INHIBITION OF THE GROWTH OF THE RAT BY L-PENICILLAMINE AND ITS PREVENTION BY AMINOETHANOL AND RELATED COMPOUNDS*

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Penicillamine (β,β-dimethylcysteine) is a degradation product of all the known naturally occurring penicillins (1). It was first reported in 1943 (2). Since that time most investigations have dealt with its preparation, chemical properties, or relationship to penicillin, and little information is available regarding its biological behavior. The purpose of the present paper is to report the results of an investigation of penicillamine as a possible metabolic antagonist. Some of these results have been reported in a preliminary note (3).

Since penicillamine bears a structural resemblance to a number of naturally occurring amino acids, particularly cysteine, methionine, valine, and threonine, it occurred to us that penicillamine might act as a metabolic antagonist toward one of these amino acids. We found that addition of L-penicillamine to the diet did inhibit the growth of young rats on a 20 per cent casein diet and eventually led to death of the animals. However, cysteine, valine, and threonine failed to overcome this inhibition. The effect of methionine upon the response to penicillamine was equivocal and appeared to be affected by the presence of choline in the basal diet. When choline was removed from the diet, additional methionine did not counteract the effect of penicillamine. However, when choline was added to the diet in sufficient amounts without the additional methionine, the effects of penicillamine were completely overcome.

To determine whether or not the quaternary ammonium structure was essential for this reversal of growth inhibition induced by penicillamine, dimethylaminoethanol and monomethylaminoethanol were investigated and were found to be as effective as choline. It then occurred to us that perhaps the methyl groups were not essential for the antipenicillamine activities of these compounds. Aminoethanol itself was tested and found to be even more effective than choline in overcoming the growth-inhibiting effects of L-penicillamine.

The administration of L-penicillamine to rats was frequently followed by

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the appearance of running fits and clonic and tonic convulsions, symptoms which could be precipitated by exposing the animals to loud or high pitched sounds. Aminoethanol and its N-methyl derivatives prevented the appearance of these symptoms.

It was of particular interest to compare the effect of D-penicillamine with that of the L isomer, since it is the D-penicillamine which is obtained when naturally occurring penicillins are degraded (1). It was found that D-penicillamine did not inhibit growth or produce the nervous symptoms associated with the action of L-penicillamine. This marked difference in the effect of these two enantiomorphs offers a striking example of stereochemical specificity.

### Table I

<table>
<thead>
<tr>
<th>Choline-Free Basal Diet</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Vitamin-free casein</td>
<td>20 gm.</td>
</tr>
<tr>
<td>Sucrose</td>
<td>55 &quot;</td>
</tr>
<tr>
<td>Salt mixture</td>
<td>4 &quot;</td>
</tr>
<tr>
<td>Vitamin mixture†</td>
<td>1 &quot;</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.15 gm.</td>
</tr>
<tr>
<td>Corn oil†</td>
<td>1.0 ml.</td>
</tr>
<tr>
<td>Vitamin A and D concentrate§</td>
<td>0.5 drop</td>
</tr>
<tr>
<td>Hydrogenated vegetable oil</td>
<td>19 gm.</td>
</tr>
</tbody>
</table>

* Osborne and Mendel (8).
† The vitamin mixture contained thiamine hydrochloride, riboflavin, nicotinic acid, pyridoxine hydrochloride, and p-aminobenzoic acid, 1 mg. each, calcium D-pantothenate 5 mg., inositol 10 mg., biotin 0.01 mg., folic acid 0.1 mg., and sucrose to make 1 gm.
‡ Corn oil containing 4.0 mg. of $\alpha$-tocopherol acetate and 0.1 mg. of 2-methyl-1, 4-naphthoquinone per ml.
§ From pharmacy of New York Hospital, 5 drops = 120 mg.; 60,000 i.u. of vitamin A per gm., 10,000 i.u. of vitamin D per gm.

A comparison was also made between L-penicillamine and the corresponding disulfide. When L-penicillamine disulfide was fed to young rats, it did not inhibit their growth. Furthermore, S-methylpenicillamine showed no growth-inhibiting properties.

