9. It is suggested that Fraction 2 is made up of unbleached parent rhodopsin and a reconstituted constant-composition mixture of rhodopsin and P486. This reconstituted material is produced by the irradiation of an intermediate substance which is unstable even at -40° .

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Adenylic Deaminase of Pea Seeds

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Adenylic deaminase catalyses the deamination of adenosine 5'-monophosphate with the formation of inosine 5'-monophosphate and ammonia. The enzyme was first reported in extracts from rabbit muscle by Schmidt (1928), who showed that it was specific for adenosine 5'-monophosphate as substrate and was distinct from adenosine deaminase. Kalckar (1947) obtained active deaminase preparations from rabbit muscle after precipitating myosin and fractionating the enzyme with ammonium sulphate. Nikiforuk & Colowick (1956) purified adenylic deaminase some 40-fold from crude bicarbonate extracts of rabbit muscle and studied a number of the properties of the enzyme. Separation from myosin and further purification enabled Lee (1957) to crystallize the rabbit-muscle enzyme.

Adenylic deaminase has been found in several other animal tissues such as heart muscle, liver and kidney but the activities are lower than in skeletal muscle (Lee, 1957). The enzyme occurs as a complex with myosin (Kalckar, 1947; Humphrey & Webster, 1951).

The occurrence of the enzyme in a higher-plant tissue was investigated. In the present investigation adenylic deaminase was found in a particulate fraction from pea seeds and a preparation free from interfering enzymes was obtained. A number of properties of the pea-seed adenylic deaminase were studied.

EXPERIMENTAL

Special chemicals. Adenine, adenosine, adenosine 5'-monophosphate (AMP), adenosine diphosphate, adenosine triphosphate, inosine 5'-monophosphate (IMP) and diphosphopyridine nucleotide were obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A., triphosphopyridine nucleotide and *p*-hydroxymercuribenzoate from Sigma Chemical Co., St Louis, Mo., U.S.A., and cysteine hydrochloride from British Drug Houses Ltd.

Preparation of 5'-adenylic acid deaminase from pea seeds. Preliminary experiments showed that the adenylic-deaminase activity was associated with a particulate fraction in pea seeds and the following preparative procedure was adopted. Pea seeds (*Pisum sativum* L., var. Laxton's Progress) were soaked overnight in water and 50 g. of the soaked seeds was blended for 3 min. at 4° with 50 ml. of 40 mM- NaHCO_3 . The homogenate was squeezed through muslin, centrifuged at 500 g for 5 min. and the supernatant centrifuged at 20 000 g for 30 min. The precipitate from the high-speed centrifuging was suspended in 10 ml. of water, centrifuged at 20 000 g for 30 min. and the resultant precipitate was dispersed in water to give a final volume of 5 ml.

The light-green enzyme preparation could be stored at -15° for 5 weeks with 10% loss of deaminase activity. Heating at 60° for 5 min. resulted in 95% loss of activity. No phosphatase activity was detected when samples of reaction mixtures containing the enzyme preparation and AMP or IMP were examined for the formation of inorganic phosphate or, on paper chromatograms, for the production of adenosine or inosine.

Reaction mixtures. The reaction mixtures were maintained at 30° . The determination of enzyme activity was a

modification of the method described by Kalckar (1947). For the assay of 5'-adenylic deaminase the following components were mixed: 1.0 μ mole of AMP, 100 μ moles of Li_2SO_4 , 24 μ moles of succinic acid buffer (brought to pH 6.2 with NaOH) and 0.05 ml. of enzyme (containing approx. 0.4 mg. of protein N); total volume, 0.25 ml. The time of incubation was 30 min. Under these experimental conditions there was not more than 40% conversion of AMP into IMP and the rate of reaction was approximately constant during the incubation period. Control reaction mixtures in which AMP was replaced by IMP were always used.

In studying the effect of inhibitors the reaction was initiated by the addition of AMP after the other components of the reaction mixture had been incubated for 15 min. at 30°.

Samples of the reaction mixtures were inactivated by the addition of perchloric acid (final concn. 3.3%, w/w) for spectrophotometric and inorganic phosphate analyses or by the addition of 2 vol. of ethanol for chromatographic studies. After inactivation the samples were centrifuged at 1000 g for 5 min.

