PANTOYLAMINOETHANETHIOL, AN ANTAGONIST OF PANTOTHENIC ACID AND PANTETHEINE ACTIVE IN VITRO AND IN THE RAT

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The compounds β-d-pantoylaminoethanethiol (PAET) and the corresponding disulfide had been prepared by Barnett (1) in an attempt to find substances similar in structure to pantoyltaurine but more favorable in their inhibitory action on pantothenic acid-requiring microorganisms. Barnett showed that, although the compounds did inhibit the growth of Lactobacillus arabinosus to about the same extent as pantoyltaurine, they were not effective in protecting rats against infection with Streptococcus hemolyticus. The effect of PAET on the growth of the microorganisms was reversible by pantothenic acid. No studies on the possible antimetabolic action of the compound on the animal were reported.

PAET was chosen for further studies as an antimetabolite, since a consideration of its structure suggests that it is an antagonist of pantetheine rather than of pantothenic acid itself.

\[
\text{HOCH}_2\cdot C(CH_3)_2\cdot\text{CHOH}\cdot\text{CO}\cdot\text{NH}\cdot CH_2\cdot CH_2\cdot \text{CO}\cdot\text{NH}\cdot CH_2\cdot CH_2\cdot SH}
\]

Pantetheine

\[
\text{HOCH}_2\cdot C(CH_3)_2\cdot\text{CHOH}\cdot\text{CO}\cdot\text{NH}\cdot CH_2\cdot CH_2\cdot SH}
\]

PAET

The structural formulae indicate that PAET contains all the functional groups of pantetheine but in a different molecular spacing due to the omission of the β-alanine part of pantetheine. It has now been demonstrated that PAET is an antagonist of the pantetheine-coenzyme A system in vitro and of the effect of pantothenic acid on the growth of the rat in vivo.

EXPERIMENTAL

Materials—β-d-Pantoylthiamedithiol and the corresponding disulfide were prepared according to Barnett (1) from d-pantolactone and β-aminoethanethiol, and bis-β-aminoethanedisulfide, respectively. The optical isomers were similarly obtained from l-pantolactone.1 In the enzymatic as well as

1 The authors are greatly indebted to A. Wilson of these laboratories for the preparation of the two isomers of PAET.

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animal experiments reported, the free thiol and the disulfide were found to be fully equivalent. In the enzymatic experiments no difference could be expected, as they are all run in the presence of a large excess of cysteine. Since no difference was found in the preliminary animal experiments, the disulfide was used in most of the experiments. The great ease with which the thiol is oxidized to the disulfide leads to the presence of some disulfide anyway, unless the experiment is performed under strong reducing conditions.

**Enzymes**

The synthesis of coenzyme A from pantetheine and its inhibition by PAET were measured with the crude pigeon liver extract system of Kaplan and Lipmann (2). A modification of Lynen et al. (3) which permits a considerable economy in the use of pigeon liver enzyme was followed in essence, except that the reaction volume was slightly increased by the addition of the antagonist and Mg$$^{2+}$$ was added to the mixture to obtain optimal response to pantetheine. The following modified procedure was used throughout.

The solution of stable reactants contained 10 ml. of 0.02 M sodium citrate, 2.5 ml. of 1.0 M sodium acetate, 8.0 ml. of 0.04 M magnesium chloride, and 10 ml. of 0.004 M sulfanilamide or p-aminobenzoate. To a small test-tube were added 0.08 ml. of pantetheine or coenzyme A solution, 0.05 ml. of antagonist solution, 0.08 ml. of a freshly prepared solution of 24 mg. of adenosine triphosphate in 3 ml. of the solution of stable reactants, 0.04 ml. of freshly prepared 0.1 M cysteine hydrochloride, 0.02 ml. of 1.0 M sodium bicarbonate, and finally 0.06 ml. of enzyme solution. The total volume was 0.33 ml. The assay mixture was incubated for 2 hours at 37° and deproteinized with 1 ml. of 5 per cent trichloroacetic acid, and the remaining free sulfanilamide was determined on a 1 ml. aliquot.

