The work described in this paper was carried out as part of the joint research programme of the Division of Food Preservation, C.S.I.R.O., and of the Botany School, University of Sydney. The authors wish to express their indebtedness to Miss S. K. Harris and Miss N. J. Eames for technical assistance; and to Dr J. R. Vickery, Chief, Division of Food Preservation, and Professor R. L. Crocker, Botany School, University of Sydney, in whose laboratories the work was carried out.

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Biochem. J. (1961) **79**, 147

Inosine 5'-Phosphate Dehydrogenase of Pea Seeds

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(Received 3 August 1960)

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,

80 μ moles of 2-amino-2-hydroxymethylpropane-1,3-diol (tris)-HCl buffer, pH 8.0, 0.2 ml. of enzyme (containing approx. 40–50 μ g. of protein); total volume, 3.0 ml. In some early experiments 5 μ moles of GSH were also added. The time of incubation and enzyme concentration were adjusted so that not more than 50% conversion of inosine 5'-phosphate occurred during the reaction period. Under these conditions the reaction rate remained approximately constant.

The oxidation of inosine 5'-phosphate was followed by observing the increase in extinction at 340 m μ due to the formation of reduced diphosphopyridine nucleotide (DPNH). Measurements were carried out in a Beckman model DU spectrophotometer with 1 cm. cuvettes. Two series of control reaction mixtures, (i) omitting inosine 5'-phosphate and (ii) omitting DPN, were always prepared. There was usually a small increase in $E_{340 \text{ m}\mu}$ in (i) and a barely significant change in (ii). The results are expressed as the increase in $E_{340 \text{ m}\mu}$ of the complete reaction mixtures after allowing for any change in the controls. In experiments where DPN was replaced by TPN, the reaction was also followed at 340 m μ . Determination of the increase in extinction at 290 m μ (Magasanik *et al.* 1957) due to the formation of xanthosine 5'-phosphate was unsatisfactory because of the high extinction of the enzyme preparation at this wavelength.

Chromatography. The nucleotides were separated on paper chromatograms, detected and eluted as described previously (Turner & Turner, 1958; Kirkland & Turner, 1959). Multiple applications of 24 μ l. portions were made on each spot.

Determination of protein. The protein concentration of the enzyme preparations was estimated by the spectrophotometric method of Warburg & Christian (1941).

RESULTS

Inosine 5'-phosphate-dehydrogenase activity

When reaction mixtures of the composition described for the assay of inosine 5'-phosphate-dehydrogenase activity were incubated, an increase in extinction at 340 m μ , which was attributed to DPNH formation, was observed.

Xanthosine 5'-phosphate was isolated from a reaction mixture of the composition described for the assay of inosine 5'-phosphate dehydrogenase. The reaction was allowed to proceed for 120 min. and stopped by heating at 95° for 5 min. After centrifuging, the supernatant was applied to Whatman no. 3MM papers which were developed for 16 hr. with the ethanol-m-ammonium acetate, pH 7.5 (5:2, v/v), solvent of Paladini & Leloir (1952). The areas corresponding to a spot moving slightly slower than inosine 5'-phosphate were cut out, eluted with water and the extract was evaporated to dryness at 50° in a stream of N₂. Two drops of water were added and the extract was applied to a Whatman no. 3MM paper, which was developed for 18 hr. with isobutyric acid-aq. NH₃ soln. (sp.gr. 0.880)-water (66:1:33, by vol.) as solvent (Pabst Laboratories, 1956). A spot which travelled

a little more slowly than inosine 5'-phosphate was eluted with 3 ml. of 50 mM-phosphate (KH₂PO₄-Na₂HPO₄) buffer, pH 7 (Kirkland & Turner, 1959). The absorption spectrum was very similar to that of xanthosine. Maxima were obtained at 248 and 276 m μ with a minimum at 267 m μ . Xanthosine 5'-phosphate prepared by the deamination of

Fig. 1. Effect of KCl concentration on inosine 5'-phosphate-dehydrogenase activity. Reaction mixtures contained 0.26 μ mole of inosine 5'-phosphate, 0.44 μ mole of DPN, 80 μ moles of tris-HCl buffer, pH 8.0, 0.2 ml. of enzyme (containing 48 μ g. of protein); total volume, 3.0 ml. Concentration of KCl was varied as shown. Time of incubation, 30 min. Temp. 25°.

