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PURIFICATION AND PROPERTIES OF THE POLYAMINE OXIDASE OF BARLEY PLANTS

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Abstract—The polyamine oxidase of barley shoots is associated with a particle which sediments in low centrifugal fields. The enzyme was removed from these particles by washing in 0·5 M NaCl and then purified about 24-fold. The purified enzyme oxidized spermine stoicheiometrically to 1,3-diaminopropane and 1-(3-aminopropyl)pyrroline (pH optimum 4·0). Spermidine was oxidized to 1,3-diaminopropane and 1-pyrroline (pH optimum 6·6). At their respective pH optima, spermine is oxidized about 30 times faster than spermidine. Hydrogen peroxide was formed in the course of the polyamine oxidation. The enzyme was not sensitive to several copper chelating reagents but 2-hydroxyethylhydrazine caused 50% inhibition at 5×10^{-4} M. The enzyme was also present in particles in the roots of barley seedlings and in extracts of the leaves of oats, maize, rye and wheat.

INTRODUCTION

THE POLYAMINES spermidine and spermine are widely distributed in animals, bacteria[1] and higher plants,[2] and enzymes concerned with the breakdown of these substances have been found in several organisms. In animals, the only well-characterized polyamine oxidase occurs in beef plasma. This enzyme oxidizes the primary amine of the propylamine residues; ammonia is released, with the formation of the propionaldehyde derivatives of the polyamines.[3] Polyamine oxidase activity is known in several micro-organisms, only the secondary amine group being attacked. In *Pseudomonas aeruginosa*[4] spermine is oxidized to spermidine and 3-aminopropionaldehyde, and the aldehyde is oxidized to 3-aminopropionic acid. Spermidine is oxidized to 1,3-diaminopropane and 1-pyrroline, and the pyrroline is further oxidized to 4-aminobutyric acid. A similar system was found in *Mycobacterium smegmatis*.[5] In another *Pseudomonas* (unspecified), an enzyme has been found which oxidizes spermidine stoicheiometrically to putrescine and 3-aminopropionaldehyde.[6] An inducible enzyme in *Neisseria perflava* oxidizes spermidine and spermine to 1-pyrroline and 1-(3-aminopropyl)-pyrroline respectively, with the additional formation in each case of 1,3-diaminopropane.[7] The formation of 1,3-diaminopropane from spermidine and spermine by *Hemophilus parainfluenzae*[8] indicates the presence of a similar enzyme in this bacterium. A highly specific enzyme in *Serratia marcescens* oxidizes spermidine, but not spermine, with the formation of 1,3-diaminopropane and 1-pyrroline.[9,10]

[1] H. TABOR and C. W. TABOR, *Pharmacol. Rev.* **16**, 245 (1964).
[2] T. A. SMITH, *Biol. Rev. Cambridge Phil. Soc.* **46**, 201 (1971).
[3] C. W. TABOR, H. TABOR and U. BACHRACH, *J. Biol. Chem.* **239**, 2194 (1964).
[4] S. RAZIN, I. GERY and U. BACHRACH, *Biochem. J.* **71**, 551 (1959).
[5] U. BACHRACH, S. PERSKY and S. RAZIN, *Biochem. J.* **76**, 306 (1960).
[6] R. PADMANABHAN and K. KIM, *Biochem. Biophys. Res. Commun.* **19**, 1 (1965).
[7] R. H. WEAVER and E. J. HERBST, *J. Biol. Chem.* **231**, 647 (1958).
[8] R. H. WEAVER and E. J. HERBST, *J. Biol. Chem.* **231**, 637 (1958).
[9] U. BACHRACH, *J. Biol. Chem.* **237**, 3443 (1962).
[10] C. W. TABOR and P. D. KELLOGG, *J. Biol. Chem.* **245**, 5424 (1970).

In higher plants the well-known amine oxidase of pea seedlings has been shown to slowly oxidize spermine to spermidine, while spermidine is quickly oxidized to 1-(3-aminopropyl)pyrroline.[11] Hill and Mann[12] found that the enzyme is most active with putrescine and cadaverine as substrates though McGowan and Muir,[13] using a modified assay, have recently claimed that spermidine is more easily oxidized than putrescine.

The present paper reviews some of the properties of a polyamine oxidase found in barley plants. This enzyme oxidizes spermine to 1,3-diaminopropane and 1-(3-aminopropyl)pyrroline[14] (E.C. 1.5.3.3; spermine : O_2 oxidoreductase) like the enzyme already described in *Neisseria* and *Hemophilus*.[7,8] The polyamines are oxidized according to the following equations:

$$\underset{\text{spermine}}{NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2} + O_2 \rightarrow \underset{\text{1-(3-aminopropyl)pyrroline}}{NH_2(CH_2)_3N\langle CH{=}CH,\ CH_2{-}CH_2 \rangle} + \underset{\text{1,3-diaminopropane}}{NH_2(CH_2)_3NH_2} + H_2O_2$$

$$\underset{\text{spermidine}}{NH_2(CH_2)_3NH(CH_2)_4NH_2} + O_2 \rightarrow \underset{\text{1-pyrroline}}{N\langle{=}CH{-}CH_2,\ CH_2{-}CH_2\rangle} + \underset{\text{1,3-diaminopropane}}{NH_2(CH_2)_3NH_2} + H_2O_2$$

In the presence of catalase, oxygen consumption is reduced to $\frac{1}{2}O_2$.

