The Inability of Sarcoma RD3 To Utilize d-Histidine for Protein Synthesis

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The search for a distinguishing biochemical property of tumor tissue as compared with normal mammalian tissue led Kögl and Erxleben (7) to investigate the nature of the amino acids which make up various tumor tissue proteins. Their findings indicated that, although most of the protein of tumors was composed of L-amino acids (the "naturally" occurring form), some tumors contained substantial amounts of the d-form of certain amino acids and that a common feature appeared to be the presence of d-glutamic acid. They were unable to isolate any appreciable amount of d-glutamic acid from the protein of normal tissues. The presence of appreciable amounts of d-amino acid in tumors has not, however, been confirmed, and the controversy has been the subject of two excellent reviews (9, 11).

In view of the fact that the mammalian diet does not normally contain appreciable amounts of d-amino acids and that d-amino acids are not detectable in the body as a result of metabolism, it seems unlikely that d-amino acids would occur in the normal or neoplastic tissues of mammals. However, the possibility remains that, if a d-amino acid is administered to a tumor-bearing animal, the tumor might accept the d-amino acid and incorporate it into its proteins, while the normal tissues may not do so. The results of Wiseman (12) show that the rat small intestine in vitro is incapable of actively concentrating d-histidine, while, on the other hand, Ehrlich mouse ascites carcinoma cells can do so (1). The experiments described below, however, show that the injected d-histidine was not incorporated into the protein of the rat RD3 sarcoma, liver, or muscle.

MATERIALS AND METHODS

TUMOR

The RD3 sarcoma used in these experiments was originally induced by 1,2,5,6-dibenzanthracene injection into the right flank of an inbred strain of albino rats and has been successfully transplanted subcutaneously in this strain for over 20 years.

All the animals in these experiments were given injections subcutaneously in the right flank of a thick, pasty suspension of tumor mince. The use of a thick paste instead of a watery suspension affords a fairly accurate and simple method of administering equal amounts of tumor material to a group of animals, as problems due to sedimenting out of cells during the course of injection do not arise. To each ml. of tumor paste was added approximately 100 mg. of streptomycin base and 50,000 units of crystalline penicillin, and the whole procedure was carried out with strict aseptic technic. There was never any macroscopic evidence of infection in any of the tumors induced by this method.

Diet

The basic diet used throughout these experiments was Diet 86, purchased from North-Eastern Agricultural Cooperative Society Ltd., Bannermill Place, Aberdeen, Scotland, and the theoretical composition is: soluble carbohydrate, 53.4 per cent; protein, 20 per cent; fat, 3.8 per cent; fiber, 3.3 per cent; ash, 5.2 per cent; moisture, 14.3 per cent. The diet was fed ad libitum to all animals.

EXPERIMENTAL TECHNIC

Male rats, weighing between 250 and 300 gm., were given inoculations of 0.2 ml. of RD3 sarcoma in the right flank. On the 5th day twelve animals with roughly equal-sized fast-growing tumors were selected and divided into four groups of three animals each. The animals in two of these four groups were given subcutaneous injections of 25 mg. d-histidine 4 times a day for 5 days. The animals in the other two groups were given injections of a similar volume of normal saline. Care was taken to inject the d-histidine into the side opposite to that on which the tumor was growing, so as to prevent direct contamination of the tumor tissue with the d-amino acid. There was no marked difference in the size or the rate of growth of the tu-
mors in the various groups during the course of the experiment. All the tumors grew at a rapid rate, and it was estimated that by the end of the 5-day period during which D-histidine was injected the tumors had increased approximately 5 times in volume. The animals were killed by a blow on the head about 18 hours after the last D-histidine injection. To obtain a satisfactory quantity of tumor tissue for analysis, the tumors of each group (three rats) were pooled, thereby providing for analysis two samples of tumor tissue which had no D-histidine available during the period of rapid growth, and two samples of tumor tissue to which no D-histidine had been made available. At the same time, samples of liver and muscle were collected and pooled from each of the four groups, care being taken to choose muscle well away from the site of injection of the D-histidine. The blood from all the D-histidine-injected animals was pooled for analysis, as was that from the control rats.

