 ml. of the 60 min. × 105,000 g supernatant of the tissue, 0.2 ml. 0.006 per cent pyridoxal phosphate, 0.2 ml. 0.05 M sodium diethyldithiocarbamate, 0.2 ml. 0.5 M a-ketoglutaric acid, pH 7.0 with NaOH, 2.0 ml. 0.2 M K2HPO4, pH 8.0 saturated with L-tyrosine, and water to 3.0 ml. Blank flasks without tyrosine were run with each flask containing the substrate. Two levels, 0.1 and 0.2 ml., of enzyme were run with each tissue sample to insure that the reaction obeyed zero order kinetics. The reaction flasks minus enzyme were preincubated at 37° C. for 5 minutes, at which time the enzyme was added and the flasks were incubated for another 15 minutes. The reaction was stopped by the addition of 1.0 ml. 0.6 N perchloric acid and the enol-borate complex determined by the method of Lin et al. (13).

For the substrate induction of tryptophan pyrrolase, the tumor-bearing animals were given injections intraperitoneally at zero time and again at 4 hours of 4.0 ml. of an 0.5 M suspension of DL-tryptophan in water and sacrificed at 7 hours after the initial injection. For cortisone induction of the enzymes 20 mg. of cortisone acetate was given intramuscularly in 5-mg. doses, spaced over 48 hours, and the animals were sacrificed 5 hours after the last injection (11). Tyrosine a-ketoglutarate transaminase was induced by a single injection of 1.2 mmoles L-tyrosine suspended in 5.0 ml. of normal saline given 5 hours prior to sacrifice (12).

In addition, one group of tumor-bearing animals was given injections intraperitoneally at zero time and at 4 hours of 1 mmole of DL-tryptophan suspended in 5 ml. of normal saline containing 2 µc. of DL-tryptophan-5-C14 (specific activity, 0.2 µc/1 µmole). At the end of the induction period the animals were sacrificed, and the tissues were prepared as described previously (18). Tryptophan pyrrolase assays were carried out on the homogenates of tumor and host liver. In addition, aliquots of homogenate were precipitated with perchloric acid (final concentration, 0.8 n), and the precipitate was washed once in 0.2 N HClO4, then 3 times in Bloom's solvent, and the ribonucleic acid was extracted in 10 per cent trichloroacetic acid at 90° C. (90). The resulting precipitate was washed once with water and dissolved in 5.0 ml. of 98–100 per cent formic acid; 0.1-ml. aliquots of this solution were removed and plated on aluminum planchets, and the radioactivity was determined in a gas-flow counter. The activity on the plates was corrected for self-absorption. Protein was determined on aliquots of the protein solution after neutralization by a modification of the method of Lowry et al. (14).

An aliquot of the acid-soluble supernatant, removed after centrifugation, was neutralized with KOH in ice, the KClO4 was removed by centrifugation, and 0.2 ml. of this supernatant was placed on aluminum planchets and the radioactivity determined in a gas-flow counter with a window; 4.0-ml. aliquots of the neutralized acid-soluble fraction were lyophilized, and the residue taken up in 0.5 ml. 0.1 N HCl. After centrifugation to remove a small amount of insoluble material, 0.1-ml. aliquots of the supernatant were placed on 1" strips of Whatman No. 1 paper, the papers dried, and the chromatograms developed by descending chromatography in the following solvent at 25° C. for 48 hours: tert-butyl alcohol (highest purity), 160 cc.; water, 40 cc.; and concentrated HCl (AR), 1 cc. In this solvent, the Rf values of kynurenine, tryptophan, and anthranilic acid are, respectively, 0.31, 0.71, and 0.97. The position and amount of radioactivity on the strips were determined by means of an automatic scanning device (15).

Sodium diethyldithiocarbamate was a product
of Eastman Chemicals. Pyridoxal phosphate, α-ketoglutaric acid, and dl-tryptophan were obtained from the California Corporation for Biochemical Research, and L-tyrosine was a product of the Nutritional Biochemicals Co. Tertiary butanol was obtained from Matheson, Coleman, and Bell Co. The dl-tryptophan-3-C\textsuperscript{14} was obtained from the New England Nuclear Corporation.

RESULTS

The effects of tryptophan and cortisone injections on the tryptophan pyrrolase activity of host liver and Hepatoma 5123 are seen in Chart 1. From the data depicted there, it is obvious that, although in the host liver the enzyme activity increased up to 30-fold over control levels after injection of tryptophan, no stimulus gave any change in the low levels seen in the tumor. That the tumor does actually possess some tryptophan pyrrolase activity has been further shown by means of a highly sensitive enzyme assay\textsuperscript{2} with tryptophan-C\textsuperscript{14} used as substrate; this demonstrates the formation of C\textsuperscript{14} kynurenine by a chromatographic separation of the two compounds, their radioactivity being determined quantitatively by means of an automatic scanning device (15). However, it cannot yet be stated that the enzyme responsible for the activity is identical with the enzyme in normal liver.

