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ACID PHOSPHATASE AND ISOCITRITASE PRODUCTION DURING SEED GERMINATION

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Abstract—The development of acid phosphatase (3.1.3.2) and isocitritase (isocitrate lyase, 4.1.3.1) activities in cotyledons during the early growth of seedlings was examined. Enzyme production in seedlings growing normally was compared with that in seedlings whose growth had been inhibited to different extents by supplying them with various concentrations of azetidine-2-carboxylic acid. This imino acid is a potent analogue of proline which, by effectively replacing proline residues in protein, leads to altered protein molecules possessing reduced biological activity. However, treatment with the analogue did not decrease phosphatase or isocitritase production and so these enzymes are presumed to arise in cotyledons by the activation of preformed macromolecules, e.g. zymogens, and not by *de novo* synthesis from amino acids.

INTRODUCTION

THE activities of many enzymes markedly increase during the first few days of seed germination and growth. For some hydrolytic enzymes, increased activity may be detected during the initial phase of water imbibition that precedes radicle growth. These early increases of activity occur within cotyledon or endosperm tissue and are associated with the onset of catabolism of seed storage compounds. Under normal growth conditions the activities of individual enzymes may continue to increase for several days as shown by Young and Varner[1] for amylase and an ATP-hydrolysing phosphatase present in pea cotyledons. These workers also showed that the development of the two enzyme activities was considerably curtailed when germinating peas were supplied with either chloramphenicol, a potent inhibitor of protein synthesis effective at a stage subsequent to the formation of aminoacyl-soluble RNA complexes, or *p*-fluorophenylalanine, one of the most useful synthetic amino acid analogues. These observations indicated that, even in cotyledons where protein degradation is predominant, *de novo* synthesis of protein from amino acids must occur to yield additional enzyme molecules; the alternative possibility that enzyme production in cotyledons is due to an activation of latent protein molecules, e.g. by partial proteolysis of zymogen-type molecules, seemed unlikely for the two enzymes studied.

In the present investigation the production of an acid phosphatase, exhibiting a pH optimum of 6·0 with *p*-nitrophenyl phosphate as substrate,[2] and of isocitritase (L_S-isocitrate glyoxylate-lyase) has been followed during the germination of several seed species. The development of enzyme activities in normal seedlings was compared with that occurring in seedlings whose growth had been inhibited to different extents by treatment with various concentrations of azetidine-2-carboxylic acid. This imino acid, the lower homologue of proline and a natural product characteristic of many liliaceous species, appears to be among the most effective analogues yet described; it can replace the proline residues of protein almost

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[1] J. L. YOUNG and J. E. VARNER, *Arch. Biochem. Biophys.* **84**, 71 (1959).
[2] D. SPENCER, *Aust. J. Biol. Sci.* **10**, 302 (1954).

completely when protein is synthesized in the presence of appropriate concentrations of analogue.[3] The resulting abnormal protein molecules lead to growth inhibitions that are more marked than those observed with comparable concentrations of *p*-fluorophenylalanine.

The positions at which proline residues occur in protein molecules constitute one factor determining the tertiary structure of these molecules because these sites are associated with breaks in the α-helical structure and with changes in the direction, i.e. bending, of the polypeptide chain. Since the spatial configuration of proline and azetidine-2-carboxylic acid differ slightly, progressive modification of the tertiary structure of proteins may be predicted as the degree of proline replacement by analogue residues is increased. Eventually the conformations of protein molecules may be so changed that they will no longer be able to exhibit enzymic properties. Therefore, if the increase in the activity of a particular enzyme in normal cotyledons is dependent upon *de novo* synthesis from amino acids, then reduced or negligible activity should develop in seedlings grown in the presence of highly inhibitory concentrations of azetidine-2-carboxylic acid. This reasoning when applied to the experimental results cited later has led to the conclusion that both the acid phosphatase and isocitritase under investigation form examples of enzymes whose production is dependent upon the conversion of latent, preformed precursors into catalytically active protein species.