**Analytical Procedures**

*Isolation of the tissue histidine.*—The tissue samples were finely chopped with the aid of scissors and were then gently boiled under a reflux condenser in 6 N HCl (6 ml/gm of tissue) for 22 hours. When the chopped tissues were washed with distilled water before hydrolysis, care was taken to prevent much loss of the sarcoma cells, which easily become suspended in the fluid. At the end of the period of hydrolysis the hydrolysate was concentrated in vacuo to a small volume, which was then twice diluted to about 100 ml with distilled water and concentrated in vacuo. The resultant dark brown fluid was boiled with decolorizing charcoal and a clear colorless filtrate obtained. The volume of this filtrate was of the order of 25 ml; the pH was below 4.

The isolation of histidine from this protein hydrolysate was based on the method of Vickery (10), which makes use of the low solubility (in the cold) of the histidine complex with 3,4-dichlorobenzensulfonic acid. The 3,4-dichlorobenzensulfonic acid was prepared from 200 gm. of 3,4-dichlorobenzensulfonyl chloride by being boiled with 33 gm. sodium hydroxide under a reflux condenser until only one phase was present. The flask contents were then cooled, and the white flaky crystals were collected, washed with a small amount of cold distilled water, and then dried in a desiccator. To obtain satisfactory yields of precipitated “histidine-sulfonate” complex, it was found necessary to dissolve, with the aid of heating, 3.0 gm. of the 8,4-dichlorobenzensulfonic acid was prepared from 200 gm. of 3,4-dichlorobenzensulfonyl chloride by being boiled with 33 gm. sodium hydroxide under a reflux condenser until only one phase was present. The flask contents were then cooled, and the white flaky crystals were collected, washed with a small amount of cold distilled water, and then dried in a desiccator. To obtain satisfactory yields of precipitated “histidine-sulfonate” complex, it was found necessary to dissolve, with the aid of heating, 3.0 gm. of the 3,4-dichlorobenzensulfonic acid in the cold (approximately) decolorized hydrolysate and to leave the solution in the refrigerator at 4° C. for about a fortnight. Shorter periods for the precipitation sometimes gave very small yields of histidine. At the end of the period allowed for precipitation the heavy crop of precipitate was harvested by centrifugation and the supernatant discarded. The precipitate was dissolved in about 20 ml of distilled water heated to about 70°-80° C. Enough saturated barium hydroxide solution was then added to the hot solution to precipitate the 3,4-dichlorobenzensulfonic acid as the barium salt, leaving the histidine behind in solution (pH 9–10). The precipitated barium 3,4-dichlorobenzensulfonate was filtered off and discarded. The barium present in the filtrate was removed by precipitation with 2 N H₂SO₄. The filtrate obtained after this procedure must remain clear on addition of further sulfuric acid, since any residual barium will interfere with the colorimetric estimation of the histidine. The filtrate, its pH brought to between 4 and 5 by the addition of 2 N NaOH and its volume adjusted to about 20–30 ml by boiling off excess water, was suitable for analysis for its content of L- and DL-histidine.

*Estimation of L-histidine.*—The L-histidine was estimated by the use of the specific L-histidine decarboxylase produced by Clostridium perfringens (strain B.W. 21). The method used for growing the organism and preparing the acetone-dried powder with the decarboxylase activity has been described by Gale (9). The decarboxylation was carried out at 37° C. in 25-ml. conventional Warburg flasks and manometers, with the main chamber containing 1 ml. sample, 1 ml. distilled water, 0.5 ml. acetic acid buffer pH 4.6, and the side-arm containing the decarboxylase powder suspended in 1 ml. 0.2 N acetic buffer, pH 4.5. It was found that the volume of gas evolved from the samples used was between 100 and 300 μl., and the estimation was complete in 1–2 hours. Each sample was subjected to four estimations.

*Estimation of total histidine.*—The total histidine (L- and D-form) was estimated by the colorimetric method of Macpherson (8), and each sample was subjected to four estimations. It is advantageous to estimate the L-form manometrically before estimating the total histidine, since this enables a good guess to be made at the degree of dilution necessary for the colorimetric estimation.

**Standards**

It is desirable to use the same sample of histidine for the standard solutions used for manometric and colorimetric estimations. The sample of DL-histidine used as standard gave the theoretical volume of gas expected when estimated manometrically, and was a 50:50 mixture of the L- and D-forms.