Several other studies have been undertaken to determine whether or not the low stable level of this enzyme in the hepatoma was an artifact of the assay procedure or a result of some defective controlling mechanism inherent in the tumor cell. The presence of the enzyme, formylase, which converts formyl kynurenine, the immediate product of tryptophan pyrrolase action, to kynurenine, the measured product, is not necessary for the assay, since under the conditions of acid deproteinization any of the formyl derivative should spontaneously hydrolyze to kynurenine (1). Preliminary studies with the Morris Hepatoma 5123 have indicated that the kynureninase activity of this neoplasm was not sufficient to explain the low, stable activity of the tumor. Addition of reducing agents, such as ascorbic acid (21), have not shown that the pH of the reaction mixture could account for the low activity in the neoplasm. Mixtures of tumor and liver whole homogenates or soluble fractions gave only additive results in the assay procedure, indicating that intrinsic inhibitors do not play an important role in the tryptophan pyrrolase activity of the hepatoma.

Since the enzymology of the neoplasm did not appear to account for its low stable pyrrolase activity, the availability of the inducer to the tumor cell became of some importance because of the inherently different blood supply of the host liver and the transplanted tumor. Buffalo strain rats bearing Hepatoma 5123 subcutaneously and intramuscularly or intramuscularly and intraperitoneally were each given injections intraperitoneally of 1 mmole of dl-tryptophan containing C\textsuperscript{14}-labeled tryptophan, as outlined in “Materials and Methods.” The results obtained are seen in Table 1. In this table one may see that, 4 hours after the injection of the labeled inducer, there was little difference in the specific activity of the acid-soluble and protein fraction of the tumor and liver, whereas the enzyme activity had increased slightly in the liver and not at all in the tumor. At 8 hours after the initial injection and 4 hours after a second injection of 1 mmole of labeled inducer, there was a marked increase in the tryptophan pyrrolase activity of the host liver but no change in this enzyme’s activity in the hepatoma, confirming the findings shown in Chart 1. Again as at 4 hours no significant difference was seen in the specific activity of the acid-soluble and protein fractions of the liver compared with that of the tumor. Although some unassimilated tryptophan was present in the peritoneal cavity of these animals at the time of sacrifice, this was carefully washed away from the tissue to be used. The subcutaneous and intramuscular tumor transplants did not, of course, have this opportunity for contamination, since they were removed before the peritoneal cavity was opened. Small aliquots of the lyophilized acid-soluble fraction were chromatographed (see “Methods”). Chromatograms from both liver and tumor showed one

\textsuperscript{2} H. C. Pitot, unpublished.
radioactive peak having an Rf of 0.72–0.78. A sample of the DL-tryptophan used for induction had an Rf of 0.73. These results point to the fact that there is no difference in the availability of the inducer to the host liver and Hepatoma 5123 when tryptophan is administered intraperitoneally to tumor-bearing animals. Thus, although the hepatoma has the capacity to form small amounts of tryptophan pyrrolase, it is unable to regulate the synthesis of the enzyme mediated by a dietary (18), substrate, or hormonal stimulus (11).

Table 2 depicts the compilation of data on the tyrosine a-ketoglutarate transaminase activity of Hepatoma 5123 and host liver under a variety of different conditions. In general, the level of activity of this enzyme in the tumor in a non-induced animal is of the same order as that found in the liver of an induced animal. The activity of the enzyme in Hepatoma 5123 does not change to a significant degree with L-tyrosine administration (P > .05), although that of the host liver increases five- to sixfold. The latter finding is in agreement with the findings of Lin and Knox (12). Adrenalectomy does not change the level of enzyme in the liver but markedly drops the activity in the tumor (P < .01). The administration of cortisol to the adrenalectomized tumor-bearing animal significantly increases the transaminase activity in the hepatoma and to a lesser degree in the liver. Thus, these data show that the tyrosine a-ketoglutarate transaminase in Hepatoma 5123 of the adrenalectomized tumor-bearing animal is low and can be increased by the administration of cortisone, indicating that the hormonal control of this enzyme in the tumor of the adrenalectomized animal is essentially normal. However, in the