RESULTS

Development of Acid Phosphatase Activity

The phosphatase activity of all the types of seedling studied increased progressively during the first four days of seedling growth. The first determinations of phosphatase in cotyledons were made between 18 and 24 hr after commencement of soaking and, during the subsequent growth period to the 92–93-hr stage, the activity in cotyledons increased about 5-fold for mung beans (*Phaseolus aureus* Roxb.), about 9-fold for cucumbers (*Cucumis sativus* L.) and about 7-fold for peas (*Pisum sativum* L.). The corresponding increase observed for cotyledons of sunflower (*Helianthus annuus* L.) between the 24- and 73 hr growth stages was about 9-fold. These increases refer to phosphatase activity present in a fixed number of cotyledons; if the activities are expressed per unit of cotyledon dry weight, then the increases tend to be slightly

TABLE 1. ACID PHOSPHATASE ACTIVITIES OF COTYLEDONS OF MUNG BEAN SEEDLINGS GROWING NORMALLY AND IN THE PRESENCE OF AZETIDINE-2-CARBOXYLIC ACID

Analogue supplied		Radicle growth	Phosphatase activity† after growth for			
mg/100 seeds	mg/g seeds	(% control)	18 hr	42 hr	66 hr	93 hr
0	0	100	77(71)	217(226)	311(416)	390(589)
0·75	0·17	60	100(94)	201(227)	310(367)	372(463)
1·5	0·33	32	98(90)	267(274)	346(377)	400(453)
3·0	0·67	14	103(100)	276(271)	350(380)	488(563)

Data expressed as μg *p*-nitrophenol formed per hr in standard reaction mixture.* Figures in parentheses refer to activity calculated per g dry wt. tissue homogenized to yield enzyme preparation

* 30 pairs of cotyledons were homogenized in 16 ml buffer and, after centrifuging, supernatant (dil. × 10), 0·5 ml, used as phosphatase preparation, was added to 0·8 ml *p*-nitrophenyl phosphate (0·05 M) and 2·7 ml buffer (pH 6·0) to give the reaction mixture.

† Values are means of two separate experiments.

[3] L. FOWDEN, *J. Exp. Botany* **14**, 387 (1963).

larger because seedling growth is accompanied by breakdown and translocation of cotyledonous materials leading to lowered dry weights (see Tables 1–4).

TABLE 2. ACID PHOSPHATASE ACTIVITIES OF COTYLEDONS OF CUCUMBER SEEDLINGS GROWING NORMALLY AND IN THE PRESENCE OF AZETIDINE-2-CARBOXYLIC ACID

Analogue supplied		Radicle growth	Phosphatase activity after growth for			
mg/100 seeds	mg/g seed	(% control)	20 hr	44 hr	68 hr	93 hr
0	0	100	35(60)	132(290)	257(632)	330(906)
1	0·33	57	35(67)	150(314)	236(614)	354(960)
2	0·67	31	37(78)	153(338)	235(552)	282(636)
4	1·33	15	31(59)	112(227)	214(504)	283(603)
5	1·67	12	35(65)	132(291)	249(603)	—

20 pairs of cotyledons were homogenized in 16 ml buffer to yield supernatant phosphatase preparation. Other conditions as in Table 1.

When azetidine-2-carboxylic acid was supplied to the seeds, the growth of mung bean and cucumber was nearly totally inhibited at the highest concentrations used. The slight radicle growth recorded, i.e. 14 per cent and 12 per cent of the normal for mung bean and cucumber respectively, is thought to represent the early growth, perhaps primarily by cell expansion, of the embryonic root that occurs before appreciable azetidine-2-carboxylic acid becomes incorporated in newly-synthesized protein molecules. This growth occurred within the first 24 hr, and most seedlings in batches receiving these treatments subsequently made little or no further growth. The limited quantity of azetidine-2-carboxylic acid available meant that the treatments of pea and sunflower seeds were restricted to lower concentrations so that each treatment could still involve 100 seeds, although each batch weighed considerably more than those of mung bean and cucumber. Reference to Tables 1–4 shows that acid phosphatase activity developed in the cotyledons of all seedlings and that the increases of activity observed during various time periods were unaffected even when severe growth inhibition resulted from azetidine-2-carboxylic acid treatment.

TABLE 3. ACID PHOSPHATASE ACTIVITIES OF COTYLEDONS OF PEA SEEDLINGS GROWING NORMALLY AND IN THE PRESENCE OF AZETIDINE-2-CARBOXYLIC ACID

Analogue supplied		Radicle growth	Phosphatase activity after growth for			
mg/100 seeds	mg/g seed	(% control)	18 hr	42 hr	68 hr	92 hr
0	0	100	54(20)	45(16)	257(99)	356(140)
2	0·07	83	68(26)	97(38)	240(91)	311(131)
4	0·13	72	68(25)	50(20)	243(97)	377(149)
8	0·27	59	71(25)	66(24)	222(86)	344(136)

10 pairs of cotyledons were homogenized in 20 ml buffer to yield supernatant phosphatase preparation. Other conditions as in Table 1.