Estimation of adenosine monophosphate and inosine monophosphate. Extracts of the reaction mixtures in perchloric acid soln. were diluted with 30 vol. of water and the extinction at 265 m μ was measured in a Beckman model DU spectrophotometer. Solutions of AMP and IMP were standardized with a molar-extinction index of 15.4×10^3 at 259 m μ and pH 7.0 for AMP (Bock, Ling, Morell & Lipton, 1956) and 12.2×10^3 at 248.5 m μ and pH 6.0 for IMP (Beaven, Holiday & Johnson, 1955). From these solutions, calibrations were prepared for the extinction at 265 m μ of the nucleotides in perchloric acid soln. The results, which are calculated from the decrease in extinction at 265 m μ , are expressed as μ moles of IMP formed per reaction mixture.

Estimation of inorganic phosphate. Extracts of the reaction mixtures in perchloric acid soln. were analysed for inorganic orthophosphate by the method of Allen (1940) as modified by Turner (1957).

Chromatography. Nucleotides in the ethanolic extracts of the reaction mixtures were separated and detected on paper chromatograms as previously described (Turner & Turner, 1958). Either isobutyric acid-aq. NH_3 soln. (sp.gr. 0.88)-water (66:1:33, by vol.) (Pabst Laboratories, 1956) or Na_2HPO_4 - NaH_2PO_4 buffer, pH 6.8 (0.1 M, 50 ml.)-(NH_4) $_2\text{SO}_4$ (30 g.)-propan-1-ol (1 ml.) (Pabst Laboratories, 1956) was used as solvent.

For further identification of IMP the nucleotide was eluted from the spot area with 3 ml. of 50 mM-phosphate (KH_2PO_4 - Na_2HPO_4) buffer, pH 7 (Kirkland & Turner, 1959). Appropriate areas of paper were taken for blank values.

Estimation of protein nitrogen. Protein in the enzyme preparation was precipitated by 5% (w/v) trichloroacetic acid and washed twice with 1% (w/v) trichloroacetic acid. Total N in the residue was determined by the micro-Kjeldahl procedure.

RESULTS

5'-Adenylic acid-deaminase activity. The deamination of AMP by the pea-seed adenylic deaminase is shown in Fig. 1. In the experiment illustrated

11.0 μ moles of AMP were added as substrate and 10.8 μ moles of IMP were formed. In experiments of this type 95-98% of added AMP could be accounted for as IMP.

Although phosphatase activity was present in the crude homogenate from pea seeds, it was not detected by the formation of inorganic phosphate, adenosine or inosine when the washed particle preparation was incubated in reaction mixtures containing AMP or IMP. There was no change in extinction during the incubation of reaction mixtures containing IMP and the adenylic-deaminase preparation. These observations indicated that the enzyme preparation was free from interfering enzymes.

IMP was isolated from reaction mixtures by applying ethanolic extracts to Whatman no. 3MM papers which were developed in the isobutyric acid-aq. NH_3 soln.-water solvent. The area corresponding to IMP was cut out and eluted. The absorption spectrum of the eluted material was identical to that of IMP.

Effect of anions. In preliminary experiments active deaminase preparations were obtained by washing the particle fraction from pea seeds with 20% saturated (NH_4) $_2\text{SO}_4$, pH 6.0. There was no activity in these preparations after dialysis for 2.5 hr. at 4° against five changes of water, but the preparation showed activity on the addition of (NH_4) $_2\text{SO}_4$ (final concn. 0.2 M).

