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AMINO ACID-ACTIVATING ENZYMES IN GERMINATING PEA SEEDLINGS

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Abstract—The properties and distribution of the amino acid-activating enzymes in young pea plants have been investigated. In leaves, the major activity was in the soluble fraction of chloroplasts and in the supernatant fraction of intact tissue homogenates. In embryonic root tissue most activity was in the microsomal and supernatant fractions. The increased activity of these enzymes during germination is largely due to *de novo* synthesis rather than activation.

Simazine, Atrazine, Diquat, 2,6-dichlorobenzonitrile, chloramphenicol, *p*-fluorophenylalanine and gibberellic acid had no effect on the activity of partly purified systems. Ribonuclease appeared to render the enzyme less precipitable at pH 5·0 rather than to inhibit the enzymatic reaction.

A short exposure to red light stimulated equally growth and activity of the amino-acid-activating system in etiolated seedlings; the effect was nullified by subsequent exposure to far-red light. If seedlings were treated with red light 1 hr before being brought into the light then no significant effect on the enzyme system could be observed.

INTRODUCTION

IT IS now well established that the first step in the biosynthesis of proteins is the activation of amino acids with the formation of amino acid–*S*-RNA complexes.[1] The overall reaction consists of two steps:

Enz.+amino acid+ATP ⇌ Enz.–amino acid–AMP+P–P
Enz.–amino acid–AMP+*S*-RNA ⇌ Enz.+amino acid–*S*-RNA+AMP

It can be followed by measuring either formation of *S*-RNA, ATP P–P exchange, or formation of amino acid hydroxamate. In the present work the last method has been used.

Considerable information is available regarding amino acid-activating enzymes (amino acyl-ribonucleic acid synthetases) in animal tissues and in micro-organisms [see e.g. Berg[2]] but much less is known about these enzymes in higher plants. They have been detected in plant tissue by several workers[3–9] and Bové and Raacke[10] showed that the enzyme of *Spinacea oleracea* was present in isolated chloroplasts.

Although no amino acid-activating enzyme has yet been fully purified, it is probable that each L-amino acid which normally occurs in proteins has its own specific activating enzyme;

[1] G. C. WEBSTER, *Ann. Rev. Plant Physiol.* **12**, 113 (1961).
[2] P. BERG, *Ann. Rev. Biochem.* **30**, 293 (1961).
[3] J. M. CLARK, *J. Biol. Chem.* **233**, 421 (1958).
[4] J. W. DAVIS and G. D. NOVELLI, *Arch. Biochem. Biophys.* **75**, 299 (1958).
[5] A. MARCUS, *J. Biol. Chem.* **234**, 1238 (1959).
[6] G. C. WEBSTER, *Arch. Biochem. Biophys.* **82**, 125 (1959).
[7] Y. HAYASHI, *Virology* **18**, 140 (1962).
[8] E. MOUSTAFA and M. H. PROCTOR, *Biochim. Biophys. Acta* **63**, 93 (1962).
[9] E. MOUSTAFA and J. W. LYTTLETON, *Biochim. Biophys. Acta* **68**, 45 (1963).
[10] J. BOVÉ and I. D. RAACKE, *Arch. Biochem. Biophys.* **85**, 521 (1958).

this has been demonstrated for L-methionine, L-valine, L-isoleucine, L-leucine,[11] L-tryptophan,[12] L-tyrosine, L-threonine,[13] L-alanine,[14] L-serine,[15] L-arginine[16] and L-aspartate.[17]

The work reported here represents an investigation into the properties and distribution of amino acid-activating enzymes in relation to germination in pea seedlings.

RESULTS AND DISCUSSION

General Properties of the Amino acid-activating Enzyme System

Previous investigations on animals and micro-organisms have shown that the amino acid-activating system was located in the soluble fraction of the tissues and that the major part

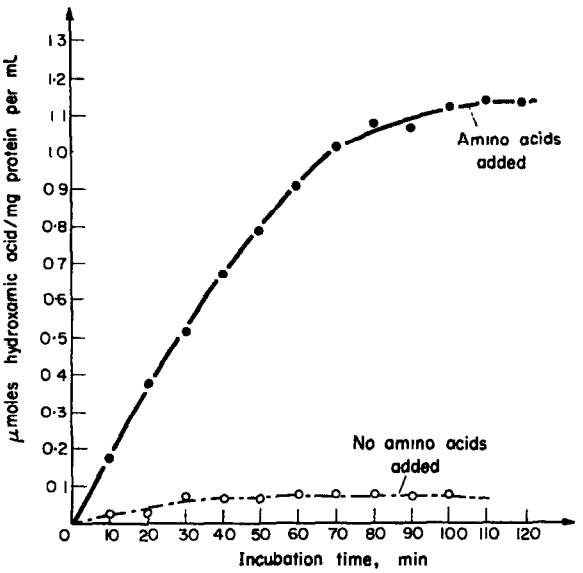

FIG. 1. TIME CURVE FOR AMINO ACID-ACTIVATING ENZYME ACTIVITY OF PEA ROOTS.

Standard assay conditions as described under "Experimental".
●, with amino acids; ○, no amino acids.

of the activity was concentrated in the protein fraction precipitated at pH 5·2. This was confirmed with an extract of the first 5 mm of the roots of 2-day-old seedlings.

