ETHIONINE INHIBITION AND MORPHOGENESIS OF EXCISED TOMATO ROOTS

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Replacement of the terminal methyl group of methionine by an ethyl group gives the substance ethionine. Ethionine has been shown to be an antimetabolite of methionine in a number of biological systems in that inhibition by ethionine is reversed by methionine (16, 35). The precise mode of action is not certain; it probably varies from one organism to another. Present evidence from work on animals (11, 14, 16, 26, 27, 35) indicates that ethionine exerts inhibitory effects upon 1) transmethylation, 2) conversion of methionine to cystine, 3) the lipotropic activity of methionine, 4) the incorporation of amino acids into proteins, and 5) the formation of certain adaptive enzymes. A recent report (20) attributes the inhibitory effect on transmethylation to the formation of S-adenosylethionine, which serves as an ethyl donor, with the consequent formation of various ethyl analogs of metabolites which are normally formed as a consequence of transmethylation from S-adenosylmethionine.

This paper records effects, inhibitory and otherwise, of ethionine on the growth of excised tomato roots in sterile culture. The effects of some antagonistic substances are also described. The purpose of the experiments was to obtain information regarding the possible metabolic relationships and physiological importance of methionine in the excised tomato root. The main interest is in the morphogenetic consequences of controlling methionine metabolism in the root. A general discussion of growth and growth correlations, upon which the morphogenesis of the excised tomato root depends, is presented elsewhere (7). The present work, which has been presented in a preliminary report (5), is, to my knowledge, the only record of the effects of ethionine on growth of plants, other than microorganisms. An exception is a paper by Schrank (22) who showed that the inhibition of growth of oat coleoptile cells by ethionine was reversed by methionine.

MATERIALS AND METHODS

The general techniques are described elsewhere (2, 3). The basal medium for all experimental media was that of White (34) except that sucrose concentration was 1.5 per cent and glycine and niacin were omitted unless stated otherwise. This basal medium contained thiamin and pyridoxin and, is referred to as TPy medium. TPy medium plus niacin is referred to as TPyN medium. Within any one experiment, initial pH's of media were adjusted where necessary so that the pH of each treatment was approximately the same and within the range 4.8 to 5.0.

The clone of excised tomato roots used as source of inocula was that designated as R5 (2). Cultures were harvested and measured on the sixth day after inoculation. Standard errors, calculated according to the method given by Snedecor (25), are included where necessary and are followed by the number of replicates in brackets.

Three features of growth were recorded: 1) the final length of the main axis, 2) number of laterals (lateral number) and 3) total length of the ten basal laterals (lateral length). In addition, in some experiments, the lateral frequency was estimated. Lateral frequency is the number of laterals per mm of main axis calculated from the number of laterals divided by the length of main axis between the most basal and most apical lateral.

All substances used as additions to the basal medium (TPy medium), with the exception of niacin and ethanolamine, were purchased from Nutritional Biochemicals, Inc. The ethanolamine was purchased from Matheson Company and the niacin from Eastman Kodak. Unless stated otherwise, all substances included in any one medium were autoclaved in that medium. Pyrex sintered glass filters were used to sterilize certain solutions by filtration. New filters were autoclaved for prolonged periods in distilled water prior to acid cleaning and subsequent use.

RESULTS

The effect of L-methionine in TPy medium was tested over a wide range of concentrations (0.004 to 4 μM) in three separate experiments. It did not stimulate growth of the main axis or lateral length; lateral frequency was decreased at 0.4 μM and higher concentrations. Inhibition of growth of the main axis commenced at 4.0 μM.

The effects of adding D,L-ethionine to TPy medium, and the effects of adding L-methionine at an inhibitory concentration of ethionine, are shown in figure 1. Corresponding treatments in TPyN medium are also shown.

