Studies of D-Amino Acid Oxidase

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The significance of mammalian D-amino acid oxidase (DAAO) has not been established. Little is known of the source of its substrates, since only the L form appears to be involved in the metabolism of amino acids in higher animals. In vivo isotope dilution studies (22) have indicated that normal mammals contain no free D-tyrosine or D-glutamic acid, although Kögl (13) has reported the presence of D-glutamic acid in tumors. The latter finding has been challenged by several investigators (18, 22) but recently has received independent support (11).

D-amino acids have been shown to occur in bacteria, molds, and, possibly, viruses. It appears possible that D-amino acids from intestinal flora are absorbed from the gut and serve as the substrate. The considerable enzymatic capacity of the DAAO in the liver and kidney, which are the enzyme's principal sites, would be expected to prevent the accumulation of D-amino acids in the plasma and might explain the failure of isotope dilution experiments to detect D-amino acids in the rat. The rapid removal of D-amino acids from the plasma by DAAO and by excretion in the urine (1, 6, 7, 10, 17, 24) has complicated studies on possible incorporation of the unnatural isomers into proteins or other compounds.

The present study was concerned with the development of in vitro and in vivo assays of DAAO and studies on the relation of intestinal flora, D-amino acid feeding, and tumor growth to the amount of enzyme found in mouse kidney. The distribution and metabolism of D-alanine in nephrectomized normal and tumor-bearing mice were also studied.

MATERIALS AND METHODS

D-alanine-L-¹⁴C.—Carboxyl-labeled D-alanine¹ was acetylated (23), the L-compound was split with hog kidney acylase,² and the resulting L-alanine and acetyl-D-alanine were separated by ion exchange (3). Acid hydrolysis yielded D-alanine with an observed activity of about 610,000 counts/min/mg, of which 6.7 per cent was not attacked by kidney DAAO. The amount of the contaminant, apparently L-alanine, could not be decreased despite considerable effort, including the use of more highly purified acylase.³

Radioactivity measurements.—Respiratory CO₂ was collected in bubble towers (9, 19) filled with 1 M NaOH. The activity in the resultant sodium carbonate solutions was determined with a liquid scintillation counter (16).

The radioactivity in tissue extracts and hydrolysates was determined by wet combustion to CO₂, which was then absorbed in NaOH and counted as above. The combustion method of Katz et al. (18) was modified, because it was found that some CO₂ was formed even at room temperature. It was therefore necessary to add the ammonium per sulfate in solution through a serum stopper, with a needle and syringe, after first evacuating the diffusion flasks.

Measurement of DAAO activity in vitro.—The mice were killed by severing the spinal cord; 0.4 ml. of a 1:10 kidney homogenate in water was incubated 10 minutes at 88° C. in a 20-ml beaker with 2 ml. of Ca-free Krebs-Ringer phosphate (5), pH 7.4, 1 ml. of 1 M D-alanine in 1 M sodium arsenite, and 2.6 ml. water; 4 ml. of 12.5 per cent trichloroacetic acid was added, the mixture was centrifuged, and 1- to 3-ml aliquots of the supernatant (diluted, if necessary, to 3 ml. with 5 per cent TCA) were analyzed for pyruvate by the direct method of Friedemann and Haugen (8). Sodium pyruvate was used as the standard. Tissue blanks gave no color, and there was no disappearance of pyruvate during the incubation.

Tumor implantation.—Ehrlich carcinoma ascites cells were carried in CPA mice by injecting intraperitoneally 0.8 ml. of the peritoneal cell suspension from a 5-day-old tumor. The tumors used in these studies were induced by the injection of 0.2 ml. of a similar suspension into an air sac on the animal's back, near the neck. The sac was produced by injecting 2 ml. of air subcutaneously.⁴

RESULTS

1. Measurement of DAAO in vitro.—A linear relation was found between amount of kidney and observed activity up to 40 or 50 mg kidney/tube. Higher amounts of kidney did not produce proportional increases in activity, probably because of the presence of natural DAAO inhibitors (9, 25). The rate of oxidation was found to decrease with an observed activity of about 610,000 counts/min/mg, of which 6.7 per cent was not attacked by kidney DAAO. The amount of the contaminant, apparently L-alanine, could not be decreased despite considerable effort, including the use of more highly purified acylase.³

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1. We are indebted to Dr. J. P. Greenstein for a sample of acylase I.


6. Obtained from Tracerlab, Boston.


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gradually with time of incubation but was reasonably constant during the first 10 minutes.