The roots were dropped into a phosphate (0·1 M) buffer solution pH 7·4 which also contained sucrose (0·5 M), EDTA (0·01 M) and $MgCl_2$ (0·01 M). The mixture was cooled to 4°, ground in a pre-cooled mortar with acid-washed silver sand, filtered through eight layers of cheese-cloth and centrifuged at 200 *g* for 5 min. The supernatant was then centrifuged at 0° at 14,000 *g* for 60 min, and the resulting supernatant brought to pH 5·2 by addition of HCl (0·1 N) dropwise with constant stirring. The white flocculent precipitate was centrifuged at

[11] F. H. BERGMANN, P. BERG and M. DIECKMANN, *J. Biol. Chem.* **236**, 1735 (1961).
[12] E. W. DAVIE, V. V. KONIGSBERGER and F. LIPMANN, *Arch. Biochem. Biophys.* **65**, 21 (1956).
[13] R. W. HOLLEY, E. W. BRUNNGRABER, F. SAAD and H. W. WILLIAMS, *J. Biol. Chem.* **236**, 197 (1961).
[14] G. C. WEBSTER, *Biochim. Biophys. Acta* **49**, 141 (1961).
[15] L. T. WEBSTER and E. W. DAVIE, *J. Biol. Chem.* **236**, 479 (1961).
[16] H. G. BOMAN, I. A. BOMAN and W. K. MASS in *Biological Structure and Function* (Edited by T. W. GOODWIN and O. LINDBERG), Academic Press, London (1961).
[17] E. MOUSTAFA and G. PETERSON, *Nature* **196**, 377 (1962).

10,000 *g* for 5 min and the supernatant discarded. The precipitate was suspended in 0·1 M Tris buffer pH 7·4 and diluted to a concentration of 10 mg protein/ml.

With preparations prepared in this way activation of amino acids increased linearly with time up to 80 min (Fig. 1). The specific activity (μmoles hydroxamic acid/mg protein/hr) of the preparations was usually of the order of 1·0 under the standard experimental conditions employed[12,18,19] (see Experimental section). The reaction rates observed under these conditions approached the maximum obtainable. When L-alanine was substituted for the mixture of fifteen amino acids* routinely used in the assay, a 40 mM solution gave the maximum reaction rate.

The pH activity curve of the pH 5·2 preparation from pea root tissue is rather flat between pH 5·0 and 7·0 with a maximum at 5·4 (Fig. 2). The unsymmetrical nature of the curve is almost certainly due to the fact that the various specific amino acid-activating enzymes present in the preparation have different optimum pH values.

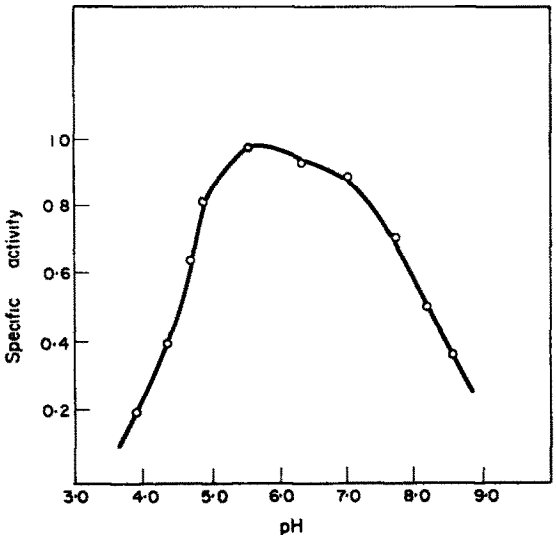

FIG. 2. THE pH-ACTIVITY CURVE FOR THE AMINO ACID-ACTIVATING ENZYMES IN PEA ROOTS.

Standard assay conditions but with pH of the Tris altered to the appropriate value.

The activity of fractions of root extracts precipitated between the pH ranges 6·0–7·0, 5·0–6·0 and 4·0–5·0 were therefore compared for their ability to activate five individual amino acids, methionine, tryptophan, isoleucine, valine and alanine; these amino acids were chosen because in pilot experiments it was found that they were activated to a greater extent than other amino acids.

Fractionation of the crude extract was carried out by adjusting the pH to the required value, collecting the precipitate by centrifugation at 0° at 15,000 *g* and resuspending it in 0·1 M Tris buffer at 7·4. Incubation and colour development were carried out under standard conditions. Figure 3 shows that whilst the enzymes concerned with the activating of methionine, isoleucine and alanine have a very similar distribution, more tryptophan-activating

* Listed in Table 4.

[18] M. B. HOAGLAND, *Biochim. Biophys. Acta* **16**, 288 (1955).

[19] J. A. DEMOSS and G. D. NOVELLI, *Biochim. Biophys. Acta* **18**, 592 (1955).

enzyme is precipitated at relatively higher pH values and relatively more valine-activating enzyme is precipitated at lower pH values. It is interesting in this connexion to note that the valine-activating enzyme from wheat germ has very recently been purified some 300 fold by Moustafa;[20] it has a pH optimum of 7·1.

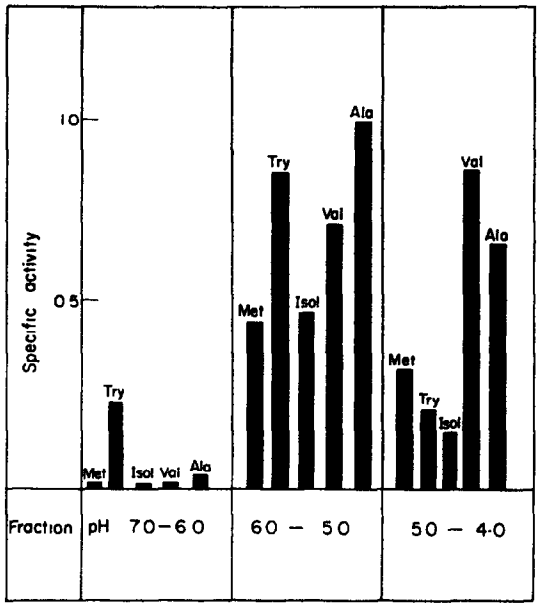

FIG. 3. THE DISTRIBUTION OF FIVE AMINO ACID (METHIONINE, TRYPTOPHAN, ISOLEUCINE, VALINE, AND ALANINE) ACTIVATING ENZYMES IN THE FRACTIONS FROM PEA ROOT TISSUE EXTRACTS PRECIPITATED OVER THE pH RANGES 4·0–5·0, 5·0–6·0 and 6·0–7·0.