The 1-(3-aminopropyl)pyrroline formed as an oxidation product of spermine by the barley polyamine oxidase has been characterized earlier,[14] although the position of its double bond is still unknown. In the present paper the identity of 1-pyrroline as a product of spermidine oxidation has been confirmed.

RESULTS AND DISCUSSION

Distribution of Polyamine Oxidase in Higher Plants

A number of different plant species were investigated for polyamine oxidase. Activity of the enzyme was determined by GLC estimation of the 1,3-diaminopropane formed on incubation of the extracts with spermine (see Experimental).

No activity was found in the aerial shoots of seedlings of cress (*Lepidium sativum* L.), pea (*Pisum sativum* L. cv. Meteor), radish (*Raphanus sativus* L.), sunflower (*Helianthus annuus* L. cv. Giant Yellow), or in the leaves of mature plants of apple (*Malus sylvestris* Mill. cv. Worcester Pearmain), marrow (*Cucurbita pepo* L. var. *medullosa* Alef., cv. Green

[11] K. HASSE and K. SCHÜHRER, *Biochem. Z.* **336**, 20 (1962).
[12] J. M. HILL and P. J. G. MANN, *Biochem. J.* **91**, 171 (1964).
[13] R. E. MCGOWAN and R. M. MUIR, *Plant Physiol.* **47**, 644 (1971).
[14] T. A. SMITH, *Biochem. Biophys. Res. Commun.* **41**, 1452 (1970).

Bush), spinach (*Spinacea oleracea* L. cv. Noorman), strawberry (*Fragaria chiloensis* Duchesne, var. *annanassa*, Bailey, cv. Cambridge Vigour), or the male fern (*Dryopteris filix-mas* Schott). The enzyme was found in oats, maize, barley, wheat and rye in order of diminishing activity. Activity in the oats was over twice as high as that in barley (Table 1). In the roots of

TABLE 1. POLYAMINE OXIDASE ACTIVITY OF VARIOUS GRAMINEAE

	1,3-Diaminopropane μmol/ hr/g fresh wt	1-(3-Aminopropyl)pyrroline / 1,3-Diaminopropane
Oats (*Avena sativa* L. cv. Black Supreme)	3·30	3·1
Maize (*Zea mays* L. cv. Hy 2X07)	2·20	3·2
Barley (*Hordeum vulgare* L. cv. Zephyr)	1·25	4·3
Wheat (*Triticum vulgare* Vill. cv. Atle)	0·48	5·6
Rye (*Secale cereale* L. cv. Lovaspatona)	0·33	3·3

Leaf extracts (2 ml) at pH 4·5 were incubated with spermine (0·5 ml of 25 mM) and catalase (100 μg in 0·1 ml H_2O) for 3 hr at 25°. Activity was estimated from the area of the 1,3-diaminopropane peak on GLC. The ratios of the peak areas of 1-(3-aminopropyl)pyrroline/1,3-diaminopropane found on GLC were also determined.

1-week-old barley seedlings and in the leaves of 10-day-old dark-grown barley seedlings the activity of the polyamine oxidase was comparable to that found in the light-grown leaves on a fresh weight basis.

Purification

The polyamine oxidase was found to be associated with particles which sedimented easily in low centrifugal fields and the enzyme could be solubilized by washing these particles in a weak salt solution. NaCl or KCl (0·5 M) were found to be equally effective for the elution of this enzyme, and both were slightly better than 0·5 M $MgCl_2$ or $CaCl_2$. For NaCl, a 0·5 M solution was more effective than 0·25 M but equally effective as 1 M. Ultrasonics, Triton X-100 or acetone were ineffective in removing the enzyme from the particles. An experiment was conducted to investigate the distribution of the enzyme on fractionation of barley tissue. Pre-frozen barley seedling leaves (7 days old, 400 g) were macerated in 800 ml of water and squeezed through muslin. The fibrous residue was suspended in pH 4·5, 0·1 M citrate buffer (1200 ml) (Table 2, fraction A) and an aliquot of the filtrate retained and dialysed against this buffer (fraction B). A further sample was frozen and centrifuged (5000 *g* for 30 min). The supernatant was dialyzed against citrate buffer (0·1 M, pH 4·5), (fraction C), and the precipitate was suspended in a solution of 0·5 M NaCl in the citrate buffer for 18 hr. The precipitate was then removed by centrifuging, and both supernatant (fraction D) and precipitate (fraction E) were made up separately to the original volume of the sample with the citrate buffer. The precipitate was dispersed with a Potter–Elvejhem macerator. The separate fractions (A–E) were incubated with spermine, and activity estimated by determination of the 1,3-diaminopropane (see Experimental) (Table 2).