TABLE 4. ACID PHOSPHATASE ACTIVITIES OF COTYLEDONS OF SUNFLOWER SEEDLINGS GROWING NORMALLY AND IN THE PRESENCE OF AZETIDINE-2-CARBOXYLIC ACID

Analogue supplied		Radicle growth	Phosphatase activity after growth for		
mg/100 seeds	mg/g seed	(% control)	24 hr	49 hr	73 hr
0	0	100	19(15)	76(68)	169(161)
1	0·10	82	19(16)	76(61)	185(159)
2	0·21	70	23(18)	66(55)	166(153)
4	0·42	51	30(24)	61(53)	159(139)

20 pairs of cotyledons homogenized in 16 ml buffer to yield supernatant phosphatase preparation. Other conditions as in Table 1.

In contrast, Table 5 shows that the production of acid phosphatase activity in the radicles, where growth must be associated with *in situ* synthesis of new cellular protein, was considerably reduced when mung bean seeds were treated with azetidine-2-carboxylic acid.

TABLE 5. ACID PHOSPHATASE ACTIVITIES OF RADICLES OF MUNG BEAN SEEDLINGS GROWING NORMALLY AND IN THE PRESENCE OF AZETIDINE-2-CARBOXYLIC ACID

Treatment	Seedling growth (hr)		
	42	66	90
Water:			
g fresh wt. (60 radicles)	1·91	4·06	6·27
Phosphatase activity	126	324	502
Azetidine-2-carboxylate (1·5 mg/100 seeds):			
g fresh wt. (60 radicles)	0·92	1·56	1·64
Phosphatase activity	56	182	222

Enzyme activities expressed as μg *p*-nitrophenol formed per hr in standard reaction mixture.*

* 60 radicles were homogenized in 16 ml buffer to yield supernatant phosphatase preparation. Other conditions as in Table 1.

Development of Isocitritase Activity

The development of isocitritase activity during the early growth of seedlings of pumpkin (*Curcurbita pepo* L.) is illustrated in Table 6. In untreated seedlings, a rapid rise in the enzyme activity present in cotyledons occurred between the third and fifth days of growth. A similar increase in activity was observed for cotyledons of seedlings receiving four different concentrations of azetidine-2-carboxylic acid, the highest of which produced severe inhibition of radicle growth.

TABLE 6. ISOCITRITASE ACTIVITIES OF COTYLEDONS OF PUMPKIN SEEDLINGS GROWING NORMALLY AND IN THE PRESENCE OF AZETIDINE-2-CARBOXYLIC ACID

Analogue supplied		Radicle growth	Isocitritase activity after growth for					
mg/100 seeds	mg/g seed	(% control)	48 hr	62 hr	70 hr	86 hr	95 hr	112 hr
0	0	100	54	144	112	158	216	376
6·6	0·25	70	58	148	140	155	209	350
13·2	0·5	49	43	—	148	—	258	—
26·4	1·0	23	47	—	151	—	241	—
52·8	2·0	11	—	158	—	158	—	324

Data expressed as μg glyoxylate formed per hr in standard reaction mixture.*

* 10 pairs of cotyledons were homogenized in 20 ml buffer and, after centrifuging, supernatant (1 ml) was added to other components (see Experimental section).

Results obtained with cucumber seedlings, although considerably less detailed, confirmed the fact that isocitritase production in cotyledons is unaffected by the presence of growth-inhibitory concentrations of azetidine-2-carboxylate (see Table 7).

TABLE 7. ISOCITRITASE ACTIVITIES OF COTYLEDONS OF CUCUMBER SEEDLINGS GROWING NORMALLY AND IN THE PRESENCE OF AZETIDINE-2-CARBOXYLIC ACID

Analogue supplied		Radicle growth	Isocitritase activity after growth for	
mg/100 seeds	mg/g seed	(% control)	62 hr	95 hr
0	0	100	58	97
1	0·33	57	54	94
4	1·33	15	62	87

40 pairs of cotyledons were homogenized in 20 ml buffer to yield supernatant isocitritase preparation. Other conditions as in Table 6.

DISCUSSION

Previously, growth inhibition of seedlings caused by treatment with azetidine-2-carboxylic acid was shown to be correlated with the degree to which the imino acid replaced normal proline residues in newly-synthesized protein molecules.[3] If the view is accepted that these altered protein molecules cannot be endowed with normal catalytic activities, then the experimental data given in Tables 1–4 and 6 and 7 must mean that both the acid phosphatase and isocitritase studied are examples of cotyledonous enzymes whose activation during the first few days of seedling growth is not dependent upon *de novo* biosynthesis from precursor amino acids.