The deaminase preparation used in the present investigation was normally prepared by washing the particulate fraction with water and possessed negligible or no activity in the absence of added salts. The effects of the addition of various salts on the deaminase activity are shown in Table 1. The

Fig. 1. 5'-Adenylic acid-deaminase activity as measured by IMP formation. The reaction mixture contained 11.0 μ moles of AMP, 1 m-mole of Li_2SO_4 , 0.24 m-mole of succinate buffer, pH 6.2, and 0.5 ml. of enzyme (containing 4.2 mg. of protein N); total volume, 2.5 ml. Temp. 30°.

sulphates of the cations tested were more effective than the chlorides but the nature of the cation also had some effect. The maximum increase was given by $(\text{NH}_4)_2\text{SO}_4$ and Li_2SO_4 in relatively high final concentration (0.4M). Citrate produced a good stimulation of deaminase activity but acetate did not increase the activity in any of the concentrations tried. Increasing the salt concentration above the optimum led to a decrease in the rate of reaction.

Effect of pH. The effect of pH on the activity of pea-seed adenylic deaminase is shown in Fig. 2. With succinate buffer the optimum pH was 6.0–6.2, but with 2-amino-2-hydroxymethylpropane-

1:3-diol (tris)-acetic acid buffer there was a broader optimum from approx. pH 5.6 to 6.8.

Effect of substrate concentration. The effect of concentration of AMP on the initial reaction velocity of the adenylic deaminase is shown in Fig. 3. With the method of Lineweaver & Burk (1934) it was calculated from these results that the Michaelis-Menten constant (K_m) for AMP was $1.3 \times 10^{-2}\text{M}$.

Effect of inhibitors. The effects of several inhibitors on adenylic-deaminase activity are shown in Table 2. The enzyme was inhibited by phosphate and fluoride and also by the metal ions Zn^{2+} , Cu^{2+} and Hg^{2+} . The enzyme was sensitive to the sulphhydryl reagents *p*-hydroxymercuribenzoate and iodoacetate. Ethylenediaminetetra-acetate inhibited the reaction. Cysteine had no effect.

Table 1. *Effect of anions on adenylic-deaminase activity*

Reaction mixtures contained 1.05 μmoles of AMP, 24 μmoles of succinate buffer, pH 6.2, 0.05 ml. of enzyme (containing 0.42 mg. of protein N); total volume, 0.25 ml. Salts were added as shown. Time of incubation, 30 min. Temp. 30°. The results are expressed as the increased formation of IMP (μmole /reaction mixture) above the control figure (0.05 μmole) obtained in a reaction mixture containing no added salt.

Addition	Final concn. (M)			
	0.01	0.1	0.4	0.8
$(\text{NH}_4)_2\text{SO}_4$	0	0.24	0.37	0.22
Li_2SO_4	0	0.07	0.34	0.11
Na_2SO_4	0	0	0.29	0.13
MgSO_4	0	0.25	0.19	0.03
NH_4Cl	0	0.03	0.20	0.09
NaCl	0	0.01	0.14	0.06
MgCl_2	0.05	0.18	0	0
Sodium citrate	0.01	0.25	0.16	0.06
Sodium acetate	0	0	0	0

Fig. 2. Effect of pH on deaminase activity. Reaction mixtures were of the composition described for the assay of 5'-adenylic acid deaminase. Succinic acid (24 μmoles)-NaOH (●) and tris (25 μmoles)-acetic acid (○) buffers of the required pH were used. Time of incubation, 30 min. Temp. 30°.

Fig. 3. Effect of substrate concentration on deaminase activity. Substrate concentration, $[S]$, is expressed as μmole of AMP/ml.; initial velocity, V , is expressed as μmoles of IMP formed/ml. per 30 min. Reaction mixtures were of the composition described for the assay of deaminase activity with the AMP concentration varied as shown. Time of incubation, 30 min. Temp. 30°.

Table 2. *Effect of inhibitors on adenylic-deaminase activity*

Reaction mixtures were of the composition described for the assay of 5'-adenylic acid deaminase. Inhibitors were added as shown. Time of incubation, 30 min. Temp. 30°.

Inhibitor	Final concn. (mm)	Inhibition (%)
NH_4F	1	38
KH_2PO_4	8	76
ZnSO_4	5	23
CuSO_4	1	100
HgCl_2	0.01	100
<i>p</i> -Hydroxymercuribenzoate	0.2	100
Iodoacetate	10	39
Ethylenediaminetetra-acetate	100	60
Cysteine	10	0

Specificity of the adenylic-deaminase preparation. The pea-seed-deaminase preparation was specific for AMP as substrate. There was no reaction when AMP was replaced by adenine, adenosine, adenosine diphosphate, adenosine triphosphate, diphosphopyridine nucleotide or triphosphopyridine nucleotide in reaction mixtures of the composition described for the assay of 5'-adenylic deaminase.