2. Assay of DAAO in vivo with D-alanine-1-C14.—In the metabolism of D-alanine by DAAO, the product of oxidative deamination, pyruvic acid, undergoes decarboxylation to yield a two-carbon fragment. Side reactions of pyruvate include transamination, reduction to lactate, and CO2 fixation. If decarboxylation reactions are quantitatively most important and are not rate-limiting, the production of C14O2 would be a measure of DAAO activity. The respiratory C14O2 is in rapid equilibration with the body bicarbonate pool (4).

To estimate DAAO by collection of respiratory C14O2, it is necessary, as in in vitro assays, to measure the reaction rate in the plateau region of the substrate-activity curve. To establish the dose of D-alanine which would fulfill these requirements, mice were given intraperitoneal injections, on successive days, of 1 ml. of solution containing 0.11 µc. of D-alanine to which increasing quantities of nonradioactive D-alanine were added. Collection of CO2 was carried out for the period 15–200 minutes after injection, and the C14 activity in the sample was measured. From the C14O2 found, the corresponding amount of D-alanine metabolized was calculated. The data obtained in studies on four mice are shown in Chart 1. The lower curve is drawn through points derived from data for three mice obtained in a single shipment and studied in August. There is a difference in the dose at which the curves reach their plateaus and in the plateau levels. While no conclusion can be drawn about the effect of season, it is of special interest that a similar difference is found in the in vitro values. The ratios of in vivo to in vitro values for the four mice are: 0.44; 0.40; 0.36; 0.47. The relative constancy of the ratio, despite an almost twofold difference in the DAAO activity measured, suggests that the in vivo method furnishes a measure of DAAO activity in the intact animal. This general approach appears to have promise as a method of measuring the activity of other enzymes in vivo.

The difference between the in vitro and in vivo results may be due to the natural proteinaceous inhibitor present (9), the inhibitory effect of the kidney ionic medium (25), the lower O2 tension in the kidney, or to side reactions with pyruvate-C14 produced.

3. Studies of factors affecting DAAO activity.—To determine whether DAAO levels in the kidney could be influenced by the addition of D-alanine to

<table>
<thead>
<tr>
<th>GROUP</th>
<th>BODY WEIGHT INCREASE (gm.)</th>
<th>KIDNEY WEIGHT (mg.)</th>
<th>DAAO ACTIVITY (per gm. kidney)</th>
<th>DAAO ACTIVITY (per kidney)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5.2</td>
<td>474</td>
<td>60 ± 10</td>
<td>29 ± 8</td>
</tr>
<tr>
<td>10 per cent DL-Alanine</td>
<td>4.8</td>
<td>517</td>
<td>57 ± 6*</td>
<td>19 ± 4</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td>5.9</td>
<td>480</td>
<td>55 ± 8</td>
<td>26 ± 5</td>
</tr>
</tbody>
</table>

* Difference from the normal value is significant (P = 0.34).

the diet or by tumor growth in the animals, 80 mice were divided into three groups: a control group received a diet of ground Rockland mouse pellets mixed with an equal quantity of water; a second group was fed the same diet to which 10 per cent DL-alanine was added; the third group was fed the control diet, but was inoculated with ascites cells on the 1st day. The animals were sacrificed on the 14th–18th days, and the kidneys were removed and assayed for DAAO in vitro. The results are given in Table 1. The tumor weight in the animals averaged 2.90 gm. (9.6 per cent of body weight). There was no statistically significant difference between the three groups of animals in the weight gain or kidney weights. Although the tumor produces no alteration in kidney DAAO level, the DL-alanine feeding actually depressed...
the level when calculated per gram of kidney (P < 0.05) but not when calculated for the total kidney (P > 0.05). Apparently, dietary D-alanine does not produce an adaptive increase in DAAO activity.

Similar studies by Westphal in rats with Walker carcinoma revealed considerable decrease in DAAO activity (26).

The kidneys of Swiss Albino mice maintained germ-free for three generations were found to oxidize D-alanine at the rate of 35 μmoles/10 min/gm of kidney. It is apparent that a considerable amount of DAAO can be found in kidneys in the absence of D-amino acids from intestinal bacteria or fungi.

4. Metabolism of D-alanine-C14 in DAAO-free mice.—No studies appear to have been made of the effects and metabolism of D-amino acids in animals deprived of their DAAO. The possibility exists that D-amino acids might enter normal metabolic pathways and give rise to toxic substances. There is a single experiment (28) with a homogenate in which the incorporation of L-leucine-C14 into protein was measured, with and without unlabeled L-leucine. The latter appeared to lower the amount of C14 incorporated, but the effect was interpreted as being too small to signify that L-leucine is utilized for protein synthesis.