Fractions C, D and E accounted for 88% of the activity in the crude extract (B). Only a small proportion of the activity was lost in the fibrous residue. It was consistently found that 15–20% of the activity remained in the supernatant on centrifuging at 5000 *g* for 30 min.

In extracts of barley leaves the enzyme was associated with a particulate fraction which consisted mainly of chloroplasts. In the roots, however, the enzyme was also found to be particulate and it seems unlikely therefore that the enzyme is chloroplastic in the leaves. In

TABLE 2. DISTRIBUTION OF POLYAMINE OXIDASE ACTIVITY ON FRACTIONATION OF BARLEY TISSUE

Fraction	nmol/hr/g fresh wt
A Fibrous residue	220
B Crude extract (filtered)	1400
C Supernatant after centrifuging	220
D NaCl eluate of precipitate	680
E Residual precipitate	320

Activity was estimated by GLC (see Experimental). The results are expressed as nmol/hr/g original fresh wt.

view of the association of polyamines with nucleic acids,[1] it seems possible that the enzyme is present in the nuclei, though it could be associated with a different sub-cellular particle. The possibility that the enzyme is adsorbed non-specifically to particles as an artefact cannot be eliminated at present, though this would seem unlikely in view of the apparent lack of association of the enzyme with the cell wall material.

Subsequently the activity in the crude extract (fraction B) was compared with that in the 0·5 M NaCl eluate of the precipitate (fraction D) on a protein basis (Table 3). It was possible to concentrate the enzyme approximately 20-fold with a mean purification factor of 24 using this technique. In experiment 3 (Table 3) the effect of dialysis on the eluate of the precipitate was investigated. Some inert protein was found to be precipitated on removal of the NaCl by dialysis. However, some of the enzyme activity was lost from the supernatant and the purification gain was quite small.

TABLE 3. PURIFICATION OF POLYAMINE OXIDASE

Experiment	Sample*	Activity μmol/hr/g original fresh wt	Relative peak area on GLC 1-(3-aminopropyl)pyrroline / 1,3-diaminopropane	μg Protein/ml	Purification factor
	Fraction				
1	B. Crude extract	1·62	4·28	2700	
	D. Purified dialysed	0·74	3·43	45	27·0×
2	B. Crude extract	1·57	4·20	3320	
	D. Purified dialysed	0·31	3·90	27	24·5×
3	B. Crude extract	1·62	3·35	3280	
	D. Purified not dialysed	0·35	3·05	38	18·4×
	D. Purified dialysed	0·23	3·50	17	26·4×

The purification factor is given on the basis of protein, relative to the frozen crude extracts, for the corresponding enzyme samples prepared by washing the precipitate obtained by centrifuging the crude extracts for 30 min at 5000 *g*, in 0·5 M NaCl, with or without subsequent dialysis against pH 4·5 buffer. Activity was estimated by GLC.

* Fractions B and D, Table 2.

In order to investigate the effect of freezing on the enzyme, a crude extract (B) of barley leaves which had not been pre-frozen was frozen and immediately thawed. No significant loss

in activity of the polyamine oxidase could be detected. However, on storage of this extract for 2 months at −15° only 50% of the activity remained.

pH Optimum

The pH optimum for spermine oxidation was determined by the manometric method using 0·1 M citric acid–NaOH buffers. The enzyme showed an optimum at pH 4·0–4·5, activity falling off more steeply at the lower pH values (Fig. 1). An almost identical relationship for a crude extract was found, using the GLC method for determining activity. A similar though somewhat broader pH optimum was found for a crude extract of maize leaves. The pH optimum for spermidine oxidation in the barley extracts was determined using citric acid–NaOH buffers below pH 7 and Tris-HCl buffers above pH 7 estimating the enzyme activity by the GLC method after 22 hr incubation with toluene as an antiseptic. The optimum was quite sharp at pH 6·6. In this experiment a small amount of 1-(3-aminopropyl)pyrroline was also formed with a pH optimum at 6–7. For the polyamine oxidase in *Neisseria perflava* the pH optimum was 6·5–7·0 with spermine as substrate.[7]

The activity of a purified extract diluted 1:50 at pH 4·5 with spermine as substrate was compared with the same extract (undiluted) at pH 6·6 with spermidine as substrate. After 22 hr incubation with toluene as antiseptic, the 1,3-diaminopropane was determined by GLC. After correction for dilution, the activity of the extract at pH 4·5 with spermine as substrate was found to be about 30 times greater than the activity in the extract at pH 6·6, with spermidine as substrate.