**Animals**

Young male rats of the Holtzman strain, weighing from 75 to 100 gm., were used for the animal experiments. Groups of eight animals of equal average weight were kept in individual wire mesh cages and fed a semisynthetic diet of 30 parts of casein (Labco), 56 parts of dextrose, 10 parts of Crisco, and 4 parts of salts (U. S. P. XV, p. 881). The diet was supplemented with all the known vitamins except pantothenic acid; the control groups received in addition a supplement of 100 mg. of calcium pantothenate per kilo of diet.

**Results**

**Enzymes**

The compound was first tested on the crude pigeon liver extract (2) which is not only capable of acetylating arylamines in the presence of coenzyme A
but also of synthesizing coenzyme A from pantetheine though not from pantothenic acid. On an equivalent basis pantetheine is only about 10 to 15 per cent as efficient as coenzyme A.

The experimental conditions chosen yield a response curve to pantetheine which is essentially identical in shape to the one described for coenzyme A. It reaches asymptotically a maximum of acetylation at about 9 to 11 \( \gamma \) of pantetheine per test. Since inhibition studies require a reasonably linear and sensitive response, the upper limit of pantetheine concentration useful for these studies is about 6 to 7 \( \gamma \).

The amount of sulfanilamide acetylated in the presence of a given amount of pantetheine alone was taken as 100 per cent, and the reduction of acetylation in the presence of the antagonist was used as a measure of inhibition.

![Graph showing inhibition of acetylation of sulfanamide by PAET.](image)

**Fig. 1. Inhibition of acetylation of sulfonamide by PAET**

A typical inhibition curve is illustrated in Fig. 1, where remaining acetylation activity on a linear scale is plotted against the molar ratio of antagonist to pantetheine on a logarithmic scale. The pantetheine concentration was held constant at 4 \( \gamma \) per test.

50 per cent inhibition was attained in this case when the molar ratio was about 1:13. The molar ratio at which 50 per cent inhibition is observed with a constant pantetheine concentration is somewhat dependent on the enzyme preparation. Under the conditions of Fig. 1 it has been observed to vary from 1:10 to 1:20.

Information on the type of inhibition involved has been obtained by determining the molar ratio of antagonist to pantetheine required for 50 per cent inhibition at different pantetheine concentrations.

The results of such an experiment with four different pantetheine concentrations in which the same enzyme preparation is used throughout are summarized in Table I. Apparently the inhibition is non-competitive in view of the 10-fold decrease in the effective molar ratio with a 20-fold increase in
pantetheine concentration. The very desirable extension of these observations to a wider range of pantetheine concentration is impossible with the methods used, since the asymptotic shape of the response curve limits the amount of pantetheine that can be used per test.

The data so far demonstrate that the PAET interferes with the acetylation capacity of a system which is synthesizing coenzyme A from pantetheine. The antagonistic action of PAET on coenzyme A itself was tested with the phosphotransacetylase system (4), which responds to coenzyme A only.

From Fig. 2 it is evident that PAET has some antagonistic action against coenzyme A itself. The molar ratio of about 1:500 necessary for 50 per cent inhibition indicates, however, that the direct effect of the antagonist on coenzyme A is weak if compared to the effect on the system synthesizing coenzyme A from pantetheine.

### Table I

<table>
<thead>
<tr>
<th>Pantetheine</th>
<th>Molar ratio of antagonist to pantetheine necessary for 50 per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>µMol</td>
<td></td>
</tr>
<tr>
<td>0.009</td>
<td>28</td>
</tr>
<tr>
<td>0.018</td>
<td>16</td>
</tr>
<tr>
<td>0.027</td>
<td>10</td>
</tr>
<tr>
<td>0.18</td>
<td>3.5</td>
</tr>
</tbody>
</table>

**Animals**

The antimetabolite activity of PAET was first demonstrated on rats on a pantothenic acid-deficient diet in order to accelerate and intensify any effects. Fig. 3 shows the growth curves of three groups of rats which are receiving, respectively, the complete diet, the pantothenic acid-deficient diet, and the deficient diet plus 10 mg. of PAET per day subcutaneously.