A group of CF1 mice, some of them bearing ascites tumors, were nephrectomized while under ether anesthesia, and 1 hour later 0.15 ml. of D-alanine-C14 (0.21 mg.) was injected into the tail vein. In two animals, the respiratory CO2 was collected for 3 hours after injection and was found to contain 2.5 and 3.7 per cent of the C14 in the initial dose. This slight activity was probably derived from the contaminating L-alanine-C14 rather than from D-alanine metabolized by extra-renal DAAO. This finding, as well as actual test of mouse liver, confirm the report by heart puncture, and organs and aliquots of the liver, pulmonary tissue, tumor for D-alanine (Table 3) appears to be about the same order of magnitude as liver, spleen, and lungs. The blood cells, in the three mice examined, contained about one-half the activity of the plasma. This was probably derived from the contaminating L-alanine-C14 rather than from D-alanine metabolized by extra-renal DAAO.

At the end of various time intervals, the animals were anesthetized with ether, blood was drawn by heart puncture, and organs and aliquots of various parts were removed for C14 analysis. Wet combustions were performed on plasma, washed red cells, a saline extract of the intestinal contents, and the trichloroacetic acid extracts of liver, tumor, muscle, brain, lungs, spleen, stomach, intestines, skin, and the remaining carcass.

We wish to thank Professor Morris Wagner of the Lobb Institute, Notre Dame University, for furnishing these kidneys.

The organs were extracted with cold and hot (20) trichloroacetic acid, and the acid was removed from the pooled solutions with ether. The liver and tumor proteins were purified further by extraction with hot alcohol-ether and treatment with mercaptoethanol (15) and were then hydrolyzed with 6 N HCl. The two femurs and the saline-insoluble contents of the intestines were also hydrolyzed. The hydrolysates were evaporated to dryness (16) to remove HCl and the residues combusted to CO2.

The liver proteins were found to contain no C14, even after 20 hours, indicating that DAAO is not needed to protect that organ from formation of proteins containing D-alanine.

The tumor proteins also were free of C14, indicating that the solid Ehrlich ascites tumor shows no general predilection for synthesizing proteins containing D-amino acids. However, there remains the possibility that a special preference, e.g., for D-glutamic acid, exists. No DAAO could be demonstrated in the tumor by in vitro assay.

The saline-insoluble intestinal contents were nonradioactive. Since considerable C14 (presumably D-alanine) was present in the intestinal contents, it would appear that the intestinal bacteria did not incorporate D-alanine into their proteins (28) under these conditions.

In presenting the data in Tables 2 and 3, in which values for representative samples of body are shown, aliquot factors were applied to calculate the percentage of the original dose in those parts. The factors were derived from the following estimates: two femurs are 8.9 per cent of total bone; skin, 16 per cent of body weight; blood (sp. gr. 1.06; hematocrit, 43 per cent), 5 per cent of body weight.

From Table 2, it can be seen that the total activity in the plasma, 100 per cent of the dose at the time of injection, drops off to a very small fraction within 1 hour, evidently by distributing itself rapidly through the body. The blood cells, in the three mice examined, contained about one-half the activity of the plasma. The columns giving the per cent of dose per gram of tissue show that the liver, lungs, and spleen appear to concentrate the radioactivity. The concentrating power of the tumor for D-alanine (Table 8) appears to be about the same order of magnitude as liver, spleen, and lung. Because of its relatively large mass, a considerable fraction of the radioactivity accumulated in the tumor tissue, reducing the amount available for distribution in the other organs. When a calculation is made of the distribution of the D-alanine present in the body outside of the tumor, an essentially normal pattern is found. This...
suggests that the tumor has no specific influence on the accumulation of D-alanine by normal tissues.

The greater C⁴ concentration in liver trichloroacetic acid extracts does not represent partial conversion of D-alanine to other compounds. This was shown by paper chromatography of an ether-extracted sample with butanol-acetic acid. A radioautograph revealed only a spot in the alanine position. This finding, the general similarity in specific activities of the various extracts, and the lack of radioactivity in liver protein indicate that D-alanine undergoes no significant metabolic reactions other than its destruction by DAAO.

SUMMARY

Studies were made of D-amino acid oxidase activity in mouse kidney by a simplified and rapid assay procedure. Feeding Dl-alanine resulted in a decreased activity per gram of kidney, but growth of a subcutaneous solid ascites tumor did not affect the activity. Oxidase activity was found in germ-free mice. The distribution of D-alanine-C⁴ in nephrectomized mice was determined in normal and tumor-bearing mice. No metabolic conversions or incorporation into liver or tumor protein were observed, although the liver, lungs, and spleen seemed to concentrate the D-alanine to a small extent. An attempt was made to measure D-amino acid oxidase activity in vivo by the injection of D-alanine-C⁴ and observation of the radioactivity in the respiratory CO₂.

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