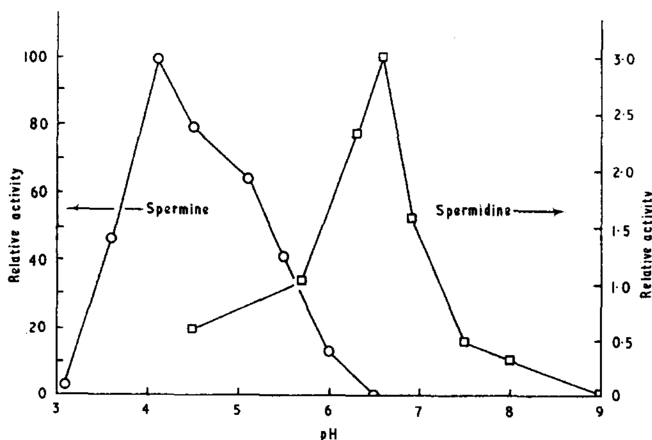

FIG. 1. EFFECT OF pH ON BARLEY LEAF POLYAMINE OXIDASE.
Purified polyamine oxidase dialysed against a range of buffers was incubated with spermine or spermidine in the presence of catalase (2 ml of enzyme; 0·5 ml of 25 mM amine substrates, as hydrochlorides; 0·1 ml of 0·1% catalase). With spermine as substrate (○), oxygen uptake over a 30-min period was determined in a Warburg. With spermidine as substrate (□) activity was determined by GLC estimation of 1,3-diaminopropane after a 22-hr incubation under toluene (0·5 ml). Activity is expressed in arbitrary units; the scale for spermine on the left, for spermidine on the right.

Effect of Inhibitors

Since all the amine oxidases which have so far been investigated for metal prosthetic groups have been shown to be copper-dependent,[15,16] the effect of a number of copper

[15] S. NARA and K. T. YASUNOBU, in *The Biochemistry of Copper* (edited by J. PEISACH, P. AISEN and W. E. BLUMBERG), p. 423, Academic Press, New York (1966).
[16] J. M. HILL and P. J. G. MANN, *Biochem. J.* **85**, 198 (1962).

TABLE 4. EFFECT OF INHIBITORS ON POLYAMINE OXIDASE ACTIVITY

Inhibitor	Final concn (M)	% Inhibition
8-Hydroxyquinoline	10^{-2}	70
Bathocuproine	10^{-2}	38
	10^{-3}	6
Bathocuproinesulphonate	10^{-2}	52
Sodium diethyldithiocarbamate	10^{-2}	36
	10^{-3}	6
Biquinoline	10^{-2}	17
Cuprizone	10^{-2}	37
	10^{-3}	0
p-Chloromercuribenzoate (suspension)	10^{-2}	11
Potassium cyanide	10^{-2}	7
Hydroxyethylhydrazine	10^{-2}	100
	10^{-3}	88
	10^{-4}	14
	10^{-5}	0
Semicarbazide	10^{-2}	63
	10^{-3}	28
Ammonium sulphate	10^{-2}	33
Sodium chloride	2×10^{-1}	0
Indole acetic acid	10^{-2}	48
Diaminopropane	$2{\cdot}5 \times 10^{-3}$	9
Diaminodipropylamine	$2{\cdot}5 \times 10^{-3}$	1
Spermidine	$2{\cdot}5 \times 10^{-3}$	28

Purified enzyme (1·75 ml) was incubated with inhibitor (0·25 ml) and catalase (100 μg in 0·1 ml H_2O) in a Warburg, prior to the addition of spermine (0·5 ml of 25 mM). Activity was estimated by oxygen uptake over a 30-min period after addition of spermine.

chelating reagents was tested, but none was found to inhibit greatly (Table 4). Although 8-hydroxyquinoline inhibited the enzyme by 70% at 10^{-2} M as measured by oxygen uptake, at 10^{-3} M oxygen uptake was stimulated due to the peroxidative oxidation of this inhibitor. Evidence for this was seen in the colour of the incubate, which became yellow. Since copper chelators were comparatively ineffective as inhibitors, either the enzyme does not depend on copper for its activity or the metal is very strongly chelated. The presence of copper in the polyamine oxidase of *Neisseria perflava* has not as yet been established.

Hydroxyethylhydrazine gave 50% inhibition of the polyamine oxidase at about 5×10^{-4} M and this enzyme is therefore much less sensitive to this compound than pea diamine oxidase which is inhibited 50% at about 5×10^{-7} M.[17,18] Similarly, semicarbazide is inhibitory to the barley polyamine oxidase only at much higher concentrations (50% at about 5×10^{-3} M) than were found to inhibit the pea diamine oxidase (50% at 5×10^{-5} M).[19] However, for the *Neisseria perflava* polyamine oxidase, 4×10^{-3} M semicarbazide gave only 27% inhibition.[7]

The enzyme was found to be inhibited significantly by 10^{-2} M ammonium sulphate and therefore ammonium sulphate was not used as a fractionating agent. Sodium chloride did not appear to inhibit below 1 M. Reduction of the citrate buffer concentration to $2{\cdot}5 \times$

[17] D. J. REED, *Science* **148**, 1097 (1965).
[18] E. F. YAMASAKI, R. SWINDELL and D. J. REED, *Biochemistry* **9**, 1206 (1970).
[19] P. J. G. MANN, *Biochem. J.* **59**, 609 (1955).