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In the absence of niacin (figure 1, top), ethionine, at concentrations below 11.9 \( \gamma \)/liter caused a marked increase in growth in length of the main axis with an even more marked increase in number of laterals per root. It was qualitatively apparent that the increased number of laterals was due, at least in part, to an increase in the lateral frequency. The actual values for lateral frequency were 0.443 \( \pm \) 0.028 \( (9) \)\(^4\) for TPy medium against 0.530 \( \pm \) 0.075 \( (9) \) for basal medium plus 5.96 \( \gamma \)/liter ethionine. At higher concentrations of ethionine, growth of the main axis was inhibited and the number of laterals per root decreased. The effect of concentration of ethionine on growth of laterals differed from that on growth of main axis. Inhibition of growth of laterals was observed at 5.96 \( \gamma \)/liter ethionine, but this inhibition was relieved at higher concentrations. Indeed, the growth of laterals became slightly greater than in the controls. This relief of the inhibition was correlated with the commencement of growth inhibition of the main axis. Inhibition by ethionine was completely reversed by an appropriate concentration of L-methionine. Note that with relief of growth inhibition of main axis, there was an increase in the inhibition of growth of laterals. Thus the progress of relief of inhibition, on adding methionine at an inhibitory level of ethionine, was the reverse of the progress of inhibition due to increasing concentrations of ethionine added to basal medium. High concentrations of methionine inhibited growth of the main axis, but this inhibition was accompanied by an increase in growth of laterals to levels greater than obtained in the controls.

The effect of L-methionine in TPyN medium was tested in two experiments using concentrations of 0.04, 0.12, 0.2, 0.4, and 1.2 \( \mu \)M. L-methionine did not increase growth of the main axis in either experiment. However, L-methionine, at all concentrations in both experiments, increased the lateral frequency and in one experiment there was a marked increase (24 \%) in the lateral length.

In the presence of niacin (figure 1, bottom), ethionine, at concentrations 11.9 \( \gamma \)/liter and below, again caused a marked increase in growth in length of the main axis with a corresponding increase in number of laterals per root. The increase in lateral number was due, in part, to an increase in lateral frequency. The actual values were 0.404 \( \pm \) 0.022 \( (9) \) for TPyN medium against 0.486 \( \pm \) 0.041 \( (9) \) in the presence of 11.9 \( \gamma \)/liter ethionine. At the lowest concentrations there was a marked stimulation in growth of laterals. This effect was not observed in the absence of niacin. Inhibition of growth of main axis occurred at lower concentrations than were required to inhibit growth of laterals. It is uncertain whether the growth of laterals at 29.8 \( \gamma \)/liter ethionine represents a removal of inhibition correlated with growth inhibition of main axis, as was the case in the absence of niacin. The probability of the difference between growth of laterals at 11.9 \( \gamma \)/liter ethionine and growth of laterals in the controls was < 0.2 but > 0.1. The probability of the difference between growth of laterals in concentrations 5.96 and 11.9 \( \gamma \)/liter, however, was less than 0.5, indicating that growth of laterals was inhibited at 11.9 \( \gamma \)/liter ethionine. Methionine at appropriate concentration, completely removed the inhibition. In contrast to the effects obtained in the absence of niacin, the effects of

\(^2\) Standard Error
\(^4\) Number of replicates

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Fig. 1. Inhibition by DL-ethionine (left) and reversal of the inhibition by L-methionine (right), in medium not containing niacin (TPy medium, above) and containing niacin (TPyN medium, below). Horizontal broken lines show values obtained with the control medium. Horizontal dot-dash lines show values obtained with control medium plus an inhibitory concentration of ethionine (0.184 \( \mu \)M \( = \) 30.0 \( \gamma \)/liter). Each point is the mean of nine replicates.

Fig. 2. Reversal of ethionine inhibition by homocysteine or homocysteine in medium containing niacin (TPyN medium). Horizontal dash lines show values obtained with the control medium. Horizontal dot-dash lines show values obtained with control medium plus an inhibitory concentration of ethionine (0.184 \( \mu \)M \( = \) 30.0 \( \gamma \)/liter). The symbols are as in figure 1. Each point is the mean of nine replicates.
increasing concentrations of methionine on growth of laterals were positively correlated with the effects on growth of main axis. However, relief of inhibition of growth of laterals occurred at lower concentrations of methionine. Furthermore, note that, with 1.2 μM methionine, the number of laterals per root was much greater than in the controls. The actual lateral frequencies were 0.419 ± 0.015 (9) for TPyN medium against 0.516 ± 0.027 (9) in the presence of ethionine and methionine.