### TABLE 1

<table>
<thead>
<tr>
<th>Time after injection (hours)</th>
<th>Tryptophan pyrrolase activity†</th>
<th>Acid-soluble radioactivity‡</th>
<th>Protein radioactivity§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host liver</td>
<td>Hepatoma 5123</td>
<td>Host liver</td>
<td>Hepatoma 5123</td>
</tr>
<tr>
<td>0</td>
<td>4.0; 2.6; 2.4; 1.8</td>
<td>0.5; 0.4; 0.2; 0.5</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4.5; 5.5</td>
<td>0.6; 0.2</td>
<td>6070; 2205</td>
</tr>
<tr>
<td></td>
<td>16.0; 12.2; 12.0</td>
<td>0.5; 0.2; 0.1</td>
<td>11,840; 3870; 2770</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3630; 2130</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5440; 3540; 2710</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>92; 72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>102; 102; 80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>129; 100; 96</td>
</tr>
</tbody>
</table>

* The values are given in order in each time period—i.e., the first values in each group in any one time period were all obtained from the same animal.
† μmoles kynurenine formed/hr/gm tissue.
‡ Counts/μmin/gm tissue.
§ Counts/μmin/mg protein.

### TABLE 2

<table>
<thead>
<tr>
<th>Stimulus†</th>
<th>No. animals</th>
<th>Host liver</th>
<th>Hepatoma 5123</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>16</td>
<td>347 ± 53</td>
<td>2040 ± 238</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6</td>
<td>1850 ± 314</td>
<td>2780 ± 205</td>
</tr>
<tr>
<td>Cortisone</td>
<td>4</td>
<td>1550 ± 117</td>
<td>3700 ± 1275</td>
</tr>
<tr>
<td>Adrenalectomy</td>
<td>5</td>
<td>380 ± 63</td>
<td>630 ± 87</td>
</tr>
<tr>
<td>Adrenalectomy + cortisone</td>
<td>4</td>
<td>610 ± 58</td>
<td>1500 ± 540</td>
</tr>
</tbody>
</table>

* All values are given in μmoles of hydroxy-phenylpyruvate/hr/gm protein ± S.E. of the mean. P values are given in the text.
† The methods of administration of the stimuli are detailed in the text.

### DISCUSSION

In the experiments with tryptophan pyrrolase reported here on substrate and hormonal induction, the repressed level of this enzyme in Hepatoma 5123 seen in the dietary experiments (18) was maintained without change regardless of the stimulus (Chart 1, Table 1). The low, unchanging level of this enzyme in the hepatoma was not a result of artifacts in the assay procedure, nor did it appear to be the result of inhibitors indigenous to the tissue or liberated during homogenization. In addition, by means of labeled inducer no difference was found in the intracellular pool of tryptophan in liver and Hepatoma 5123 after induction by labeled tryptophan, nor was there any difference in the amount of this amino acid incorpor-
rated into the protein of the tumor and host liver (Table 1). In view of these findings the basis for the repression of tryptophan pyrrolase in the Hepatoma 5123 appears to lie within the molecular constitution of the tumor cell itself.

There have been several previous reports on the attempted induction of tryptophan pyrrolase and related enzymes in other rat hepatomas. Chan et al. (5) recently reported that two transplanted hepatomas and a number of primary liver neoplasms in the rat had negligible tryptophan pyrrolase activity. Administration of tryptophan resulted in no change in the activity present in the transplanted neoplasms but gave variable results with the primary tumors, some of them showing slight activity after induction. No study was made to rule out the presence of contaminating liver tissue in the primary hepatomas. Auerbach and Waisman (2) have demonstrated the absence of tryptophan pyrrolase and threonine dehydrase activities in the Novikoff hepatoma and showed that administration of the substrates, tryptophan and threonine, did not induce activity in that tumor. In view of recent work (17) suggesting that the cell of origin of the Novikoff hepatoma may be more closely related to biliary epithelium than to parenchymal liver cells, these results are difficult to evaluate. At about the same time, however, Ichii (10) reported that primary carcinomas of the liver in rats produced by feeding 0.06 per cent dimethylaminoazobenzene possessed tryptophan pyrrolase activity which increased one- to threefold after injection of histidine or tryptophan. However, the non-neoplastic host liver gave three- to ninefold increases in this enzyme's activity by the same stimuli, and again no histologic studies were done to show the degree of contamination of the neoplasms by liver tissue. Pitot (16) has also shown that primary hepatic neoplasms induced in the rat by the feeding of ethionine or S'-methyltrimethylnitrosobenzene will not respond to tryptophan administration in vivo by an increase in tryptophan pyrrolase activity, although the neoplasms used possessed a low level of the enzyme. In this case, a histologic examination of the tissues was made, and only those neoplasms showing negligible contamination by normal liver were used. The transplantable Dunning hepatoma showed a similar phenomenon in vivo and in vitro (16).