10^{-2} M did not affect activity. The reduction in activity due to spermidine (28% at $2 \cdot 5 \times 10^{-3}$ M) may be attributed to competitive inhibition between similar substrates.

Effect of Pyridoxal Phosphate

Since pyridoxal phosphate may be a co-factor of some amine oxidases[15] and as the barley polyamine oxidase was inhibited by hydroxyethylhydrazine, a potential carbonyl reagent, the effect of pyridoxal phosphate on the activity of the enzyme was investigated. The enzyme was dialysed exhaustively against 3 changes of buffer for 2 days, and activity determined using the manometric method in the presence and absence of 1 mg of pyridoxal phosphate. No significant difference in activity was found on addition of pyridoxal phosphate.

Stoicheiometry

The precipitate, to which the enzyme was adsorbed, obtained by freezing the extracts of barley leaves, was incubated with and without spermine in the Warburg apparatus. Oxygen uptake was found to be quite high in the absence of spermine and this uptake was greatly enhanced on adding spermine. The difference in the amount of oxygen consumed was frequently about 3–4 times greater than the expected volume on adding spermine (on the basis of $\frac{1}{2}$ mol O_2 per mol spermine, in the presence of catalase). The 1,3-diaminopropane and 1-(3-aminopropyl)pyrroline in this experiment were at the stoicheiometrically expected concentration. It therefore seems likely that two processes are operating simultaneously in the particulate preparation. Firstly, the accelerated oxidation of an endogenous substrate induced by spermine, or by the products of spermine oxidation, and secondly the oxidation of spermine by the polyamine oxidase. The spermine-accelerated oxidation was independent of buffer composition at pH 4·5, being equally effective in citrate, phthalate or acetate buffers; neither was any CO_2 produced. It was therefore unlikely that the buffer was the substrate for the oxidation.

One explanation for the supra-stoicheiometric oxygen uptake on adding spermine may be found in a peroxidative oxidation of a natural substrate, utilizing the hydrogen peroxide generated by the process of enzymic polyamine oxidation. Although it was found experimentally that addition of hydrogen peroxide to the particles will not stimulate the oxygen uptake, it is possible that enzymically-generated peroxide has properties which differ from those of hydrogen peroxide.[20] Evidence in favour of the 'peroxide-hypothesis' was given on adding spermine to a 0·5 M NaCl extract of the particles prior to dialysis. The oxygen uptake here was greater than that required for stoicheiometry (119%, 122%) while the amount of 1,3-diaminopropane formed was that expected (94%, 102%) (Table 5); simultaneously the solutions became yellow, indicating a peroxidative oxidation of a naturally-occurring phenol.

On exhaustive dialysis the values for oxygen consumption became stoicheiometric (90%, 101%) like the diaminopropane (98%, 104%) and these extracts did not become yellow. Similarly, with a preparation subjected to acetone precipitation prior to dialysis, the stoicheiometry was 97% for oxygen and 94% for diaminopropane. These results suggest that the supra-stoicheiometric oxygen consumption may be due to the coupled peroxidative oxidation of a natural substrate which may be eliminated by dialysis.

Another explanation for the supra-stoicheiometric oxygen uptake may be that spermine *per se* induces the oxidation of a natural substrate. This phenomenon has already been

[20] R. J. Nash, T. A. Smith and R. L. Wain, *Ann. Appl. Biol.* **61**, 481 (1968).

Table 5. Stoicheiometry of the polyamine oxidase

Extract preparation	Oxygen Theoret. = 100% (%)	1,3-Diaminopropane Theoret. = 100% (%)	Ratio of peak area on GLC 1-(3-aminopropyl)pyrroline / 1,3-diaminopropane
1 Not dialysed	119	94	3·4
	122	102	3·3
2 Further purified by	97	92	2·8
acetone precipitation; dialysed		98	2·8
		89	2·7
3 Dialysed	90	98	3·0
	101	104	3·1
4 Dialysed	92	94	2·9

The enzyme samples were eluted from the precipitate with 0·5 M NaCl. This purified enzyme (2 ml) was incubated with spermine (0·5 ml of 25 mM) and catalase (100 μg in 0·1 ml H_2O). Oxygen uptake was measured manometrically and the amines determined by GLC (see Experimental).

described in other systems. For instance, spermine has been shown to accelerate oxidation by rat liver mitochondria[21] and of lipid and glucose oxidation in the fat cells of the rat.[22] Moreover, spermidine and other amines have also been shown to accelerate the oxidation of an endogenous substrate in micro-organisms.[4,5,23] It is possible that either or both phenomena are contributing to the excessive O_2 uptake in the particulate preparations.