Ethanolamine, choline, and glycine-betaine, at concentrations 0.4, 4.0, 20.0, 40.0, 200.0 and 400.0 μM, were tested for antagonism toward inhibition by ethionine. The concentration of ethionine was 29.8 μ/liter in TPyN medium. Neither choline nor betaine relieved the inhibition. Both substances were inhibitory at concentrations 200.0 and 400.0 μM. Addition of either choline or betaine to TPyN medium showed inhibition at 0.4 μM. Ethanolamine showed no effect, inhibitory or otherwise, on growth of main axis or lateral number in medium containing the inhibitory concentration of ethionine. Inhibition of growth of laterals, however, was partly relieved at concentrations 20.0, 40.0 and 200.0 μM.

The effects of dl-homocysteine (free base) and dl-homocystine on inhibition by ethionine in TPyN medium are shown in figure 2. The homocysteine was filtered sterile and the homocystine autoclaved in the medium. Homocystine relieved the inhibition completely at 80.0 μM. The concentration required for complete relief of growth inhibition of laterals was lower than that required for main axis. Homocysteine did not relieve the inhibition completely at any concentration tested. This result may have little significance, however, in view of the concentration at which homocysteine was toxic.

Because of the known instability of the sulfur containing amino acids the effect of sterilization by filtration was tested on the activity of homocystine. The results of two representative experiments are shown in table I.

The concentration of ethionine was 17.9 μ/liter in TPyN medium; thus the degree of inhibition of lateral root growth was slight. Homocystine showed maximum activity only when autoclaved in the medium. When the filtered solution was added to the medium and then autoclaved, the activity was the same (at least as measured by growth of main axis) as when the homocystine was added without prior filtration. Therefore, it is unlikely that the lower activity of filtered homocystine was due to loss on the filter or introduction of an inhibitory factor from the filter. Furthermore, the activity obtained by autoclaving the homocysteine occurred whether the

**Table I**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
<th>Final length of main axis/root (mm)</th>
<th>Number laterals/root</th>
<th>Length of ten basal laterals/root (mm)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>145.8 ± 5.15* (8)**</td>
<td>48.5 ± 2.69</td>
<td>155.4 ± 7.46</td>
</tr>
<tr>
<td></td>
<td>Ethionine</td>
<td>54.0 ± 1.48 (8)</td>
<td>20.6 ± 1.13</td>
<td>129.8 ± 5.22</td>
</tr>
<tr>
<td></td>
<td>Ethionine + homocystine (A)</td>
<td>154.9 ± 4.88 (8)</td>
<td>67.3 ± 2.40</td>
<td>182.9 ± 6.25</td>
</tr>
<tr>
<td>1</td>
<td>Ethionine + homocystine (F) added after autoclaving</td>
<td>126.9 ± 5.14 (7)</td>
<td>38.4 ± 1.72</td>
<td>147.3 ± 15.47</td>
</tr>
<tr>
<td></td>
<td>Ethionine + homocystine (F) added before autoclaving</td>
<td>155.0 ± 2.87 (8)</td>
<td>67.0 ± 2.34</td>
<td>165.3 ± 5.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>108.7 ± 3.31 (9)</td>
<td>36.9 ± 2.44</td>
<td>150.2 ± 14.01</td>
</tr>
<tr>
<td></td>
<td>Ethionine</td>
<td>51.9 ± 1.81 (8)</td>
<td>18.9 ± 0.72</td>
<td>124.4 ± 13.34</td>
</tr>
<tr>
<td>2</td>
<td>Ethionine + homocystine (A)</td>
<td>127.9 ± 4.80 (7)</td>
<td>50.7 ± 1.82</td>
<td>141.9 ± 9.85</td>
</tr>
<tr>
<td></td>
<td>Ethionine + homocystine (A) but autoclaved separately</td>
<td>136.9 ± 4.36 (9)</td>
<td>39.9 ± 1.58</td>
<td>164.2 ± 15.01</td>
</tr>
</tbody>
</table>