The only previous study describing the effect of an amino acid analogue upon higher plant enzymes seems to be that of Young and Varner[1] mentioned previously. Many similar examples are encountered in studies involving animal or microbial systems. *p*-Fluorophenylalanine and β-2-thienylalanine, both analogues of phenylalanine, and ethionine and selenomethionine,

two methionine analogues, are among the most widely investigated substances. In favourable circumstances, high percentage replacements of the normal amino acid by analogue have been observed to occur in particular enzyme molecules, e.g. in β-galactosidase, exopenicillinase and α-amylase (see reviews[4,5]). Replacement of phenylalanine residues in the two latter enzymes of bacteria by the *p*-fluoro derivative leads to lowered enzyme activities. However, incorporation of these analogues frequently does not seriously impair the catalytic properties of enzymes. For example, β-galactosidase of *Escherichia coli* retained full enzymic activity when at least 95 per cent of its phenylalanine residues were replaced by β-2-thienylalanine;[6] similarly, the replacement of one-third of the methionine residues of bacterial α-amylase by ethionine left the enzyme activity unimpaired.[7]

Richmond[5] suggests that the effectiveness of an analogue in decreasing the activity of an enzyme may depend upon the following factors: (*a*) the degree to which the analogue differs structurally from the natural amino acid, (*b*) whether a residue situated at the active centre is replaced, and (*c*) whether a residue involved in maintaining the tertiary structure of the enzyme is implicated. The different bond angles encountered in the heterocyclic ring structures of proline and azetidine-2-carboxylic acid provide the underlying reason for predicting that changes in protein tertiary structure will be associated with the incorporation of this analogue[8] and that the abnormal proteins resulting will be ineffective as enzymes. The analogues discussed in the previous paragraph are unlikely to cause any fundamental alteration in tertiary structure and therefore are less likely to affect enzyme activities. In those cases where significant reductions of activity were observed, analogue residues probably were introduced at positions in the enzyme molecules that form part of the active site.

The nature of the precursors that yield active acid phosphatase and isocitritase molecules are not known. Presumably, since the present experiments indicate that elaboration from unbound amino acids is not involved, the precursors must exist as relatively high molecular-weight polymers. One suggestion is that they are proteins of the trypsinogen type. The precursors then would have molecular weights slightly higher than those of the active enzymes and the increase in the activity of an enzyme could be ascribed to the fission of a peptide fragment, that originally masked the active centre, by the action of specific proteases. A simpler explanation of the increase in enzyme activity without new synthesis may be based upon a transition of protein from an insoluble to a soluble, active form. Protein is known to exist as particulate inclusions in the cells of many dormant seeds of which the best known are perhaps the aleurone grains of cereals. In this highly-conjugated form, protein can possess little enzymic capacity; however, these grains gradually disappear during growth of the seed and this solubilization process may lead to the formation of many catalytically active entities.

Alternate explanations for the lack of effect of azetidine-2-carboxylic acid upon the development of enzyme activity may be advanced. One would require that isocitritase and acid phosphatase do not contain proline residues but this seems very improbable: each dimer molecule of alkaline phosphatase from *Escherichia coli* is known to contain 36 proline residues. A second would assume that proline residues occur in the enzyme molecules at positions remote from the active centres and therefore changes of tertiary structure following analogue incorporation may not alter their catalytic properties. Unfortunately this type of uncertainty is encountered in all amino acid analogue work until very detailed information is available

[4] H. K. KING, *Science Progress* **49**, 703 (1961).
[5] M. H. RICHMOND, *Bact. Rev.* **26**, 398 (1962).
[6] J. JANECEK and H. V. RICKENBERG, *Biochim. et Biophys. Acta* **81**, 108 (1964).
[7] A. YOSHIDA and M. YAMASAKI, *Biochim. et Biophys. Acta* **34**, 158 (1959).
[8] L. FOWDEN and M. H. RICHMOND, *Biochim. et Biophys. Acta* **71**, 459 (1963).

concerning the entire conformation of an enzyme molecule in relation to its amino acid sequence. Again the closest comparison possible would seem to be with the phosphatase of *E. coli* whose activity is reduced when molecules of 3,4-dehydroproline replaced the normal proline residues (Neale and Tristram[9]). Finally it is possible that, although the enzyme molecules are synthesized *de novo*, residues of azetidine-2-carboxylic acid are not incorporated into these proteins. The present evidence cannot refute this view but, since replacement of proline by azetidine-2-carboxylic acid has been observed in a number of very different systems synthesizing protein, it would appear improbable.

The present suggestion that the acid phosphatase under investigation is formed by the activation of a preformed protein differs from the conclusion of Young and Varner[1] regarding the phosphatase of peas. However, there is no certainty that the same enzyme was under investigation because Roberts[10] has shown that the phosphatase activity of wheat plants may consist of a complex of different enzymes showing quite similar substrate specificities. Until more details are available concerning individual enzymes of this complex, the two investigations should not be considered to have produced mutually contradictory results.