In order to determine the stoicheiometry of the 1-(3-aminopropyl)pyrroline production, a barley polyamine oxidase preparation was incubated with spermine (0·5 ml of 25 mM), and a pea cotyledon extract, dialysed to pH 7·0 (2 ml) was incubated with spermidine (0·5 ml of 25 mM), both in Warburg flasks. Catalase was added to each incubate. In each case when oxygen uptake ceased (94% of the expected volume for barley and 98% of the expected volume for pea), the incubates were subjected to GLC and the relative peak areas of the amine products compared with authentic 1,3-diaminopropane as standard, peak area 100 units. In the barley incubate 1,3-diaminopropane was 96 units, and 1-(3-aminopropyl)-pyrroline was 314 units; this compound in the pea incubate was 321 units. Since the amount of 1-(3-aminopropyl)pyrroline produced by the pea and barley systems was almost identical, it is likely that both are produced stoicheiometrically.

The mean ratios of the area of the peaks found on GLC for 1-(3-aminopropyl)pyrroline/ 1,3-diaminopropane were 3·9 for the crude extracts and 3·2 for the purified preparations. This difference is statistically significant at the 1% level of probability, and might imply that further metabolism of the 1,3-diaminopropane occurs in the crude preparations.

The Enzymic Oxidation of Spermidine

A purified barley leaf extract was dialysed against pH 7·2 Tris-HCl buffer (0·1 M) and incubated for 24 hr at 25° with 0·5 ml of 25 mM spermidine and catalase (100 μg in 0·1 ml water) under toluene (0·5 ml). After adding 0·5 ml of N HCl the solution was evaporated to dryness at 60° *in vacuo* and taken up in 0·25 ml of 4% acetic acid. Controls having water in place of spermidine and Tris buffer in place of the extract were included. Aliquots (10 μl) of each incubate were chromatographed by TLC (see Experimental) and 1-pyrroline detected

[21] P. Bjorntorp, *Exptl Cell Res.* **41**, 630 (1966).
[22] D. H. Lockwood, J. J. Lipsky, F. Meronk and L. E. East, *Biochem. Biophys. Res. Commun.* **44**, 600 (1971).
[23] E. F. Gale, *Biochem. J.* **36**, 64 (1942).

with the *o*-aminobenzaldehyde reagent. Only in the sample in which spermidine was incubated with the barley leaf extract could 1-pyrroline be detected. Identity was confirmed by co-chromatography with authentic 1-pyrroline. Extracts of spermine incubated at pH 4·5 with the barley polyamine oxidase gave no colour with *o*-aminobenzaldehyde, and 1-(3-aminopropyl)pyrroline apparently does not react with this compound.

Apparent Loss of Enzyme Activity on Incubation with Spermine

The activity measured manometrically declined progressively while the enzyme oxidized the spermine to completion in the standard assay (Fig. 2). Incubation at 25 mM, 5 mM (the concentration normally used) and 1 mM spermine showed no difference in initial activity and it seems possible that this decline is not due only to substrate limitation, since the K_m is probably less than 1 mM. An alternative explanation for the loss of activity is that the enzyme is inactivated or inhibited by the reaction products. To test the possibility that enzyme inactivation could account for this non-linearity, the enzyme was incubated with and without 12·5 μmol of spermine in the presence of catalase until oxygen uptake ceased. Further spermine (12·5 μmol) was then added to both incubates and the activity shown to be reduced by about 30% in the extract previously incubated with spermine (Fig. 2). It therefore seems likely that the enzyme suffers inactivation on being incubated with spermine.

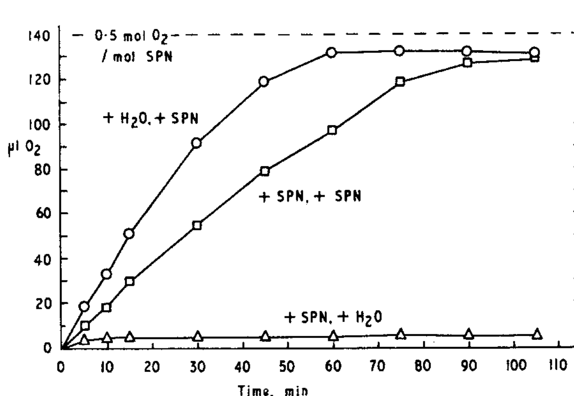

Fig. 2. Effect of pre-incubation with spermine on enzyme activity.
Polyamine oxidase activity was determined with spermine as substrate by manometry after incubation with water (+H_2O + SPN) or with spermine (+SPN + SPN). The control (+SPN + H_2O) was incubated with spermine prior to adding water. The expected stoicheiometric oxygen uptake equivalent to $\frac{1}{2}$mol O_2/mol spermine was 140 μl.