* Standard Error = \[\frac{\text{variation}}{\sqrt{\text{number of replicates}}}\] (Snedecor (15))

** Number of replicates
homocystine was autoclaved in the medium or separately. The effect of autoclaving, therefore, was not dependent upon the presence of the medium. Note that growth in media containing autoclaved homocystine was significantly greater than in the controls.

A chromatographic analysis of fresh and autoclaved solutions of homocystine was made as follows: 25 mg aliquots of DL-homocystine were weighed into three separate, graduated test tubes. To two of the tubes, double distilled water, and to the third tube TPyN medium was added to give a total volume of 10 ml. The tube containing TPyN medium plus homocystine and one of the tubes containing water plus homocystine were then autoclaved for 30 minutes at fifteen pounds pressure. After cooling, the volume was readjusted. All three solutions were then centrifuged and the supernatant decanted off. Repeated applications, each of approximately equal volume, of each solution were then made on Whatman No. 1 paper and chromatograms run by the ascending method at room temperature (approximately 32° C.) for 16 hours using phenol:water (4:1) as the solvent and a solution of sodium cyanide in the chamber. The chromatograms were developed with ninhydrin.

The fresh solution, at high concentrations on the paper, produced two spots. One spot was large and presumably homocystine; the other spot was much smaller and relatively faint with an Rf value of 0.79. Mixed chromatography indicated that this smaller spot was methionine. The two autoclaved solutions both produced the spots corresponding to homocystine and methionine, and in addition a third spot with Rf value approximately 0.54. This spot was more marked with homocysteine autoclaved in the standard medium. It has not been identified as yet. It did not correspond to homocysteine, or to products formed on oxidation of methionine, homocysteine, or homocystine by treatment with hydrogen peroxide and ammonium molybdate (9). There was no apparent increase in the intensity of the methionine spot after autoclaving the homocystine. Fresh or aged solutions of homocysteine, at high concentrations on the paper failed to show the presence of methionine or any spot corresponding to the unknown in autoclaved homocystine. There were, however, two unknown spots, each with pronounced tailing continuous to the origin. The approximate Rf values for the two spots were 0.63 and 0.87. In the solvent system used here, homocysteine and homocystine show approximately the same Rf values. It was not, therefore, possible to determine whether the homocysteine contained homocysteine or whether homocysteine was produced when homocystine was autoclaved.

To assist in interpreting some of the data presented above, figure 3 shows the results of an experiment in which desthiobiotin, an anti-metabolite for biotin (35), was added in a range of concentration to TPyN medium. The experiment was done twice with the same result on each occasion. Desthiobiotin increased the growth of the main axis and the lateral roots. Furthermore, at concentrations which did not affect, or which stimulated the growth of the main axis, desthiobiotin gave a marked increase in the lateral frequency.

**Discussion**

The antagonism toward ethionine inhibition shown by methionine, homocysteine, and to a slight extent, ethanalamine indicates that these substances are related metabolically in the excised tomato root. Such relationships are well established in animals and microorganisms (8, 32). In the work reported here choline and betaine failed to relieve inhibition by ethionine even though to some extent, ethanalamine would do so. This is unexpected in light of work on biosynthesis and function of choline (4). It is probably a consequence of the low concentrations at which choline and betaine are toxic to the clone of excised roots used here.