The growth of the rats receiving PAET stops after about 6 days, and their weight begins to decline after 2 weeks. In this experiment two rats died on the 20th day and one died on the 21st day, at which time the experiment was terminated. In general, in a group of animals receiving this dose of PAET on a pantothenic acid-free diet, deaths occur about the 18th day and most animals are dead before the 23rd day.

The coenzyme A content was determined in the livers of the rats killed on the 21st day of the experiment in Fig. 3 (see Table II).

The coenzyme A content of the liver reflects the conditions as seen from
the growth curves, while the coenzyme A content of the brain remained constant even in the most severely deficient animals. Apparently death occurs as soon as the liver coenzyme A falls to about one-half of its normal value.

Complete gross and microscopic examinations were made on the rats which had received 10 mg. of PAET daily. Except for the presence of a moderately extensive ulceration in the large intestine of one of the rats, there were no appreciable abnormalities referable to pantothenic acid deficiency. Alopecia, chromodacryorrhea, and "hemorrhagic necrosis" of the adrenals appear to be quite reproducible consequences of advanced pantothenic acid deprivation. None of these lesions was found in PAET-injected rats at a time (3 weeks on injections) that represented the terminal stage of this type of pantothenic acid deficiency. Differences in the pathology of nutritional diseases, depending on whether they develop gradually or are precipitated by the use of an antimetabolite, have been observed in other instances (5).

Reversibility of the effect of PAET by pantothenic acid administration in vivo is demonstrated in Fig. 4.

Two groups of eight rats each were kept on the deficient diet, and one group received in addition 10 mg. of PAET per day subcutaneously. After 15 days the deficiency and the effect of the antagonist are clearly demon-
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stralable, but deaths do not occur. At this time the animals were put on the complete diet, which contains a large excess of pantothenic acid. After a 3 day lag period, the normal rate of growth, shown by the control group

TABLE II
Coenzyme A Content of Liver and Brain

<table>
<thead>
<tr>
<th>Coenzyme A</th>
<th>Pantothenic acid- deficient diet + 10 mg. antagonist</th>
<th>Pantothenic acid- deficient diet</th>
<th>Complete diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units per gm.</td>
<td>units per gm.</td>
<td>units per gm.</td>
</tr>
<tr>
<td>Liver</td>
<td>60 (39-86)</td>
<td>92 (67-113)</td>
<td>123 (105-134)</td>
</tr>
<tr>
<td>Brain</td>
<td>31 (26-40)</td>
<td>35 (28-41)</td>
<td>27 (23-32)</td>
</tr>
</tbody>
</table>

Fig. 4. Reversibility of the effect of PAET on rat growth by pantothenic acid. Curve 1, complete diet; Curve 2, pantothenic acid-deficient diet; Curve 3, pantothenic acid-deficient diet + 10 mg. of PAET subcutaneously daily. On the 15th day the animals in the groups shown by Curves 2 and 3 were switched to the complete diet and the administration of PAET to the group of Curve 3 was continued.

animals, is resumed by the deficient animals as well as those receiving PAET.

Since the effect of PAET is readily reversed by pantothenic acid in vivo, the approximate effective ratio of pantothenic acid to PAET has been determined for a dose level of 10 mg. of antagonists per day per rat (see Table III).
The data indicate that the administration of 10 mg. of PAET is effective even if 64 \( \gamma \) of pantothenic acid are administered simultaneously but is ineffective if 90 \( \gamma \) are administered. Therefore, for the rat receiving 10 mg. of PAET, the limiting effective molar ratio is about 1:150.

Doses of PAET smaller than 10 mg. per day have little effect in the rat even in the absence of pantothenic acid. With a dose of 5 mg. per day subcutaneously, a slight effect is demonstrable after about 20 days, while with 2.5 mg. no effect is apparent even after 30 days. On the other hand, increasing the dose to 30 mg. per day leads to an earlier plateau, a more pronounced fall in weight, and a somewhat earlier death of the animals.