**Trial of analytical procedure**

To test the method of precipitation of the histidine, a trial experiment was done with solutions of pure L-histidine, D-histidine, and DL-histidine. Table 1 shows the amount of histidine used and the percentage recoveries. It also shows that no racemization occurred during this procedure, that the recovered L-histidine was free from D-histidine, that the recovered D-histidine was free from L-histidine, and that the DL-histidine recovered was still a 50:50 mixture of the L- and D-forms.

**Results**

The results in Tables 2 and 3 show that there was no D-histidine in tumor, liver, or muscle of tumor-bearing rats, even though the animals of Table 3 had been given injections of D-histidine during the phase of marked growth of the tumor. The tissues of Table 3 were washed free of gross blood contamination before being hydrolyzed.

Table 4 shows that the blood of tumor rats which had been given D-histidine for the 5 previous days did, in fact, contain D-histidine in appreciable amounts, but none was found in the blood of the untreated animals. As would be expected from this result, Table 5 shows that the D-histid-
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Dine may be recovered from tissues which are not washed free of blood if D-histidine is present in that blood. It is interesting to note that the very vascular tumor and liver contained D-histidine as a result of blood contamination, but the less vascular muscle did not.

The results as a whole show that there is no D-histidine present in the blood, tumor, liver, or muscle of tumor-bearing rats unless D-histidine is specially given. The D-histidine may then be present in the blood of the animals but is not in the actual protein of the tumor tissue, liver, or muscle of such animals.

DISCUSSION

In recent years the advances in analytical techniques have provided several new satisfactory methods for the investigation of the amino acid content of protein. It is now often possible to determine accurately not only the total amount of an amino acid present, but also the amounts of the L- and D-forms individually, even though they are present in the same solution. The availability of such analytical tools makes the problem of the amino acid composition of the proteins of tumors a little easier to decide, especially in relation to the possible content of the n-form of amino acids in such proteins. That tumor proteins may contain D-amino acids was first suggested by Kögl (4, 5) and Kögl and Erxleben (7). On the other hand, since 1939, numerous other investigators in this field have been unable to confirm these findings (see 3, 9, 11). The experiments of Christensen et al. (1) show that the Ehrlich mouse ascites carcinoma cells in vitro are able to concentrate actively many D-amino acids intracellularly, and, although Christensen and his co-workers (1) do not suggest the incorporation of the D-amino acids into the

### TABLE 1

**RECOVERY OF L- AND D-HISTIDINE FROM AQUEOUS SOLUTIONS BY THE METHOD EMPLOYED**

<table>
<thead>
<tr>
<th>Form</th>
<th>DL-form estimated colorimetrically (mg.)</th>
<th>L-form estimated colorimetrically (mg.)</th>
<th>D-form estimated colorimetrically (mg.)</th>
<th>n-form by difference (mg.)</th>
<th>Per cent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-form</td>
<td>11.1</td>
<td>5.4</td>
<td>8.8</td>
<td>5.6</td>
<td>5.5</td>
</tr>
<tr>
<td>L-form</td>
<td>5.4</td>
<td>5.3</td>
<td>None</td>
<td>None</td>
<td>53</td>
</tr>
<tr>
<td>D-form</td>
<td>None</td>
<td>None</td>
<td>8.8</td>
<td>8.8</td>
<td>88</td>
</tr>
</tbody>
</table>

### TABLE 2

**HISTIDINE RECOVERED FROM HYDROLYZED TUMORS, LIVERS, AND MUSCLES OF Untreated TUMOR-BEARING RATS**

<table>
<thead>
<tr>
<th>Weight of tissue obtained (total from three rats) (gm.)</th>
<th>Total DL-histidine recovered (mg.)</th>
<th>Total L-histidine recovered (mg.)</th>
<th>DL-histidine present (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>17.3</td>
<td>30.1</td>
<td>31.0</td>
</tr>
<tr>
<td>Liver</td>
<td>20.9</td>
<td>15.4</td>
<td>18.2</td>
</tr>
<tr>
<td>Muscle</td>
<td>19.3</td>
<td>8.9</td>
<td>9.3</td>
</tr>
</tbody>
</table>

### TABLE 3

**HISTIDINE RECOVERED FROM HYDROLYZED TUMORS, LIVERS, AND MUSCLES OF TUMOR-BEARING RATS**

The rats were given 25 mg. D-histidine subcutaneously 4 times a day for 5 days. The tissue was chopped and well washed with distilled water before hydrolysis.