High concentrations of homocysteine or homocysteine were required to effect the reversal of inhibition by ethionine. That their activity was not due to contamination by methionine is indicated by the differences in their morphogenetic effects. Furthermore, no methionine was detected chromatograph-

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*Fig. 3. Effect of concentration of desthiobiotin added to TPyN medium, on excised tomato roots grown for 6 days from 10 mm tips. Each point is the mean of nine replicates.*
ically in the homocysteine and it seems unlikely that the higher activity of autoclaved, compared with sterile-filtered, homocystine could be due to an increase in content of methionine. No such increase was apparent on the chromatograms. Whether the unknown substance detected in the autoclaved homocystine can account for the anti-ethionine activity of homocystine, remains to be determined. If the unknown is the effective agent, then it is possible that, in the excised tomato root, homocystine is not converted to homocysteine either at all, or at a rate great enough to provide an effective level of homocysteine. It has indeed been shown previously (4) that although methionine or homocysteine replaced vitamin B₆ in the nutrition of the clone of roots used here, homocystine would not do so.

The high activity of autoclaved homocystine, and the appearance therein of an unidentified, ninhydrin-sensitive decomposition product, may be relevant to some observations made with microorganisms. Thus under conditions where cystine was required for growth of Lactobacillus casei, replacement of the cystine by homocystine was much greater if the homocystine was autoclaved (21). The nature of the active principle has not been determined (private correspondence). Evidence has also been presented (13) for a factor (not B₁₂ or choline) from liver which enables Tetrahymena gelleii to utilize homocystine.

Until recently, very little attention has been directed toward the use of cultures of excised roots for the study of factors controlling the morphology (or similarly the anatomy) of the root. The morphology is, in its grosser aspects, dependent upon the frequency of production of lateral roots, the growth rates of main apex and lateral roots relative to each other, and the degree of persistence of growth of individual meristems. Various surgical or nutritional treatments, including treatment with growth regulators, have revealed the existence of hormonal, correlative mechanisms in the excised root (7, 31). First, the growth of lateral roots is inhibited by the main apex and younger parts of the main axis. Second, the growth of the main apex becomes limited by the accumulation of an inhibitor(s) produced in the older parts of the root; certain antiauxins (7, 29) overcome the limiting effect of this endogenous inhibitor(s), which suggests that it is an auxin-type growth regulator. Third, the production of lateral roots is, presumably, under hormonal control because both 3-indolylacetonitrile (IAN) (30) and naphthaleneacetic acid (NAA) (7) increase the lateral frequency at concentrations which do not decrease cell length in the main axis.

Ethionine, or various ratios of methionine (or homocystine) to ethionine, markedly affected the morphology of the root. The results suggest that ethionine affects the hormonal mechanisms in the root either directly or through an effect upon the metabolism of methionine in the root. Thus certain concentrations of ethionine reduced the growth of the main apex but simultaneously increased the growth of lateral roots. In other words, ethionine reproduces the effect obtained when the main apex is excised. This effect is also obtained with dichloroanisole (7), 2-diethylaminoethanol (6) and benimidazolate (unpublished) but not with desthiobiotin (figure 3), sulfanilamide and thiouracil (unpublished). Thus this morphogenetic effect of ethionine is not simply a consequence of inhibition of the growth of the apical meristem. It may be attributed to an effect upon the hormonal system controlling growth of lateral roots.

The effects of ethionine on growth in length of the main axis also indicate that ethionine affects the hormonal system which limits the growth of the main axis. At non-inhibitory concentrations of ethionine, growth of the main axis in the absence of niacin, and growth of both main axis and laterals in the presence of niacin was much greater than in the controls. Exogenous methionine did not decrease growth when added to either TPy medium or TPyN medium even at concentrations (1.2 μM) which removed ethionine inhibition. Therefore, it is unlikely that the stimulation of growth by ethionine was due to counteracting the effects of an inhibitory accumulation of endogenous methionine in the apex. Stimulation of growth by antimetabolites is not uncommon (35); it has been observed with some, but not all, of a number of antimetabolites tested on the clone of roots used here. It is significant that growth of the main axis of excised tomato roots was stimulated by the antiauxins a-(1-naphthyl-methylsulphide)-propionic acid (29) and dichloroanisole (compare above). Growth was also stimulated by desthiobiotin, an antimetabolite of biotin. Biotin is known to affect auxin action (19) and γ-(3:4-ureylene cyclohexyl) butyric acid, another antimetabolite of biotin, inhibits tryptophane utilization by Neurospora crassa (23). It would, therefore, affect indole metabolism. In contrast, four other antimetabolites tested on the clone used here did not stimulate growth when added to TPyN medium. These antimetabolites were 2-diethylaminoethanol (6), sulfanilamide, benimidazolate, and thiouracil (unpublished results). With the possible exception of benzimidazolate, the metabolites corresponding to these four antimetabolites are not known to affect auxin action. It is concluded that ethionine, or a product thereof, is antagonistic toward or affects the production of the endogenous auxin-type inhibitor(s) which limits the growth of the main axis (7, 31).