One rather surprising physiological fact emerges from this investigation, namely that the development of enzyme activities in cotyledons of seedlings apparently continues unimpaired even though the growth of radicles ultimately may become greatly inhibited. Water imbibition and the onset of the germination process trigger off processes in the cotyledons, which, once started, do not seem to be co-ordinated with the growth of the embryo.

EXPERIMENTAL

Plant materials. Acid phosphatase activity was determined in seeds of mung bean (green variety), pea (var. Laxton's superb), cucumber (Carter's var. Best-of-All), and sunflower (Carter's). Isocitritase studies used cucumber and pumpkin (Carter's var. King of the Mammoth) seed.

Growth of seedlings. Batches of seed were soaked at 26° for 18 hr either in water or in L-azetidine-2-carboxylic acid solutions. The volume of liquid was limited so that complete uptake was effected in this time. The seedlings were then planted in moistened vermiculite maintained at 26° in the dark until harvested for enzymic activity determinations.

Radicle fresh weights were determined after 72 hr growth and these were used as an index of the growth inhibition caused by azetidine-2-carboxylic acid. The degree of inhibition produced by the imino acid is known to vary between species being dependent in part upon the endogenous amounts of free proline present in different types of seed.[3]

Enzyme assays. Normally, enzymic activities were determined in extracts of cotyledons removed from seedlings at different stages of development. Isocitritase activity is known to be negligible in tissues having low fat contents[11] and therefore no determinations were made with radicles of cucumber or pumpkin. Acid phosphatase activity was determined occasionally in radicles detached from mung bean seedlings.

Assay of acid phosphatase. Cotyledons were macerated for 2 min in ice-cold 0·05 M KH phthalate-KOH buffer (pH 6·0) using a Nalco homogenizer (Measuring and Scientific Equipment Ltd., London). The slurry was squeezed through muslin and centrifuged at 2° for 30 min at 12,000 *g*. Acid phosphatase activities were determined using the clear supernatant fractions obtained.

[9] S. Neale and H. Tristram, *Proc. Intern. Cong. Biochem.* N.Y. (1964).
[10] D. W. A. Roberts, *J. Biol. Chem.* **219**, 711 (1956).
[11] W. D. Carpenter and H. Beevers, *Plant Physiol.* **34**, 403 (1959).

The reaction mixtures contained: 0·8 ml *p*-nitrophenyl phosphate solution (0·05 M, pH 6·0), 0·5 ml supernatant fraction (diluted × 10), and 2·7 ml KH phthalate-KOH buffer (0·05M, pH 6·0). Normally, phosphatase activity was allowed to proceed at 30° for 1 hr; the rate of *p*-nitrophenol production was constant over this period. Enzyme action was stopped by the addition of 0·1 N NaOH (6 ml). *p*-Nitrophenol formation was determined by measuring extinctions at 450 mμ and comparing them with a standard colour curve obtained using a Unicam S.P. 1400 spectrophotometer.

Assay of isocitritase. Cotyledons were macerated in ice-cold 0·05 M KH_2PO_4–K_2HPO_4 buffer (pH 7·5) and a supernatant fraction was prepared as above.

The reaction mixtures contained: 0·6 ml sodium DL-isocitrate (0·01 M), 0·3 ml $MgCl_2$ (0·1 M), 0·3 ml reduced glutathione (0·05 M), 1 ml supernatant fraction, 3 ml K phosphate buffer (0·2 M, pH 7·5), and water to a final volume of 9 ml. When older seedlings were used, the supernatant was diluted × 2 or × 4 before use. The amounts of reaction occurring at 30° after 30, 45, 60, 75 and 100 min was determined by withdrawing 1·5 ml portions from the mixtures for determination of glyoxylic acid production. In this way a mean reaction rate was determined for each preparation from the gradients of graphs relating glyoxylate production with time.

The quantity of glyoxylate produced was determined after pipetting the 1·5-ml samples into 0·6 ml of 100% (w/v) trichloroacetic acid solution to terminate the enzymic reaction. Dinitrophenylhydrazine (0·1% in 2 N HCl, 1·2 ml) was added to each sample and the mixtures were kept for 30 min to complete the formation of the dinitrophenylhydrazone derivative of glyoxylic acid. Finally, 2·5 N NaOH (3 ml) was added and, after clarifying by centrifuging, glyoxylic acid production was calculated from measurements of extinctions at 445 mμ after comparison with a standard colour curve.

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