DISCUSSION

This investigation has established that active 5'-adenylic-deaminase preparations free from interfering enzymes may be readily obtained from pea seeds. From the range of compounds tested the enzyme was specific for AMP as substrate and had no action on adenine, adenosine, adenosine diphosphate or adenosine triphosphate.

From the data illustrated in Fig. 1 it can be concluded that the reaction was essentially irreversible and proceeded virtually to completion. Kalckar (1947) was unable to detect any reversal of the reaction in the presence of a relatively high concentration of NH_4^+ ions, and similar results were obtained by Lee (1957).

The stimulatory effect of ions on the pea-seed deaminase was very pronounced and most preparations possessed no detectable activity in the absence of added salts. Ammonium sulphate, lithium sulphate and sodium sulphate were the most effective of the salts tested and the effect appeared to be largely due to the anion, as ammonium chloride and sodium chloride gave substantially less stimulation. The addition of sodium citrate increased the rate of reaction but no detectable increase over the control value was obtained with sodium acetate. Humphrey & Webster (1951) found that the deaminase activity of preparations of rabbit-muscle myosin was accelerated in the presence of 10 mM-calcium chloride or -magnesium chloride. The effect of anions on rabbit-muscle deaminase was studied by Nikiforuk & Colowick (1956). These authors found that citrate, chloride, acetate and lactate stimulated deaminase activity but sulphate had no effect. The enzyme preparation used by Nikiforuk & Colowick was active without added salts and the maximum stimulation of activity obtained (approximately 150%) was much lower than that obtained with pea-seed deaminase. The salt concentration employed by these authors was lower than that found to be optimum in the present investigation. No effect of chloride, citrate or acetate was found by Lee (1957), with the crystalline deaminase from rabbit muscle. The optimum pH for pea-seed deaminase in succinate buffer (pH 6.0-6.2) agrees well with the figures obtained for the rabbit-muscle enzyme. Kalckar (1947) and Nikiforuk & Colowick (1956)

reported a value of 5.9, and Lee (1957) found that the optimum pH for the crystalline rabbit-muscle enzyme was 6.4 in succinate buffer. The K_m for AMP with the pea-seed deaminase was $1.3 \times 10^{-2} \text{ M}$ and this value is considerably greater than the K_m of $6.0 \times 10^{-5} \text{ M}$ for the rabbit-muscle enzyme reported by Nikiforuk & Colowick (1956), but is closer to that of $1.4 \times 10^{-3} \text{ M}$ reported by Lee (1957) for the crystalline enzyme.

In the present investigation it was found that the deaminase was inhibited by fluoride and inorganic orthophosphate and this agrees with observations with the enzyme from animal tissues (Nikiforuk & Colowick, 1956; Lee, 1957). Zn^{2+} ions inhibited the pea-seed deaminase and this is in agreement with the results of Lee but not with those of Nikiforuk & Colowick, who found no inhibition by Zn^{2+} ions. Hg^{2+} and Cu^{2+} ions strongly inhibited the plant deaminase. The sensitivity of the pea-seed enzyme to Hg^{2+} ions suggested that sulphhydryl groups were essential for the enzyme activity and this was confirmed by the inhibition by *p*-hydroxymercuribenzoate and iodoacetate. Neither Nikiforuk & Colowick nor Lee found iodoacetate inhibition, although Lee observed inhibition by *p*-mercuribenzenesulphonic acid. Ethylenediaminetetra-acetate inhibited the enzyme used in the present investigation. This suggests that the pea-seed deaminase may have a requirement for a bivalent metal ion and this is supported by the fluoride inhibition. Nikiforuk & Colowick and Lee did not, however, observe inhibition by ethylenediaminetetra-acetate.

SUMMARY

1. 5'-Adenylic acid deaminase was found in a particulate fraction obtained from pea-seed extracts. A preparation free from interfering enzymes was obtained.