Distribution of Amino Acid-activating Enzymes in Pea Tissues

(*a*) *In subcellular fraction of leaves.* Leaves from plants grown in a greenhouse in a soil–sand compost mixture for 14 days were used, because at this stage of growth they were reasonably free from hard, fibrous tissue. The subcellular fragments were prepared as shown in Table 1 and as described in the experimental section. To minimize contamination by soluble components, each particulate fraction was washed three times with 0·1 M Tris buffer at 4°. The pH 5·2 enzyme was then extracted from each particulate fraction as described in the previous section. Table 2 demonstrates that essentially all the chloroplast activity is associated with the soluble fraction; no activity could be detected in the washed grana. In this aspect the distribution resembles that in the cytoplasmic membranes of *Bacillus megaterium* in which most of the enzyme activity is in the membrane fraction but sonication of the membrane releases the bulk of the enzymes in soluble form.[21] There is considerable activity in the microsome fraction and none in the mitochondria. The high activity in the microsome fraction may be due to the presence of plastids and plastid primordia. It is not possible to say with certainty whether any of the considerable activity in the supernatant fraction is due to the leaking out of enzyme from subcellular particles. The fact that chloroplast fragments still contain activity suggest that the amino acid-activating enzymes are bound in some way to a surface and do not leak out on preparation of chloroplasts.

[20] E. MOUSTAFA, *Biochim. Biophys. Acta* 76, 280 (1963).
[21] G. D. HUNTER, P. BROOKES, A. R. CRATHORN and J. A. V. BUTLER, *Biochem. J.* 73, 369 (1959).

TABLE 1. THE PREPARATION OF SUBCELLULAR FRACTIONS OF LEAF TISSUE

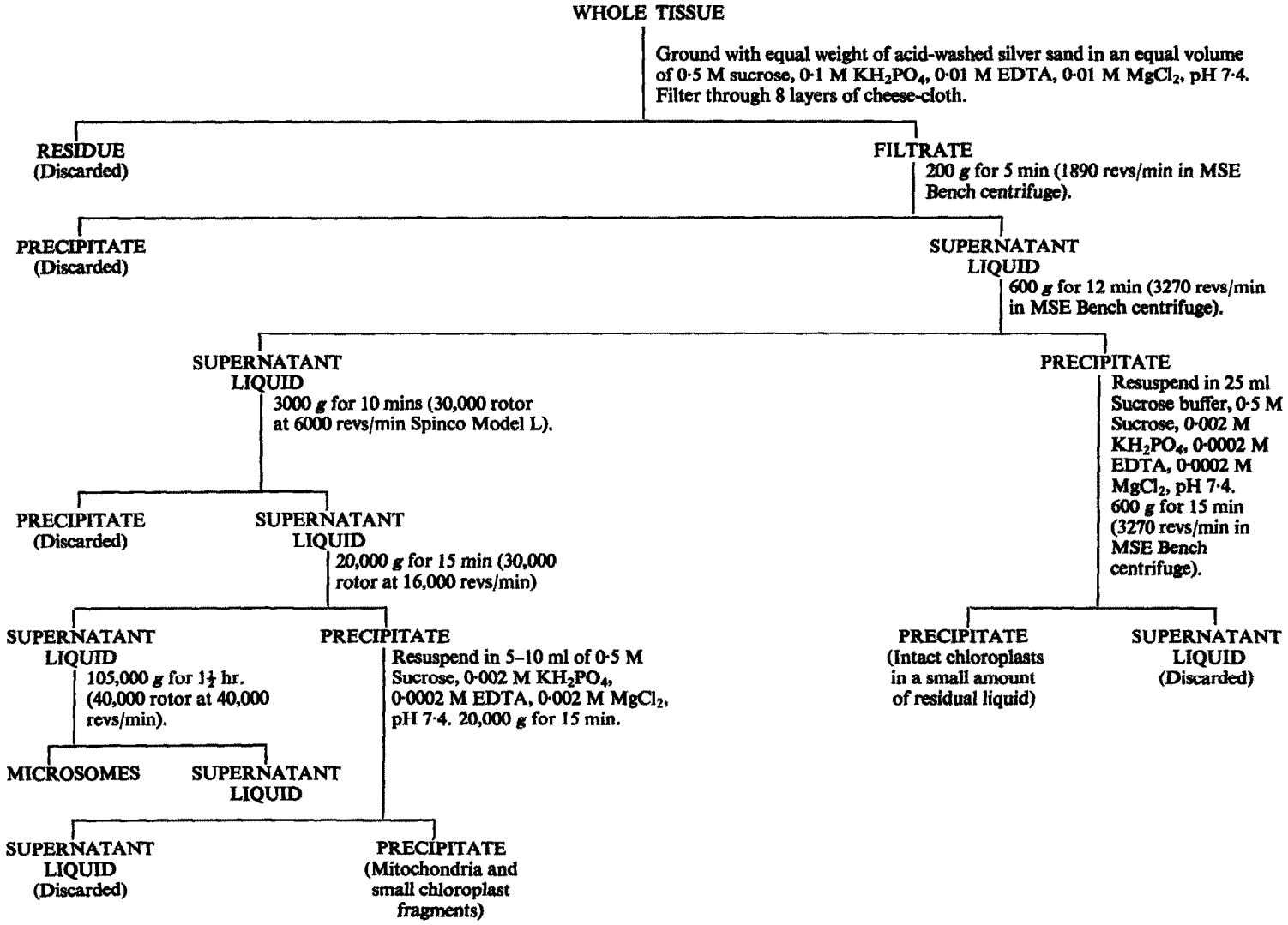

TABLE 2. SUBCELLULAR DISTRIBUTION OF AMINO ACID-ACTIVATING ENZYMES IN PEA LEAVES*

Subcellular fraction	Protein content of extract (mg/ml)	Specific activity†
Whole chloroplasts	25·0	0·23
Chloroplast fragments	17·0	0·23
Washed grana	23·0	trace
Chloroplast supernatant	3·2	0·87
Mitochondria	3·0	0·0
Microsomes	2·1	0·83
Supernatant from whole-cell homogenate	6·0	0·66