Fig. 2. Effect of pH on inosine 5'-phosphate-dehydrogenase activity. Reaction mixtures contained 0.22 μ mole of inosine 5'-phosphate, 0.45 μ mole of DPN, 100 μ moles of KCl, 80 μ moles of tris-HCl buffer of required pH, 0.2 ml. of enzyme (containing 42 μ g. of protein); total volume, 3.0 ml. Time of incubation, 15 min. Temp. 25°.

guanosine 5'-phosphate by nitrite (Abrams & Bentley, 1959) gave maxima at 248 and 276 $m\mu$ and a minimum at 267 $m\mu$. Magasanik *et al.* (1957) found that xanthosine 5'-phosphate formed by the oxidation of inosine 5'-phosphate by the enzyme from *A. aerogenes* had maxima at 248 and 277 $m\mu$ with a minimum at 265 $m\mu$ at pH 7.

In some preliminary experiments a slight stimulation of DPNH formation was obtained on the addition of GSH. Subsequent experiments failed to confirm those observations and GSH was therefore omitted from the reaction mixtures.

Cofactor requirements. The enzyme was largely inactive in the absence of a suitable univalent cation. Fig. 1 shows the effect of K^+ ion concentration on inosine 5'-phosphate-dehydrogenase activity. The reaction rate reached a maximum at a concentration of between 33 and 66 mM-KCl; further increase in KCl concentration to 0.13 M did not affect the rate of reaction.

When KCl (final concn. 33 mM) in the reaction mixture was replaced by the same concentrations of NH_4Cl or NaCl the rates of reaction were 47 and 6% respectively of those obtained with KCl. The addition of NaCl (33 mM) to a reaction mixture containing KCl (33 mM) had no effect.

Effect of pH. The activity of the enzyme was studied in a series of buffers containing tris (80 μ -moles) and HCl over the range pH 6.7–8.6 (Fig. 2). The pH-activity curve showed a sharp optimum at approximately pH 8.0.

Effect of concentration of substrates. The effect of increasing DPN concentration on the rate of reaction is shown in Fig. 3. The optimum concentration was greater than 0.6 mM and from a Line-

Fig. 3. Effect of DPN concentration on inosine 5'-phosphate-dehydrogenase activity. Reaction mixtures contained 0.26 μ mole of inosine 5'-phosphate, 100 μ moles of KCl, 80 μ moles of tris-HCl buffer, pH 8.0, 0.2 ml. of enzyme (containing 48 μ g. of protein); total volume, 3.0 ml. Concentration of DPN was varied as shown. Time of incubation, 30 min. Temp. 25°.

weaver & Burk (1934) plot the K_m for DPN was calculated to be approximately 0.25 mM.

Variation in inosine 5'-phosphate concentration also affected the reaction velocity (Fig. 4); a Lineweaver-Burk plot gave K_m approximately 0.026 mM for inosine 5'-phosphate.

Effect of inhibitors on inosine 5'-phosphate-dehydrogenase activity. The effects of several inhibitors on the rate of reaction are shown in Table 1. Mercuric chloride, *p*-hydroxymercuribenzoate and iodoacetate strongly inhibited inosine 5'-phosphate-dehydrogenase activity. Phosphate, arsenate and fluoride were without effect.

Specificity of the pea-seed inosine 5'-phosphate dehydrogenase. Inosine could replace inosine 5'-phosphate to a small extent as substrate for the

Fig. 4. Effect of inosine 5'-phosphate concentration on inosine 5'-phosphate-dehydrogenase activity. Reaction mixtures contained 0.41 μ mole of DPN, 100 μ moles of KCl, 80 μ moles of tris-HCl buffer, pH 8.0, 0.1 ml. of enzyme (containing 22 μ g. of protein); total volume, 3.0 ml. Concentration of inosine 5'-phosphate was varied as shown. Time of incubation, 30 min. Temp. 25°.