### EXPERIMENTAL

**Composition of Diets**—The composition of the basal diet is given in Table I. A choline-free basal ration was used. To this basal ration the various compounds to be tested were added at the expense of an equal weight of sucrose. When either L-penicillamine hydrochloride hydrate or choline chloride was used, 1 equivalent of sodium bicarbonate was also added to the diet.
Growth Experiments—Young male albino rats of the Rockland strain were used for all experiments. The animals were fed the basal ration ad libitum for 1 week before being placed on the experimental diets. The average weight gain was about 25 gm. during this period.

Effect of L-Penicillamine upon Growth—When a level of 0.35 per cent L-penicillamine hydrochloride hydrate was added to the diet, immediate weight loss and decrease in food consumption occurred. In the course of studying a variety of compounds for their ability to counteract the action of penicillamine, forty-two animals have been fed the penicillamine diet as controls for periods up to 10 weeks. Thirty-seven of them died within the 10 week period; twenty-two of them died after they had received the penicillamine for 4 weeks or less. Forty of the rats lost weight during the 1st week following the addition of penicillamine to the diet. The weight losses ranged from 5 to 38 gm., with an average loss of 20.6 gm. In subsequent weeks the animals which survived generally lost weight more slowly or showed erratic weight changes. The average daily food consumption on the basal ration was 8.8 gm. per rat; it fell to 3.3 gm. during the week following the addition of penicillamine to the diet.

Fits or convulsions were observed in twenty-three of the rats. These symptoms did not usually appear until the animals had been receiving L-penicillamine for at least a week. No gross pathological symptoms were consistently observed at autopsy.

Effect of Choline and Related Compounds on Growth of Penicillamine-Treated Animals—After exploratory experiments had been conducted with aminoethanol, monomethylaminoethanol, dimethylaminoethanol, and choline, an experiment was carried out in which the four compounds were tested simultaneously. The animals were fed the basal ration for the 1st week, and for the 2nd week the diet containing 0.35 per cent L-penicillamine hydrochloride hydrate. After this period, an appropriate level of the compound to be tested was added to the diet containing penicillamine. The growth curves of these animals, shown in Fig. 1, illustrate the prompt growth response to the choline-ethanolamine series of compounds under these conditions.

Another type of experiment was performed in which the preventive substance and penicillamine were added simultaneously to the diet. Aminoethanol and its N-methyl derivatives were again found to be effective in overcoming the growth-inhibitory action of L-penicillamine. Of the thirty-six animals tested, six served as controls receiving penicillamine alone. Although three of the control animals died before the end of the 10th week, none of the rats receiving the preventive compounds did so. When the N-methyl compounds and penicillamine were added to the diet, temporary cessations of growth lasting for a few days were observed in every instance.
before the resumption of growth comparable to that of animals on the basal diet in the absence of penicillamine. However, no inhibition of growth occurred when aminoethanol and penicillamine were added simultaneously to the diet.

Effect of D-Penicillamine upon Growth—Both curative and preventive experiments demonstrated clearly that D-penicillamine did not possess the
growth-inhibitory power of the L form. Fig. 2 shows the growth curves from a typical experiment. Further investigation showed that n-penicillamine produced no observable toxic effects, even when large doses (660 mg. per kilo) were injected intraperitoneally into rats. Fits, convulsions, and death almost invariably followed the injection of half this dose of the L isomer.

**Effect of L-Penicillamine Disulfide upon Growth**—When L-penicillamine disulfide was added to the basal diet at a level of 0.27 per cent, no breaks
were observed in the growth curves of the rats to whose diets it was added. When the level of the disulfide was doubled, there was still no effect on growth.

Effect of S-Methyl-DL-penicillamine upon Growth—When S-methyl-DL-penicillamine was added at a level of 0.5 per cent to the diets of rats, no significant growth inhibition occurred over a period of 2 weeks. Neither S-methylpenicillamine nor penicillamine disulfide produced the nervous symptoms associated with the administration of L-penicillamine.

Sources of Compounds—The aminoethanol and dimethylaminoethanol used in these experiments were Eastman products and were fractionally distilled before use. The choline chloride was a gift from the American Cyanamid Company. To prepare monomethylaminoethanol, the method of Schotte, Priewe, and Roescheisen (4) was modified by dissolving the phosgene in toluene and carrying out the first two steps of the synthesis in this solvent. This eliminated the sealed tube reaction used in the original method. The L-penicillamine disulfide was made by a procedure previously described (5). The methods used for making D- and L-penicillamine and S-methylpenicillamine are given in detail.