<table>
<thead>
<tr>
<th>Wt. of tissue obtained (total from three rats) (gm.)</th>
<th>Total DL-histidine recovered (mg.)</th>
<th>Total L-histidine recovered (mg.)</th>
<th>DL-histidine present (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>30.3</td>
<td>26.6</td>
<td>27.2</td>
</tr>
<tr>
<td>Liver</td>
<td>21.2</td>
<td>16.6</td>
<td>16.6</td>
</tr>
<tr>
<td>Muscle</td>
<td>19.8</td>
<td>20.0</td>
<td>20.8</td>
</tr>
</tbody>
</table>

### TABLE 4

**HISTIDINE RECOVERED FROM HYDROLYZED Whole BLOOD OF Untreated and D-HISTIDINE-Injected TUMOR-BEARING ANIMALS**

<table>
<thead>
<tr>
<th>Vol. of blood (total from six rats) (ml.)</th>
<th>Total DL-histidine recovered (mg.)</th>
<th>Total L-histidine recovered (mg.)</th>
<th>DL-histidine present (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated animals</td>
<td>7.1</td>
<td>50.5</td>
<td>49.2</td>
</tr>
<tr>
<td>D-histidine-injected animals</td>
<td>13.2</td>
<td>92.3</td>
<td>77.6</td>
</tr>
</tbody>
</table>

### TABLE 5

**HISTIDINE RECOVERED FROM HYDROLYZED TUMORS, LIVERS, AND MUSCLES OF TUMOR-BEARING RATS**

The rats were given 25 mg. D-histidine subcutaneously 4 times a day for 5 days. The tissues were chopped and drained of blood but not washed with distilled water before hydrolysis.

<table>
<thead>
<tr>
<th>Wt. of tissue obtained (total from three rats) (gm.)</th>
<th>Total DL-histidine recovered (mg.)</th>
<th>Total L-histidine recovered (mg.)</th>
<th>DL-histidine present (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>21.5</td>
<td>42.8</td>
<td>38.6</td>
</tr>
<tr>
<td>Liver</td>
<td>17.1</td>
<td>63.9</td>
<td>58.2</td>
</tr>
<tr>
<td>Muscle</td>
<td>15.9</td>
<td>12.0</td>
<td>11.7</td>
</tr>
</tbody>
</table>

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tumor protein, the possibility that this could happen must be considered in view of these cells’ capability to capture and concentrate D-amino acids from their environment.

In considering an approach to this problem we decided to use histidine as a test amino acid, since it can be satisfactorily isolated from a protein hydrolysate and the amount of the L- and D-forms accurately estimated. The experimental groups of animals received repeated injections of D-histidine subcutaneously during the period of active growth of the tumor. Subcutaneous injection of the D-histidine allows the amino acid to reach the tumor via the blood stream without having first to pass through the liver. It will be seen from Table 4 that the D-histidine content of the blood of the animals receiving this amino acid was reasonably high. However, the sarcoma tissue showed no appreciable D-histidine content, provided that the tissue was washed free of gross blood contamination. The inability to obtain D-histidine from the washed sarcoma tissue shows not only that the sarcoma cannot incorporate this D-amino acid into its protein but also suggests the possibility that the sarcoma cells are unable to concentrate appreciably the D-histidine even in the free state. This latter finding with sarcoma tissue in vivo is especially interesting, since the Ehrlich mouse ascites carcinoma cells in vitro appear to concentrate free D-histidine reasonably well (1).

It seems important for future work to decide whether this apparent difference in ability to concentrate free D-histidine intracellularly is due to inherent characteristics of the sarcoma and carcinoma cells, or whether the in vitro findings do not normally occur or are for some other reason undemonstrable in vivo. It is possible that in vivo the relatively avascular portions of the tumor may not concentrate amino acids very efficiently for the simple reason that they are not freely available to it. The in vivo experiments would then indicate the average activity of the whole tumor and not the concentrating power of its most vascular portions, and the in vitro experiments might be taken to reflect a potentiality which is not always real-

ized in vivo. Nevertheless, it must be pointed out that in vivo findings may show up important biochemical reactions that cannot be easily demonstrated in vitro but which may account for the difference in behavior of tissues in the intact animal.

SUMMARY

Rats bearing rapidly growing RD3 sarcoma were given injections of 25 mg. D-histidine subcutaneously 4 times each day for 5 days, but the D-amino acid was not incorporated into the tumor protein.

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