The effects of ethionine and methionine on lateral frequency also indicate an influence of these substances on growth regulators in the root. Ethionine increased lateral frequency in both TPy medium and TPyN medium. In contrast, l-methionine decreased lateral frequency in TPy medium but increased it in TPyN medium. This indicates that in TPy medium ethionine increased lateral frequency because it antagonized an inhibitory level of endogenous methionine. This cannot be the case in TPyN medium in which methionine and ethionine had the same effect, which suggests that both ethionine and methionine give a product which has the same effect as the growth regulators naphthaleneacetic acid (NAA) (7) and
3-indolylacetonitrile (IAN) (30). That exogenous ethionine may have the same effect as the growth regulators is of added interest in view of the relationship between auxin action and sulphydryl groups in shoot tissues (12, 15, 18, 24, 33).

The stimulation of root growth by ethionine and desthiobiotin warrants comment in relation to a general problem of tissue and organ culture. Although tissues and roots of many different species have been successfully maintained in culture, many failures have been reported, particularly with roots of monocotyledonous species, even after detailed nutritional studies. It is possible that the limitation of growth may be due not to a deficiency of a metabolic intermediate but to production of a toxic quantity of a natural metabolite. Under certain conditions, a supply of an appropriate anti-metabolite could then permit growth to occur. Therefore, where a question of culturability is involved, routine testing of anti-metabolites on the growth system may prove of value.

In this connection it is worth noting that, with the clone of roots used here, the largest roots yet grown in a six day passage were in a medium containing an inhibitory concentration of ethionine and 80 µM homocystine autoclaved in the medium (Table I). An example of apparent accumulation of a metabolite in excised roots was found in the case of excised pea roots (10). Growth of the intact root on decorticated pea seedlings was less than that of the excised root. In the presence of adenine, however, growth of the seedling roots equaled that of the excised roots. The simplest interpretation of this is that adenine, which would otherwise be lost to the shoot, accumulated in the excised root and ceased to limit growth.

Summary

Ethionine inhibited growth of excised tomato roots. The inhibition was relieved by methionine, homocysteine, homocystine, and, to a slight extent, ethanolamine. Choline and glycine-betaine were ineffective. The antagonism by homocystine was much greater when the homocysteine was autoclaved either in the medium or separately than when filtered sterile. Chromatographic analysis of autoclaved and fresh solutions of homocystine showed the formation of a ninhydrin-sensitive substance on autoclaving. This substance has not been identified.

Growth of the roots was markedly increased by low concentrations of ethionine. This was due to an antagonism toward an inhibitory level of endogenous methionine and may represent an antagonism toward a native growth regulator.

Ethionine and various ratios of methionine (or homocystine) to ethionine exerted profound effects on morphology of the roots, including an increase in frequency of production of lateral roots. Morphological effects of ethionine varied with the presence or absence of niacin.

Desthiobiotin, an antimetabolite for biotin, increased the growth of both main axis and lateral roots; it also markedly increased the frequency of production of lateral roots.

The results are discussed in relation to the metabolic relationships of methionine and hormonal, correlative mechanisms involved in root growth. It is suggested that excised roots and tissues, which cannot as yet be cultured, may accumulate metabolites to toxic levels and that culture might be achieved by supplying an appropriate antimetabolite.

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Literature Cited