* Mean of five experiments; average differences ±15%.
† Corrected for endogenous activity which varied from zero (mitochondria) to 0·27 (microsomes).

(*b*) *In subcellular fractions of embryo root tissue.* Embryo root tissue was then examined. Peas (200 for each experiment) were soaked overnight and the embryos dissected out and sliced with a fine scalpel under 0·1 M $CaCl_2$. They were allowed to stand for 30 min at 4° and then ground up with a glass mortar and Teflon pestle. The various fractions were then prepared as

TABLE 3. SUBCELLULAR DISTRIBUTION OF AMINO-ACTIVATING ENZYMES IN PEA EMBRYO ROOT TISSUE*

Subcellular fraction	Protein content of of extract (mg/ml)	Specific activity†
Mitochondria	5·7	0
Microsomes	10·2	0·38
Supernatant	3·0	0·73
Nuclei	2·3	0·06
Nucleoli	1·5	trace

* Mean of ten experiments: average differences ±15%.
† Corrected for endogenous activity which varied from 0·16 (microsomes) to 0·22 (cytoplasm).

described under "Experimental" and analysed for amino acid-activating enzymes. Table 3 summarizes a number of experiments and shows that under the conditions specified the supernatant contains most of the activity. The microsomes are much less active than leaf microsomes, which tends to support the view that some of the activity in leaf microsomes is due to the presence of plastids and plastid primorida. No significant activity was observed in mitochondria, or nuclei or nucleoli; this appears to agree with the situation in animal tissues

where no activity is reported in mitochondria,[22] but differ from it in that animal nuclei have considerable activity.[23] On sonication of mitochondria, however, activity reappears.[22]

(*c*) *In different tissues.* In order to see if the "spectrum" of amino acid-activating enzymes differed in tissues with different physiological function, the activity of pH 5·0 extracts of leaf stem and root for 15 amino acids was examined. No significant differences were recorded (Table 3) and the quantitative similarity between the three tissues is extremely close.

TABLE 4. THE ACTIVITY OF AMINO ACID-ACTIVATING ENZYMES OF LEAF, STEM AND ROOT FOR 15 AMINO ACIDS*

Amino acid	Specific activity		
	Leaf	Stem	Root
Alanine	0·85	0·79	0·83
Arginine	0·42	0·44	0·44
Asparagine	—	0·18	—
Glycine	0·35	0·30	0·35
Histidine	0·21	0·18	0·20
Isoleucine	0·80	0·79	0·83
Leucine	0·20	0·20	0·22
Lysine	0·40	0·42	0·41
Methionine	0·88	0·80	0·86
Phenylalanine	0·42	0·37	0·42
Proline	0·22	0·28	0·30
Threonine	—	—	—
Tryptophan	0·90	0·80	0·91
Tyrosine	0·56	0·58	0·54
Valine	0·75	0·77	0·80

* pH 5 extracts prepared in the usual way and assayed under standard conditions with the amino acid mixture replaced by individual DL-amino acids.

Amino Acid-activating Enzymes and Germination

The specific activity of the activating enzymes in the cotyledons of peas has been followed over the first 8 days of germination together with that of the protease enzyme for comparison. Figure 4 demonstrates that the activity of the amino acid-activating enzyme increases considerably whilst that of protease remained essentially constant.

The next aim was to decide whether the increase in amino acid-activating enzymes was due to protein synthesis or not. The effect of the two specific inhibitors of protein synthesis *p*-fluorophenylalanine[24] and chloramphenicol[25,26] on both the amino acid-activating system and protease was examined by allowing the seeds to germinate in 1×10^{-6} M solutions of the inhibitors instead of water. Although germination rate and growth were reduced (Fig. 5), sufficient material was obtained to examine the enzyme activities; the activity of protease

[22] V. M. CRADDOCK and M. V. SIMPSON, *Biochem. J.* **80**, 348 (1961).
[23] J. W. HOPKINS, *Proc. Nat. Acad. Sci. U.S.* **45**, 1461 (1959).
[24] E. F. GALE and J. P. FOLKES, *Biochem. J.* **53**, 493 (1953).
[25] C. L. WISEMAN, F. E. HAHN, H. HOPPS and J. E. SMADEL, *Federation Proc.* **12**, 466 (1953).
[26] G. C. WEBSTER, *Arch. Biochem. Biophys.* **82**, 125 (1959).