In contrast to the complete unresponsiveness of tryptophan pyrrolase in Hepatoma 5123, tyrosine a-ketoglutarate transaminase, an enzyme that can be induced by cortisone or by substrate in the intact animal but not in the adrenalectomized animal (12), possessed a peculiar type of responsiveness in the tumor. In the intact animal the level of this enzyme in Hepatoma 5123 was high—i.e., completely derepressed—and could not be changed conspicuously by substrate or hormonal administration. However, in the adenalec-tomized animal the level of this transaminase in Hepatoma 5123 sank to near the normal repressed level, and this activity was increased after administration of cortisone to the host (Table 2). A possible explanation for these peculiar reactions in the tumor is that the mechanism controlling the activity of this enzyme in the neoplasms is overly sensitive to the hormone, that the neoplastic cell is sequestering the adrenal hormones to a very high degree, or that it is not destroying circulating hormone as rapidly as liver does. In unpublished work, Dyer 3 has shown that glutamic oxalacetic transaminase activity is high in Hepatoma 5123 as well as in the serum of the tumor-bearing animal. The effect of adrenalectomy is unknown in this case as yet. However, Rosen et al. (19) have shown that cortisone administration does increase the activity of a related enzyme, glutamic-pyruvic transaminase, in Walker carcinoma 256. Several other transaminase activities in Hepatoma 5123 are presently under investigation in this laboratory to determine whether or not all transaminase activity in the tumor is derepressed.

In trying to find a pattern to explain the faulty controlling mechanisms in the Morris Hepatoma 5123, at least two facets become clear: (a) in the enzymes studied—tryptophan pyrrolase, threonine dehydrase, and tyrosine transaminase—substrate control is completely lacking for these enzymes in this neoplasm in that there was failure to change either a previously low or a previously high level, and (b) the adrenal hormones, particularly cortisone, still have a definite, but altered, effect on the dehydrase and transaminase activities but no effect on tryptophan pyrrolase activity. Thus, this hepatoma has some aspects, at least at the molecular level, of a hormonally dependent neoplasm perhaps more analogous to testosterone-dependent prostatic cancer in man than to the experimental estrogen-dependent pituitary neoplasms whose existence is dependent on abnormally high levels of estrogens (8) in rats. However, in the case of the Hepatoma 5123, the adrenal hormones may not be essential to growth, as evidenced by failure of the tumor to regress significantly after adrenalectomy, whereas in the experimental dependent neoplasms, growth itself is hormonally dependent. Thus, it would appear from studies with this neoplasm that neither rapid growth nor complete hormonal independence are

3 H. M. Dyer, unpublished. The authors are grateful to Dr. Dyer for permission to discuss her work here.
indispensable for biologic malignancy. Numerous examples from the study of neoplastic disease in the human bear this out (9).

The lack of substrate control of specific enzyme synthesis may not pertain to all enzymes in this tumor. Recently, Conney has shown that dimethylxylase activity (6,7) may be induced in the microsomal fraction of host liver and Hepatoma 5123 by administration of methylcholanthrene in vivo. This induction does not occur in primary hepatomas induced by feeding 3'-methyldimethylaminolozobenzene (6), suggesting that Hepatoma 5123 is actually more similar to normal liver than are the primary tumors that were studied.

As was emphasized in the first paper of this series, no qualitative enzyme deletion has as yet been found in the Morris Hepatoma 5123. The major alterations from normal seen in the tumor are related to the control of enzyme synthesis and/or activity. Herein, at present, the defective substrate derepression of specific degradative enzymes, tryptophan pyrrolase, and threonine dehydrogenase appears to be a primary fault. If two generalizations can be made from studies with microorganisms and applied to mammalian cells—i.e. (a) in general, degradative enzymes are induced by their substrates and synthetic enzymes repressed by their products and (b) the basic mechanisms for enzyme induction (derepression) and repression involve similar if not identical components—then a simple working hypothesis can be suggested to explain the malignancy exhibited by Hepatoma 5123. The hypothesis merely states that certain repressor mechanisms governed by substrates and products are partly or completely inoperative in the neoplastic cell. Thus, certain inducible enzymes are repressed in the tumor, and certain repressible enzymes are derepressed. An example of the former is tryptophan pyrrolase; of the latter may be thymidylate kinase, a synthetic enzyme derepressed in regenerating liver (4) and in those malignant tumors studied (3). If the initial generalizations are valid, then a single basic lesion in the repressor mechanism could result in the biologic malignancy expressed by the Morris Hepatoma 5123.

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REFERENCES


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