Effect of Catalase

In common with other amine oxidases, barley seedling polyamine oxidase evolves hydrogen peroxide as a product of amine oxidation. Since the hydrogen peroxide may cause inactivation of amine oxidases, catalase is normally added to decompose it.[24] Figure 3 shows the effect of catalase on the oxygen uptake. In the presence of catalase the stoicheiometric value of oxygen (140 μl) is consumed, corresponding to $\frac{1}{2}O_2$. In the absence of added catalase, hydrogen peroxide accumulated and the oxygen uptake is correspondingly higher, approaching the theoretical consumption of 1 molecule of oxygen. After taking up oxygen for 100 min, oxygen was evolved, suggesting that the hydrogen peroxide was being broken down,

[24] R. H. Kenten and P. J. G. Mann, *Biochem. J.* **50**, 360 (1952).

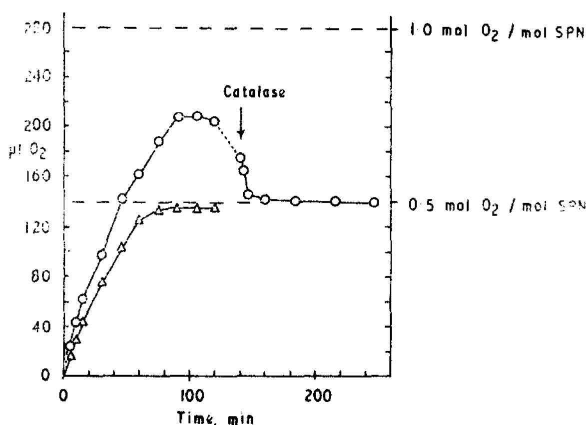

FIG. 3. EFFECT OF CATALASE ON OXYGEN UPTAKE BY BARLEY LEAF POLYAMINE OXIDASE. Polyamine oxidase was incubated with spermine as substrate, with (△) or without (○) catalase. Catalase was added after 140 min incubation. Oxygen uptake was measured by manometry. The upper dashed line represents the volume expected for a stoicheiometric uptake equivalent to 1 molecule of oxygen/mol spermine; the lower line for 1 atom of oxygen/mol spermine.

possibly by endogenous catalase. On adding catalase the remaining peroxide is quickly decomposed and the theoretical value of $\frac{1}{2}O_2$ is obtained. Since the flasks were opened for catalase addition, the change in volume over the period from 120 to 140 min is somewhat arbitrary and the final volume has been adjusted to coincide with the $\frac{1}{2}O_2$ value (140 μl).

The effect on enzyme activity of omitting the catalase was investigated in two further experiments. In the first, the activity in the presence of catalase measured by determination of both 1,3-diaminopropane and 1-(3-aminopropyl)pyrroline by GLC was found to be indistinguishable from the activity in its absence. In the second, using the Warburg apparatus, the enzyme was incubated with and without spermine in the absence of catalase. When oxygen uptake ceased, spermine and catalase were added to each. Activity of the sample pre-incubated with spermine in the absence of catalase was not greatly reduced in comparison with the activity of the extract pre-incubated with spermine in the presence of catalase. The reason for the loss of activity on incubating with spermine therefore cannot easily be attributed to enzymically generated peroxide.

On coupling the peroxide formed by putrescine oxidation (using pea seedling diamine oxidase) with the peroxidative oxidation of hydroquinone, Kenten and Mann found that the predicted uptake of 1 mol of oxygen is attained.[24] An experiment was designed to investigate the oxygen consumption on coupling this peroxidase system with the peroxide formed by spermine oxidation using the barley polyamine oxidase. Spermine was added from the side arm to a reaction mixture containing 1·5 ml of enzyme at pH 4·5, 0·5 ml of 0·05 M hydroquinone and 0·1 ml of peroxidase (100 μg), in the absence of catalase. On completion of the reaction the incubate was magenta and oxygen uptake was 94% of the theoretical value, corresponding to 1 mol/mol substrate. The diaminopropane and 1-(3-aminopropyl)-pyrroline were 92 and 80% of the expected amounts respectively.

The coupled catalatic oxidation of ethanol was not achieved. In this experiment the incubation was effected at pH 4·5, the optimum for the barley polyamine oxidase, and this pH may have been too low for the catalase-mediated ethanol oxidation.

Effect of Temperature

Samples of unpurified barley leaf extract at pH 4·5 were heated in a water bath at 50, 60, 70 and 80° for 10 min, and immediately cooled in iced water. After incubation with spermine at 25°, activity was determined by the GLC method. Maximal activity was retained on heating at 50 and 60° and activity was reduced to 10–15% of this at 70 and 80°. The purified extract was similarly heated at a range of temperatures extending from 50 to 70° for 15 min. Using the Warburg, half maximal activity was found at 60° and the energy of activation for the inactivation was 53 800 (s.d. 4650) cal/mol over the range 55–65°.

The energy of activation with spermine as substrate at 0° (4 hr incubation) and 25° (2 hr incubation) determined by measuring the 1,3-diaminopropane by GLC was found to be 4850 cal/mol (s.d. 345 cal/mol).