Table 1. Effect of inhibitors on inosine 5'-phosphate-dehydrogenase activity

Reaction mixtures contained 0.22 μ mole of inosine 5'-phosphate, 0.44 μ mole of DPN, 100 μ moles of KCl, 80 μ moles of tris-HCl buffer, pH 8.0, 0.2 ml. of enzyme (containing 42 μ g. of protein), total volume, 3.0 ml. Inhibitors were added as shown. Time of incubation, 30 min. Temp. 25°.

Inhibitor	Final concn. (mM)	Inhibition (%)
Inorganic phosphate	10	0
Arsenate	10	0
Fluoride	10	0
HgCl ₂	0.5	100
<i>p</i> -Hydroxymercuribenzoate	1	97
Iodoacetate	10	98
Iodoacetate	5	85
Iodoacetate	1	25

enzyme. When inosine 5'-phosphate ($0.2\mu\text{mole}$) was replaced by inosine of the same concentration in reaction mixtures of the composition described for the assay of inosine 5'-phosphate dehydrogenase the rate of reduction of DPN was 8% of that given with inosine 5'-phosphate as substrate.

Inosine 5'-phosphate dehydrogenase was essentially specific for DPN as substrate. When TPN ($0.4\mu\text{mole}$) was substituted for DPN ($0.4\mu\text{mole}$) in reaction mixtures of the composition used for the assay of inosine 5'-phosphate dehydrogenase, the rate of reaction was only 2% of that given by DPN; this may have been due to the presence of DPN as impurity in the TPN preparation.

DISCUSSION

The present investigation has shown that active preparations of inosine 5'-phosphate dehydrogenase may be readily obtained from pea seeds.

The inosine 5'-phosphate dehydrogenase from pea seeds has similar properties to the enzyme from *Aerobacter aerogenes* (Magasanik *et al.* 1957). The pH-activity curves for the two enzymes are very similar, the optimum pH for the pea-seed enzyme being pH 8.0 and for the bacterial enzyme pH 8.1. The inosine 5'-phosphate dehydrogenase of pea seeds required a suitable univalent cation for activity; K^+ ions were the most effective of those tested and NH_4^+ ions in the same concentration gave approximately half the rate given by K^+ ions. Sodium ions had only a slight effect and were not inhibitory in the presence of the optimum concentration of K^+ ions. Magasanik *et al.* (1957) found that K^+ or NH_4^+ ions were required for activity of the inosine 5'-phosphate dehydrogenase from *A. aerogenes*. These authors found that the latter enzyme was not active with either inosine or TPN as substrates: with the pea-seed enzyme inosine and TPN gave 8 and 2% respectively of the rates obtained with inosine 5'-phosphate and DPN as substrates.

The strong inhibition of pea-seed inosine 5'-phosphate dehydrogenase by *p*-hydroxymercuribenzoate and iodoacetate indicated that the enzyme possessed sulphydryl groups essential for activity. Although Magasanik *et al.* (1957) did not report inhibition by sulphydryl reagents they found that glutathione and cysteine stimulated the activity of the inosine 5'-phosphate dehydrogenase from *A. aerogenes*.

The role of inosine 5'-phosphate dehydrogenase in bacteria in the formation of nucleic acid guanine was indicated by the work of Magasanik *et al.* (1957) and Moyed & Magasanik (1957) with purineless mutants, and the enzyme may perform the same function in plants in the formation of guanine derivatives. Guanosine triphosphate has been

shown to participate in several reactions, including the formation of succinyl-coenzyme A (Sanadi, Gibson & Ayengar, 1954; Sanadi, Gibson, Ayengar & Jacob, 1956), the oxaloacetate-carboxylase reaction (Kurahashi, Pennington & Utter, 1957) and the synthesis of adenylosuccinate (Lieberman, 1956). Other derivatives such as guanosine diphosphomannose and guanosine diphosphofucose may be of significance in the metabolism of those plants which accumulate polysaccharides containing mannose and fucose.