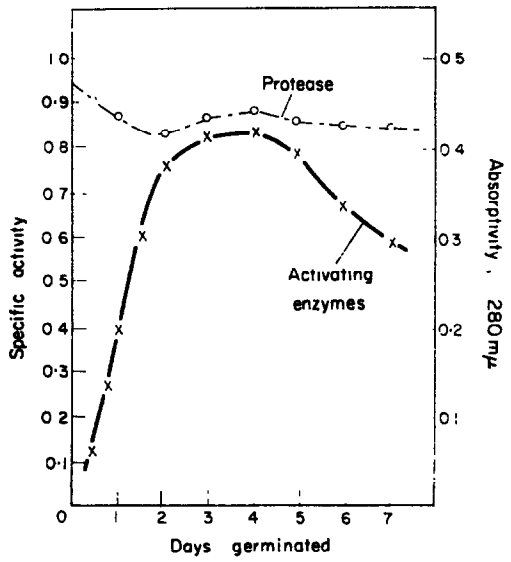

FIG. 4. THE CHANGE WITH TIME IN THE PATTERN OF ENZYME ACTIVITY IN THE COTYLEDONS OF GERMINATING PEAS.

X, amino acid-activating enzyme (left-hand ordinate); ○, protease (right-hand ordinate).

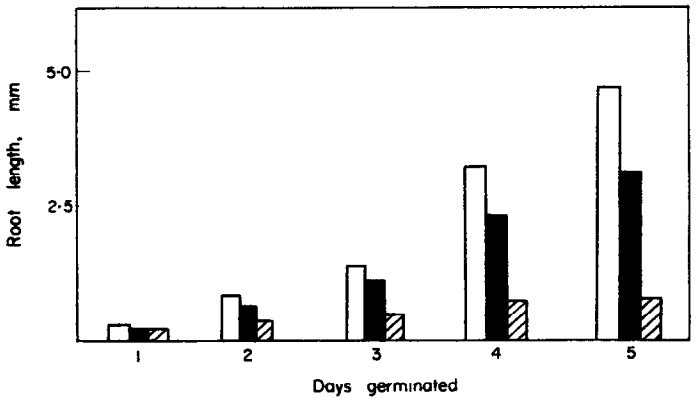

FIG. 5. THE EFFECT OF *p*-FLUOROPHENYLALANINE AND CHLORAMPHENICOL ON GROWTH OF PEA SEEDLINGS.

□, water; ■, *p*-fluorophenylalanine; ■, chloramphenicol.

was unaffected by either inhibitor (Fig. 6) whilst that of the amino acid-activating enzyme remained at a low level throughout the period (Fig. 7). As *p*-fluorophenylalanine does not inhibit the activity of amino acid activating enzymes *per se* (Table 5) it must be concluded that (*a*) the increased activity of these enzymes during germination is due to to a *de novo* synthesis of protein and (*b*) *p*-fluorophenylalanine inhibits protein synthesis at a step other than the first.

Although this represents strong evidence for enzyme synthesis rather than activation, it was decided to see if more enzyme could be extracted from sonicated microsomes than from

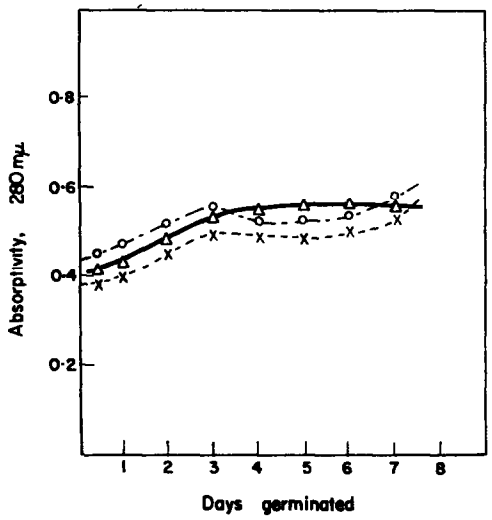

FIG. 6. THE EFFECT OF *p*-FLUOROPHENYLALANINE AND CHLORAMPHENICOL ON THE PROTEASE ACTIVITY OF GERMINATING PEA SEEDLINGS.

○, water; △, *p*-fluorophenylalanine; X, chloramphenicol.

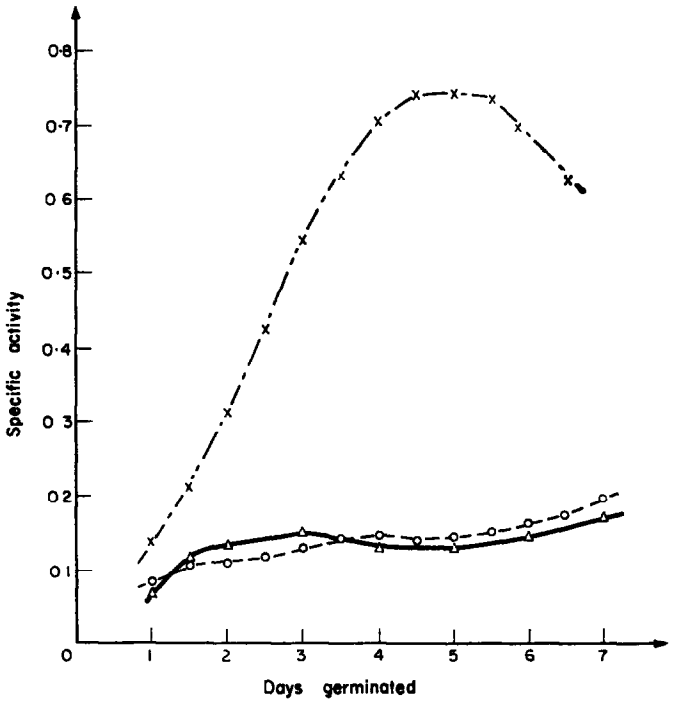

FIG. 7. THE EFFECT OF *p*-FLUOROPHENYLALANINE AND CHLORAMPHENICOL ON THE AMINO ACID-ACTIVATING SYSTEM OF GERMINATING PEA SEEDLINGS.

X, water; △, *p*-fluorophenylalanine; ○, chloramphenicol.