**Table III**

*Effective Ratio of Antagonist to Pantothenic Acid in Vivo*

Eight rats with an average starting weight of 98 gm. were used for each group. The experiment was terminated after 15 days.

<table>
<thead>
<tr>
<th>Diet and supplement</th>
<th>Average final weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm.</td>
</tr>
<tr>
<td>Complete</td>
<td>173</td>
</tr>
<tr>
<td>Deficient</td>
<td>161</td>
</tr>
<tr>
<td></td>
<td>129</td>
</tr>
<tr>
<td>+ 10 mg. PAET</td>
<td>131</td>
</tr>
<tr>
<td>+ 16 mg. PAET + 8 ( \gamma ) pantothenic acid</td>
<td>140</td>
</tr>
<tr>
<td>+ 32 mg. PAET + 16 ( \gamma ) pantothenic acid</td>
<td>130</td>
</tr>
<tr>
<td>+ 64 mg. PAET + 90 ( \gamma ) pantothenic acid</td>
<td>160</td>
</tr>
<tr>
<td>+ 120 mg. PAET + 160 ( \gamma ) pantothenic acid</td>
<td>157</td>
</tr>
</tbody>
</table>

The peptide linkage of the antagonist is apparently not hydrolyzed in the intestine, since the antagonist is fully active if given orally.

Two groups of eight animals each were kept on the deficient diet, and one group received 30 mg. of PAET daily by stomach tube (Table IV). The effect on the growth curve of the animals and the death of the first animal on the 14th day is comparable to that obtained by the same dose on parenteral administration. The average level of liver coenzyme A was also reduced to a value of about 60 units per mg.

The effect on the growth of rats of the enantiomorph of PAET was tested and compared to that of the enantiomorph of pantetheine (see Table V). The \( l \) form of PAET was inactive as an antimetabolite, exactly as the analogous \( l \) form of pantetheine was inactive as a vitamin. The weight of the rats receiving the \( l \) form of PAET was somewhat lower than that of the animals on the deficient diet alone. This difference probably represents
biological variation, but the possibility of slight non-specific chemical toxicity of the $l$ form of PAET cannot be excluded.

The biological specificity of the antimetabolic action of PAET is emphasized by the lack of action of the enantiomorph. Together with the reversibility by pantothenic acid, these results seem to exclude the possibility that the effects in rats could be due to a non-specific chemical toxicity. Similar specificity with respect to one enantiomorph has been observed for the action of pantoyltaurine against $L. arabinosus$ (6).

Previous experience with other vitamin antagonists has shown that deoxypyridoxine and galactoflavin (and isoriboflavin) are capable of suppressing the growth of transplanted lymphomata in mice and rats (7, 8).

### Table IV

**Oral Administration of PAET**

Control group on the deficient diet; other group given 30 mg. of PAET daily by stomach tube. The results represent the average weight of eight rats per group in gm.

<table>
<thead>
<tr>
<th>Days</th>
<th>Deficient + 30 mg. PAET</th>
<th>Deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>106</td>
<td>105</td>
</tr>
<tr>
<td>4</td>
<td>116</td>
<td>118</td>
</tr>
<tr>
<td>7</td>
<td>121</td>
<td>130</td>
</tr>
<tr>
<td>11</td>
<td>109</td>
<td>148</td>
</tr>
<tr>
<td>14</td>
<td>90*</td>
<td>169</td>
</tr>
</tbody>
</table>

* One animal died on the 14th day.

### Table V

**Influence of Isomer of PAET on Growth of Rats**

Eight rats with an average starting weight of 72 gm. were used for each group. The experiment was terminated after 19 days. All daily supplements were injected subcutaneously.