2. The enzyme was essentially inactive in the absence of an added salt. The sulphates of univalent cations were most effective in stimulating the reaction; the effect was largely due to the anion component.

3. The effects of pH and concentration of substrate on adenylic-deaminase activity were studied. Under the experimental conditions the Michaelis-Menten constant for adenosine monophosphate was $1.3 \times 10^{-2} \text{ M}$.

4. The enzyme was inhibited by fluoride, inorganic phosphate, Zn^{2+} ions, Cu^{2+} ions, Hg^{2+} ions, *p*-hydroxymercuribenzoate, iodoacetate and ethylenediaminetetra-acetate.

5. The enzyme was specific for adenosine monophosphate.

6. The properties of the pea-seed adenylic deaminase have been considered in relation to those of the enzyme from rabbit muscle.

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Inosine 5'-Phosphate Dehydrogenase of Pea Seeds

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Inosine 5'-phosphate (hypoxanthine riboside 5'-phosphate) has been implicated as a precursor of nucleic acid purines by the work of Greenberg (1952) and Buchanan & Schulman (1953). Adenosine 5'-phosphate may be formed from inosine 5'-phosphate by way of adenylosuccinate (Carter & Cohen, 1955, 1956; Lieberman, 1956). Investigations have shown that a derivative of xanthosine (xanthine riboside) is an intermediate in the formation of guanosine 5'-phosphate from inosine 5'-phosphate by extracts of rabbit bone marrow and pigeon liver (Abrams & Bentley, 1955*a, b*; Lagerkvist, 1955, 1958). The first reaction involved, the enzymic conversion of inosine 5'-phosphate into xanthosine 5'-phosphate, was demonstrated by Abrams & Bentley (1955*a*) in a soluble extract from rabbit bone marrow and by Gehring & Magasanik (1955) in fractionated extracts of *Aerobacter aerogenes*; diphosphopyridine nucleotide was reduced in the reaction. The enzyme involved, inosine 5'-phosphate dehydrogenase, was purified from extracts of *A. aerogenes* by Magasanik, Moyed & Gehring (1957).

In the present investigation inosine 5'-phosphate dehydrogenase was found in a higher plant and some properties of the enzyme from pea seeds were studied. It was shown previously that adenosine 5'-phosphate is deaminated to inosine 5'-phosphate by an adenylic deaminase present in pea-seed extracts (Turner & Turner, 1961).

MATERIALS AND METHODS

Substrates. Inosine, inosine 5'-phosphate, guanosine 5'-phosphate and diphosphopyridine nucleotide (DPN) were obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A., xanthosine from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., triphosphopyridine nucleotide (TPN) from Sigma Chemical Co., St Louis, Mo., U.S.A., and glutathione (GSH) from C. F. Boehringer und Soehne, Mannheim, Germany.

Preparation of inosine 5'-phosphate dehydrogenase from pea seeds. A crude extract was prepared from dried pea seeds (*Pisum sativum* L., var. Cannons' Perfection) as previously described (Turner, 1957) except that toluene was omitted. The crude extract was centrifuged at 20000 g for 20 min. at room temperature and the supernatant treated at 4° with saturated (NH₄)₂SO₄ which had been neutralized with aq. NH₃ soln. The fraction obtained between 34 and 43% saturation from 45 g. of dried-pea powder was dissolved in 12 ml. of water and dialysed with rocking for 4.5 hr. at 4° against water. Fractionation at low temperatures with acetone or ethanol yielded less active enzyme preparations.

The clear, straw-coloured enzyme preparation could be stored at -15° for 2 weeks with 10% loss of activity and with 40% loss after 6 weeks. Heating for 10 min. at 50° and 55° resulted in 32 and 95% loss respectively of inosine 5'-phosphate-dehydrogenase activity.

Measurement of enzyme activity. The reaction mixtures were maintained at 25°. For the assay of inosine 5'-phosphate-dehydrogenase activity the following components were mixed: 0.2 μmole (approx.) of inosine 5'-phosphate, 0.4 μmole (approx.) of DPN, 100 μmoles of KCl,