TABLE 5. THE EFFECT OF *p*-FLUOROPHENYLALANINE ON THE ACTIVITY OF THE AMINO ACID-ACTIVATING SYSTEM OF PEA SEEDLINGS

Concentration *p*-fluorophenylalanine (μg/ml)	Enzyme activity (specific activity)	
	Normal system	System preincubated with *p*-fluorophenylalanine for 10 min at 30°
0	0·68	0·67
1	0·68	0·67
5	0·74	0·72
10	0·75	0·71
15	0·76	0·72

untreated microsomes; that is if the observed synthesis was due to liberation during germination of a bound enzyme which could not be extracted by our usual procedure. Douglas and Munro[27] had shown that this happens with pancreatic amylase. Microsomes were prepared from cotyledons at various times after germination. The pellet was resuspended in 0·1 M Tris buffer pH 7·4 containing 0·1 mM $MgCl_2$ and divided into two portions, one was assayed for

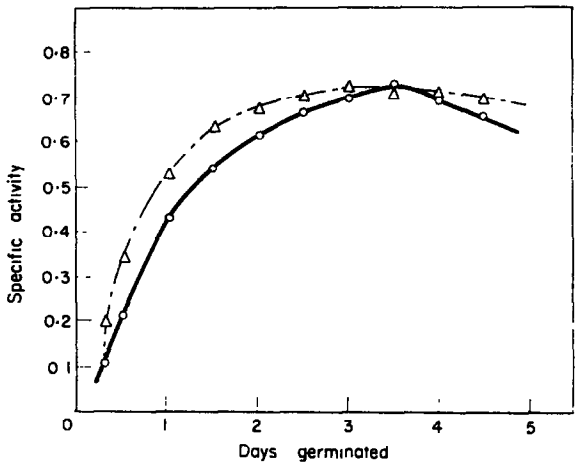

FIG. 8. THE AMOUNT OF ENZYME EXTRACT FROM SONICATED AND NON-SONICATED MICROSOMES FROM PEA COTYLEDONS.

△, sonicated; ○, untreated.

amino acid activating enzymes in the usual manner whilst the other was sonicated in a MSE-Mullard sonic disintegrator before examination. The suspension was cooled in ice and exposed to three bursts (20, 20, 10 sec) with a pause of 10 sec between each exposure. The pH 5 fraction was then prepared in the usual manner. Figure 8 shows that in the early stages of germination sonication does increase the amount of enzyme present until after 4 days the difference between the extract from the sonicated and non-sonicated microsomes disappears.

[27] T. A. DOUGLAS and H. N. MUNRO, *Expt. Cell Res.* **16**, 148 (1960).

Although there is therefore some liberation of bound enzyme, this is insufficient to account for all the increase in activity observed during germination.

Action of Some Plant Growth Inhibitors on the Amino Acid-activating Enzymes

The triazine herbicides simazine and atrazine, 2,6-dichlorobenzonitrile and diquat were examined for their effect on the activity of the amino acid-activating enzymes. Each herbicide at concentrations up to 20 ppm was examined either by adding it directly to the enzyme assay system or by preincubating it with the enzyme for 10 min at 30° prior to the addition of substrate. In no case was any inhibitory effect observed, so the detailed results are not recorded.

The effect of ribonuclease on the enzyme activity as measured by hydroxamate formation was also examined because Ogata *et al.*[28] showed that this material inhibited the enzyme activity of guinea-pig liver extracts when measured by PP–ATP exchange. They suggested

TABLE 6. THE EFFECT OF PREINCUBATION OF THE pH 5 FRACTION WITH RIBONUCLEASE (1·5 μg/ml) FOR 10 MIN AT 30° FOLLOWED BY REPRECIPITATION OF THE FRACTION

Specific activity of enzyme preparations*		
Control	Ribonuclease treated and reprecipitated	Ribonuclease treated and reprecipitated +1 mg original pH 5 fraction
0·87	0·58	0·79
0·75	0·47	0·70
0·66	0·42	0·61
0·72	0·50	0·69

* Results from four separate experiments.

that this is due to the decreased precipitability of the ribonuclease-treated protein. Table 6 shows that a similar effect is observed with the plant enzyme system. Incubation with ribonuclease (1·5 μ/ml/10 min 30°) reduced the activity of the pH 5 fraction after reprecipitation by some 35 per cent. Addition of a small amount of unprecipitated fraction returns the activity essentially to normal. If the enzyme is incubated with ribonuclease and assayed before precipitation then reduction in specific activity is slight. As the specific activity is reduced on precipitation it seems reasonable to conclude, with Ogata *et al.*, that more inactive than active enzyme is precipitated after ribonuclease treatment.

The Effect of Gibberellic Acid on Amino Acid-activating Agents

Seeds were soaked overnight in a solution (5 ppm) of gibberellic acid and then allowed to germinate. The activity of the pH 5 fraction of the resulting plants was some 5–8 times greater than that from normal seedlings. However, gibberellic acid added to the assay system was without effect on the enzyme. Incidental to this work, it was confirmed that the concentration of the amino acids in the soluble fraction of tissue extracts was considerably higher in

[28] K. OGATA, H. NOHARA, K. ISHIKAWA, T. MORITA and H. ASAOKA, in *Protein Biosynthesis* p. 163 (Edited by R. J. C. HARRIS) Academic Press, New York (1961).

gibberellic acid-treated plants than in normal plants. This was first observed by Herich.[29] We also demonstrated by paper chromatography that qualitatively the extracts were the same in both cases. These observations lend some support to the view of Mariani *et al.*[30] that the activity of the amino acid-activating enzymes is controlled by the level of free amino acids in the cell.