SUMMARY

1. Inosine 5'-phosphate dehydrogenase, which catalyses the reaction

was found in pea-seed extracts.

2. Potassium ions were necessary for the reaction; NH_4^+ and, to a slight extent, Na^+ ions could partly replace K^+ ions as cofactor for the enzyme.

3. The effects of K^+ ion concentration, pH and concentration of substrates on inosine 5'-phosphate dehydrogenase activity were studied.

4. The enzyme was strongly inhibited by sulphydryl reagents.

5. The rate of reaction was very small when inosine 5'-phosphate was replaced by inosine or when diphosphopyridine nucleotide was replaced by triphosphopyridine nucleotide.

The work described in this paper was carried out as part of the joint-research programme of the Division of Food Preservation, C.S.I.R.O., and of the Botany School, University of Sydney. The authors wish to express their indebtedness to Dr J. R. Vickery, Chief, Division of Food Preservation, and Professor R. L. Crocker, Botany School, University of Sydney, in whose laboratories the work was carried out.

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Biochem. J. (1961) **79**, 151

Cytochrome Pigments from the Green Photosynthetic Bacterium *Chlorobium thiosulphatophilum*

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(Received 17 October 1960)

The early notion that the cytochrome pigments of living tissues are associated closely with the reduction of molecular oxygen has had to be modified because of two main findings. Hill & Scarisbrick (1951) demonstrated that green plants contain cytochrome *f*, a pigment specifically associated with photosynthetic tissues and not found in other parts of the plant. Postgate (1954) showed that the strictly anaerobic bacterium *Desulphovibrio desulphuricans* is rich in cytochrome *c*₃.

Elsden, Kamen & Vernon (1953) isolated cytochrome from the purple non-sulphur bacterium *Rhodospirillum rubrum*, which is both photosynthetic and predominantly anaerobic in its mode of life. Vernon & Kamen (1954) subsequently demonstrated the presence of cytochrome pigments in extracts of the strictly anaerobic photosynthetic sulphur bacteria *Chromatium* and *Chlorobium*. In these organisms the simplest type of photosynthetic cytochrome system might be expected, uncomplicated by any possibility of oxygen metabolism, and it therefore appeared to be of interest to examine the cytochromes in these organisms more closely. The pigment from *Chromatium* has been prepared, and its properties investigated, by Newton & Kamen (1956).

The present paper describes the isolation and properties of two cytochromes, resembling each other in many of their properties, from the green sulphur bacterium *Chlorobium thiosulphatophilum*.

A preliminary account of part of this work has appeared (Gibson & Larsen, 1955).

METHODS

Organisms and culture methods. A strain of *Chlorobium thiosulphatophilum* kindly supplied by Dr J. Lascelles of Oxford was used for the main part of the work. Small-scale experiments with a second strain, obtained from Dr C. B. van Niel, showed that the two component pigments were again present.

The maintenance and culture of the organisms has been fully described by Larsen (1952). For large-scale growth Larsen's medium, modified so as to contain 0.2% of Na₂S₂O₃ and 0.4% of NaHCO₃ (both w/w), was used to fill completely 25 l. carboys, whose necks were sealed with rubber bungs. Five vessels were illuminated with 12 60 w incandescent bulbs, and the circulation of air round them was limited so as to produce a temperature of about 25° in the medium. The vessels were filled the day before they were inoculated with 200 ml. of a 2-day culture of the organisms grown in a medium containing 0.1% of Na₂S₂O₃ and 0.2% of NaHCO₃ (w/w). Growth was complete in 4–5 days, when the cells were harvested with a Sharples supercentrifuge. The yield ranged from 40 to 60 g. wet wt. equivalent to 14–20 g. of dry matter. The bacterial paste was stored as such at –20°, or spread thinly on glass plates and dried *in vacuo* over H₂SO₄ at room temperature or freeze-dried. The frozen cells and the dried preparations were both stable over a period of many months.

Phosphate buffers. These were prepared from KH₂PO₄ solutions adjusted to the required pH with NaOH, a Pye pH meter being used.