<table>
<thead>
<tr>
<th>Diet and supplement</th>
<th>Average final weight (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td></td>
</tr>
<tr>
<td>&quot; + d-pantetheine equivalent to 100 $\gamma$ pantothenic acid</td>
<td>167</td>
</tr>
<tr>
<td>&quot; + $l$-pantetheine</td>
<td>102</td>
</tr>
<tr>
<td>&quot; + 10 mg. $d$-PAET</td>
<td>73*</td>
</tr>
<tr>
<td>&quot; + 10 $l$-PAET</td>
<td>93</td>
</tr>
</tbody>
</table>

* All but one animal died before the 19th day. Average weight recorded for this group on the 14th day.
Deficiencies in thiamine and niacin induced by means of potent antagonists (neopyrithiamine, acetylpyridine) failed to exert this effect. The same failure attended attempts to influence the growth of the Murphy rat lymphosarcoma in rats on a pantothenic acid-deficient diet injected over 14 days with 10 mg. of PAET daily. At the 14th day of treatment the tumors attained an average size of 30.9 gm. against one of 30.3 gm. in untreated controls.

DISCUSSION

It is obviously not possible to compare PAET activity with all the pantothenic acid antagonists reported in the literature (9). Most of these antagonists were found to inhibit the growth of microorganisms only. The effectiveness of PAET in inhibiting the growth of *L. arabinosus* has already been reported by Barnett and was found to be about equal to that of pantoyltaurine. Figs. 3 and 4, however, demonstrate that PAET is also an effective and reversible antagonist, at least in the rat, while pantoyltaurine is probably ineffective in the mammal (10, 11).

PAET and \(\omega\)-methylpantothenic acid not only seem similar in their preservation of the essential structure of the nutrilite, but also seem to be similar in their biological action (12). Both are active *in vivo* and are reversible by pantothenic acid; however, \(\omega\)-methylpantothenic acid not only is optically unresolved, but contains a new center of asymmetry not present in pantothenic acid or pantetheine. PAET, on the other hand, is readily prepared in an optically active form from intermediates used in the synthesis of pantothenic acid.

The inhibition *in vitro* of the pantetheine-coenzyme A system by PAET shows the peculiar phenomenon of a decreasing inhibition index with increasing concentrations of the normal metabolite. In spite of the non-competitive nature of the inhibition *in vitro*, complete reversibility is found in the animal.

The data on the type of inhibition found *in vitro* do not provide a clue to the mechanism of the action of the antagonist. The ultimate result of the action of the antagonist is a reduction of the amount of coenzyme A available *in vitro* and *in vivo*. Two possible mechanisms seem of primary interest: (1) that the antagonist simply prevents the synthesis of coenzyme A from pantetheine and (2) that an analogue of coenzyme A, containing the PAET moiety instead of pantetheine, is enzymatically synthesized. This latter possibility is now being investigated with an enzymatic system more efficient in the synthesis of coenzyme A. A similar formation of an analogue of a coenzyme has been recently demonstrated (13) to occur by an enzymatic exchange reaction between the nicotinamide moiety of DPN and acetylpyridine. It should be pointed out that these two possible mecha-
nisms could also apply to ω-methylpantothenic acid, which also contains intact all of the groups necessary for coenzyme A formation, although the reactivity of the terminal hydroxyl group is probably reduced by conversion from a primary to a secondary hydroxyl.

SUMMARY

The compound β-d-pantoylaminoethanethiol (PAET) was found to be an antagonist of the pantetheine-coenzyme A system in vitro and of the effect of pantothenic acid in vivo for the rat.

The inhibition of the synthesis of coenzyme A from pantetheine in vitro is apparently non-competitive, since the molar ratio of antagonist necessary for 50 per cent inhibition varies from 1:4 to 1:30 with decreasing concentrations of pantetheine.

PAET also inhibits coenzyme A itself in vitro but at a higher molar inhibition ratio of about 1:500.

PAET inhibits the growth of rats on a pantothenic acid-deficient diet and leads to death within about 3 weeks. This effect is reversible by pantothenic acid. The limiting effective molar ratio of pantothenic acid to PAET in rats is about 1:150. PAET is effective both parenterally and orally.

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