The Effect of Red and Far-red Light

Investigations by Hendricks and his co-workers[31] on the photocontrol of a number of developmental activities of light-sensitive seedlings have indicated that the photoreceptor for

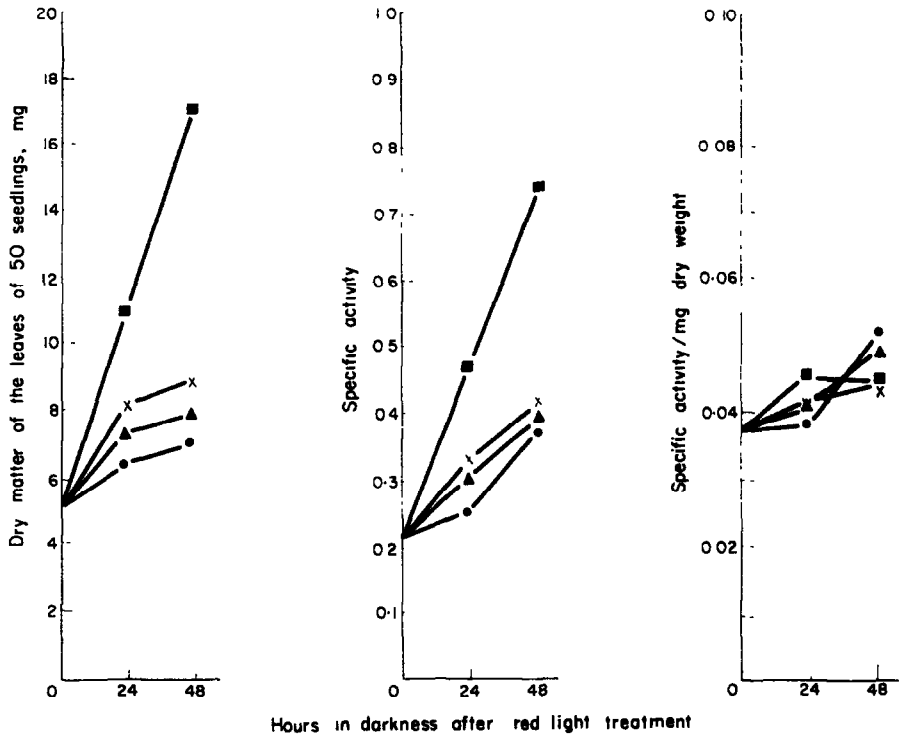

FIG. 9. THE EFFECT OF RED AND FAR-RED LIGHT ON THE AMINO ACID-ACTIVATING ENZYMES OF ETIOLATED PEA SEEDLINGS.

■, red; X, red and far-red; ▲, far-red; ●, control.

these reactions is phytochrome, a water-soluble chromoprotein. A major characteristic of all phytochrome-mediated reactions is that a small dose of red light (660 mμ) stimulates responses whilst a following dose of far-red light (780 mμ) nullifies the effect.

The synthesis of chlorophyll is a phytochrome-controlled reaction. When etiolated barley seedlings are brought into the light there is a lag period of some hours before chlorophyll synthesis began; if the seedlings are given a short dose of red light 4–6 hr before illumination

[29] R. HERICH, *Physiol. Plant.* **14**, 111 (1961).
[30] A. MARIANI, M. A. SPADONI and G. TOMASSI, *Nature* **199**, 378 (1963).
[31] S. B. HENDRICKS, in *Comparative Biochemistry of Photoreactive Systems* (Edited by M. B. ALLEN) Academic Press, New York (1962).

then the lag period is abolished.[32] Later workers confirmed these observations and showed that illumination with far-red light nullified the effect.[33,34] These results implied the stimulation of, and thus synthesis of, enzymes. The effect of red and far-red light on the amino acid-activating enzyme of etiolated pea seedlings was therefore examined.

Figure 9 demonstrates that if 4-day seedlings are exposed to 10 min of red light, then 24 and 48 hr after there has been a marked stimulation of enzyme activity in leaf tissue compared with control seedlings which were not exposed to red light. There is a concomitant increase in growth (dry weight) and this results in little difference between the enzyme activity per unit dry weight for red light treated and control seedlings. The stimulatory effect of red light on enzyme activity and growth is nullified by a following exposure to far-red light. These effects are very similar to those reported for growth and carotenoid synthesis in maize coleoptiles.[35]

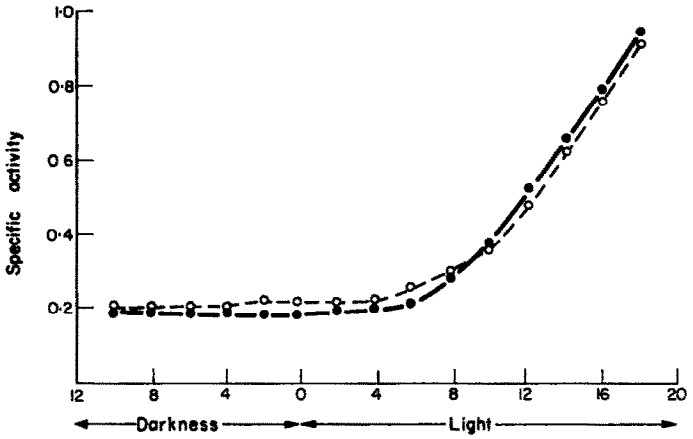

FIG. 10. THE EFFECT OF A SHORT EXPOSURE (10 MIN) TO RED LIGHT (660 mμ) ONE HOUR BEFORE DARK-GROWN SEEDLINGS ARE CONTINUOUSLY ILLUMINATED WITH WHITE LIGHT, ON THE RATE OF SYNTHESIS OF THE ENZYME-ACTIVATING SYSTEM.

●, control; ○, red.