D- and L-Penicillamine Hydrochloride Hydrate—These compounds were prepared by a modification of the procedure described previously (6) for the preparation of d-penicillamine hydrochloride. 50 gm. of S-benzyl-N-formyl-L-penicillamine1 ((5) p. 462) and 9.6 gm. of sodium were added alternately in small portions, with stirring, to liquid ammonia (800 ml.). At the end of the reduction, the blue color owing to the excess sodium was discharged upon the addition of ammonium chloride (27 gm.). The ammonia was then evaporated by gently warming the mixture, the last traces being removed at water pump pressure. The residue was dissolved in 150 ml. of water and the resulting solution was extracted with three 50 ml. portions of U.S.P. ether. The aqueous layer was then cooled to 10° and made acid to Congo red with 6 N hydrochloric acid. After the mixture had been allowed to stand for 1 hour in an ice-salt bath, the precipitate was removed by filtration, washed with a little cold water, and pressed dry on the filter.

This N-formyl-L-penicillamine was dissolved in 180 ml. of 1 N hydrochloric acid by heating, and then heated under a reflux for 1 hour. The resulting yellow solution was decolorized completely with Darco, filtered, and evaporated under reduced pressure. The crystalline residue was dissolved in 50 ml. of commercial absolute ethanol and filtered. The filter was washed with a little ethanol. The combined filtrate and washings were

1 The authors are indebted to Parke, Davis and Company for placing at their disposal a supply of S-benzyl-DL-penicillamine, which served partially as a source of S-benzyl-N-formylpenicillamine used in this investigation.
then added in small portions, with scratching and stirring, to 1800 ml. of ether containing several drops of concentrated hydrochloric acid. After this mixture had been allowed to stand overnight at 8°, a crystalline product was removed by filtration, washed with a little cold ether, and dried in vacuo over sulfuric acid. A yield of 30.2 gm. of L-penicillamine hydrochloride hydrate (79 per cent of the theoretical amount) was obtained; $[\alpha]_D^{22} = -46°$ (1 per cent in 1 N sodium hydroxide).

D-Penicillamine hydrochloride hydrate, prepared in an identical manner, had a rotation of $[\alpha]_D^{21} = -45°$ (1 per cent in 1 N sodium hydroxide).

S-Methyl-DL-penicillamine—This compound was made by the following modification of the methionine synthesis of Patterson and du Vigneaud (7). S-Benzyl-DL-penicillamine (20 gm.) was reduced in 300 ml. of liquid ammonia with 4.5 gm. of sodium. The blue color owing to the excess sodium was discharged with 0.5 ml. of methyl iodide, and an additional 5.5 ml. of methyl iodide were added with stirring. After the mixture had been stirred for an additional 5 minutes, the ammonia was allowed to evaporate, the last traces being removed by gentle warming at water pump pressure. The residue was dissolved in 50 ml. of water and extracted with a little ether. A 15 per cent aqueous solution of hydriodic acid was then added until the aqueous layer was barely alkaline to litmus. After the resulting mixture had stood overnight at 5° the crystalline product was removed in a sintered glass funnel, washed with a little cold water, and dried in vacuo over phosphoric anhydride. An additional small amount of product was obtained by concentration of the mother liquors. The total yield was 12.8 gm. (94 per cent of the theoretical amount) of a product possessing a micro melting point of 250°, with some darkening at 220°. After two recrystallizations from water, and drying in vacuo at 80° over phosphoric anhydride, the product was analyzed.

\[ C_{6}H_{12}O_{2}NS. \text{ Calculated. C 44.15, H 8.02, S 19.64} \]
\[ 163.2 \text{ Found. C 44.14, H 8.18, S 19.84, 19.55} \]

**SUMMARY**

L-Penicillamine has been fed to growing albino rats and has been found to cause loss of weight and death in these animals. The toxicity of this compound is also displayed by the appearance of peculiar nervous symptoms in animals to which it is administered. These responses to the inclusion of L-penicillamine in the diet are prevented by the addition of aminoethanol or any of its N-methyl derivatives. The influence of structure upon the toxicity described above was investigated in experiments with D-penicillamine, penicillamine disulfide, and S-methylpenicillamine. None of these three compounds was found to inhibit the growth of the rat.
BIBLIOGRAPHY

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