EXPERIMENTAL

Plant material. Barley seedlings (*Hordeum vulgare* L. cv. Zephyr) and pea seedlings (*Pisum sativum* L. cv. Meteor) were grown at 22° under fluorescent lamps (1000 lm, 16 hr day) watered with a full nutrient solution. Seedlings of other plants were grown in a greenhouse during June 1971 for 10 days. Mature plants were grown locally.

Extraction. Throughout this study pH 4·5, 0·1 M citrate buffer was used unless stated otherwise. In a typical extraction the prefrozen shoots of 7- to 14-day-old barley seedlings (800 g) were macerated in 1600 ml H_2O in a chilled homogenizer, and the homogenate squeezed through washed muslin. The filtrate was then frozen overnight. On thawing, the particles settled out and the lower portion of the extract containing the sediment was centrifuged at 5000 *g* for 30 min. The green precipitate was suspended in 40 ml of 0·5 M NaCl in buffer. After 18 hr at 2°, the ppt. was removed by centrifuging and the supernatant dialysed exhaustively against citrate buffer at 2°. In one experiment the enzyme was precipitated with 2 vol. of acetone at −15° and redissolved in buffer (see Table 5). In a survey of plant species designed to determine the distribution of the enzyme and in determining the activity in barley leaves, sampled at different ages, the tissue (10 g) was homogenized in a cooled blender in 40 ml of water and dialysed against buffer. The unfractionated extract was sampled for determination of enzyme activity.

Determination of activity—manometric method. This was used primarily for the purified enzyme preparations. The extract (2 ml) was placed in the main compartment of a Warburg flask together with 0·1 ml of catalase (1 mg/ml). Substrate (0·5 ml of 25 mM, HCl salt) was added from the side-arm and oxygen uptake determined at 25°. In order to establish stoicheiometry an additional 0·5 ml of H_2O was added to the side arm when oxygen uptake had stopped, to recover residual spermine; the additional uptake of oxygen was recorded on tipping. Flasks containing enzyme but no substrate, and substrate without enzyme, were included routinely. In the experiment designed to determine stoicheiometry, on completion of the oxidation, 0·5 ml of N HCl was added to the main vessel and any ppt. was removed by centrifuging. KOH (0·2 ml of 5 N) was added to a 1-ml sample of the supernatant prior to GLC.

Determination of activity—GLC method. Preparations with low activity were assayed at 25° in 25 ml stoppered conical flasks, shaken at 100 cycles/min. Samples (2 ml) of the extracts with 0·1 ml of catalase (1 mg/ml) were incubated with 0·5 ml of 25 mM substrate. Flasks containing enzyme but no substrate, and substrate with no enzyme were routinely included. At the end of the incubation period, normally 3 hr (22 hr with spermidine as substrate), 0·5 ml of N HCl was added to each flask, the contents were centrifuged and 0·2 ml of 5 N KOH was added to samples (1 ml) of the supernatant prior to GLC. Activity was approximately linear over a 3-hr period.

Although it would have been preferable to determine activity on the basis of 1-(3-aminopropyl)pyrroline (since for equimolar quantities this amine has a peak 3 times the area of 1,3-diaminopropane on GLC), authentic 1-(3-aminopropyl)pyrroline was not available, while 1,3-diaminopropane was. Activity was therefore expressed in terms of 1,3-diaminopropane produced/hr/g fresh wt.

Diamine oxidase. Pea cotyledons from 10-day-old seedlings were washed and extracted in water (2 vol.). The extract was centrifuged and dialysed against pH 7·0 Tris–HCl buffer.

Gas chromatography. The column parameters were as cited previously.[25] 'Ghost' peaks were found to be reduced by coating the inside of the glass column with KOH prior to packing. Samples (5 or 10 μl) of the aqueous alkaline solutions were injected. The peaks of 1,3-diaminopropane (R_t 2·5 min) and 1-(3-aminopropyl)pyrroline (R_t 5 min) were integrated electronically and the amount of 1,3-diaminopropane was determined by comparison with an authentic sample of known concentration.

[25] T. A. Smith, *Anal. Biochem.* 33, 10 (1970).

1-Pyrroline. 1-Pyrroline was separated by TLC on MN 300 cellulose with BuOH–AcOH–H_2O (4:1:1) as solvent. After drying at 100°, 1-pyrroline was detected as a yellow spot (R_f 0·4) on spraying with 0·1% *o*-aminobenzaldehyde in acetone. Authentic 1-pyrroline was prepared enzymatically by the method of Mann and Smithies.[26]

Protein. Protein was determined by the Folin–Phenol method,[27] using bovine serum albumin as standard.

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Key Word Index—Hordeum vulgare; Gramineae; barley; spermidine; spermine; polyamine oxidase; diaminopropane; pyrroline; aminopropylpyrroline; peroxide.