A dose of red light 1 hr before etiolated seedlings are brought into white light has no significant effect on the lag period for the synthesis of enzyme systems (Fig. 10); however, in simultaneous experiments, to be published elsewhere, it was found that it does reduce the lag period for the initiation of chlorophyll synthesis.

The failure to observe stimulation was probably due to the overwhelming effect of white light on the development of the chloroplast and thus on the synthesis of amino acid-activating enzymes; the red-light effect would thus be swamped.

EXPERIMENTAL

Peas. "Pilot" peas (Carters Ltd., Raynes Park, Middlesex) were used throughout. Considerable difficulty was encountered in germinating the peas without excessive fungal contamination. Eventually the simplest method tried was found to be the most effective.

[32] H. I. VIRGIN, *Physiol. Plant.* **10**, 445 (1957); **11**, 347 (1958).
[33] K. MITRAKOS, *Physiol. Plant.* **14**, 497 (1961).
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The seeds were shaken in a jar with a commercial anti-fungal preparation (Seed-Saver, I.C.I. Ltd.) and when well coated with the fungicide they were planted ½ in. deep in vermiculite in trays and then moistened with distilled water. The water content of the trays (3:1, vermiculite: water) was kept constant by daily weighing. The seeds grew well at the guaranteed germination rate (80 per cent). Before the germinated peas were used in an experiment, the adhering fungicide was washed off with deionized water and the plants were dried by gentle shaking in layers of cheese-cloth.

Plant growth inhibitors. Simazine, atrazine, and 2,6-dichlorobenzonitrile were presented by the Shell Chemical Co. Ltd.; diquat was presented by Plant Protection Ltd.

Cell Fractionation

The tissues were ground with acid-washed silver sand in a pre-cooled mortar under one volume of a sucrose–phosphate buffer (0·5 M sucrose, 0·1 M KH_2PO_4, 0·01 M EDTA, 0·01 M $MgCl_2$) pH 7·2. The homogenate was then separated into subcellular fractions by a system of differential centrifugation (Table 1) based on the methods of Holm-Hansen *et al.*[36] and Park and Pon.[37]

Resuspension of the various pellets in the appropriate buffer solution was effected with a glass homogenizer fitted with a "Teflon" pestle having a clearance of 1·0 mm.

The fraction labelled "intact chloroplasts" (Table 6) was further purified by the simplified density gradient technique of James and Das.[38] The green layer obtained by this method contained over 95 per cent of intact chloroplasts when viewed in the phase-contrast microscope; the unpurified fraction contained only 50 per cent of intact chloroplasts.

Nuclei and nucleoli were prepared by the methods of Johnston *et al.*[39, 40]

Disintegration of Chloroplasts

An MSE-Mullard 20KC ultrasonic disintegrator was used. A suspension (5 ml) of chloroplasts was pipetted into a 25 ml centrifuge tube which was surrounded by iced water. The probe was immersed to a depth of 1–2 mm and the suspension sonicated in three bursts of 45 sec with an interval of 30 sec between each burst. The suspension was then centrifuged at 20,000 *g* for 20 min; the supernatant was only slightly contaminated with membranes and grana when viewed in the phase-contrast microscope.

Protein Determinations

Determination of soluble protein was carried out by the method of Lowry *et al.*[41]

The micro-Kjeldahl method was used for microsomal pellets and the ammonia produced determined by the method of Conway.[42]

Assay of Activating Enzymes

The method used was that of Schweet *et al.*[43] with some minor modifications. The reaction mixture usually contained 10 μmoles each of 15-DL-amino acids (listed in Table 4), 110

[36] O. HOLM-HANSEN, N. G. PON, K. NISHIDA, V. MOSES and M. CALVIN, *Physiol. Plant.* **12**, 475 (1959).
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[41] O. H. LOWRY, N. J. ROSEBROUGH, L. A. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[42] E. J. CONWAY, *Microdiffusion Analysis and Volumetric Error*, Crosby, Lockwood & Son Ltd., London (1957).
[43] R. S. SCHWEET, R. W. HOLLEY and E. H. ALLEN, *Arch. Biochem. Biophys.* **71**, 311 (1957).

μmoles of Tris buffer pH 7·1, 30 μmoles of ATP neutralized to pH 7·1 with 0·01 N KOH, 15 μmoles $MgCl_2$, 3 m-moles of hydroxylamine neutralized just before use with KOH to pH 7·0, 0·5 ml enzyme extract (10 mg/g) in Tris buffer and water to a final volume of 3 ml. Incubation was for 60 min at 37° with occasional careful shaking. To the reaction mixture was added 1·4 ml 100% trichloracetic acid (pH 0·9) and 0·6 ml of 2 M $FeCl_3$. The protein precipitate was removed by centrifugation and after 15–20 min the absorptivity at 520 mμ was measured in a spectrophotometer.

In order to construct a standard graph for the reaction, glycine hydroxamic acid was prepared by the method of Safir and Williams.[44] The crystalline material had a m.p. 142° (Safir and Williams quote 140°) and on chromatograms ran essentially as one spot, although a very faint ninhydrin-positive spot could be detected which ran in front of the hydroxamate spot. Recrystallization did not remove this trace impurity.

Assay of Protease Activity

The 140,000 *g* supernatant (2 ml) was added to 2% casein (pH 7·1) (3 ml) and the mixture incubated for 60 min at 37°. A 2 ml sample was then pipetted into 15% trichloroacetic acid (2 ml) and the optical density at 280 mμ measured. The increase observed over the value obtained with a control measured at zero time was taken as an index of protease activity.[45]

[44] S. R. Safir and J. H. Williams, *J. Org. Chem.* **17**, 1298 (1952).
[45] N. C. Davis and E. L. Smith, *Methods of Biochem. Anal.*, **2**, 215 (1955).