Biochem. J. (1963) **89**, 167

Inhibition of Inosine 5′-Phosphate Dehydrogenase from Ehrlich Ascites-Tumour Cells by 6-Thioinosine 5′-Phosphate

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(*Received* 18 *March* 1963)

Inosine 5′-phosphate–NAD oxidoreductase (IMP dehydrogenase; EC 1.2.1.14) catalyses the oxidation of inosine 5′-phosphate to xanthosine 5′-phosphate by NAD. The enzyme has been found in bacteria (Gehring & Magasanik, 1955; Magasanik, Moyed & Gehring, 1957), in pea seeds (Turner & King, 1961), in animal tissues (Abrams & Bentley, 1955; Lagerkvist, 1955, 1958) and in L cells and Ehrlich ascites cells (McFall & Magasanik, 1960). Salser, Hutchison & Balis (1960) found that catalysis of oxidation of inosine 5′-phosphate by an enzyme from pigeon liver was inhibited by 6-thioinosine 5′-phosphate but the nature of the inhibition was not studied.

6-Mercaptopurine inhibits division of Ehrlich ascites cells and is converted into 6-thioinosine 5′-phosphate by these cells *in vivo* and *in vitro* (Paterson, 1959). A resistant strain was much less effective than the sensitive strain in catalysing this conversion (Paterson, 1960; Paterson & Hori, 1962). Paterson (1962) has suggested that this decreased synthesis of nucleotide results from decreased transport of 6-mercaptopurine across the cell membrane and has reviewed the evidence that inhibition of growth by 6-mercaptopurine is due to the toxicity of 6-thioinosine 5′-phosphate.

This paper describes the extraction of inosine 5′-phosphate dehydrogenase from Ehrlich ascites cells and its use in studies of purified 6-thioinosine 5′-phosphate as an inhibitor of the oxidation of inosine 5′-phosphate to xanthosine 5′-phosphate. The purpose of this work was to find if 6-thioinosine 5′-phosphate was a competitive inhibitor, to measure its K_i and to compare this with the K_m of inosine 5′-phosphate with dehydrogenase from tumour cells which are sensitive to 6-mercaptopurine.

The results obtained show that 6-thioinosine 5′-phosphate is an effective competitive inhibitor of the dehydrogenase and indicate the potential importance of this inhibition in the biological action of 6-mercaptopurine.

EXPERIMENTAL

Abbreviations. Thio-IMP, 6-thioinosine 5′-phosphate; thio-IMP disulphide, bis-(9-β-D-5′-phosphoribofuranosylpurin-6-yl) disulphide; XMP, xanthosine 5′-phosphate.

Preparation of nucleotides

6-Thioinosine 5′-phosphate. Barium thio-IMP (Montgomery & Thomas, 1961) was freed of IMP and thio-IMP disulphide by anion-exchange chromatography in the presence of 2-mercaptoethanol. The crude barium salt (0·1 g.) was suspended in 10 ml. of 0·2% mercaptoethanol at 2° and brought to pH 2 with Amberlite CG-120 (H^+ form). The suspension was poured on a column (4 cm. × 1 cm.^{2}) of Amberlite CG-120 (K^+ form) and the combined resins were washed with water (3 × 5 ml.). The combined effluent (pH 4) was passed through a column of ECTEOLA-cellulose (formate form) at about 20° at 2 ml./min. and the anion exchanger was washed with 5 mM-mercaptoethanol until the extinction of the effluent at 280 mμ was less than 0·05 when measured against 5 mM-mercaptoethanol in a 1 cm. cell. Elution with a linear gradient, obtained by mixing 400 ml. of 5 mM-mercaptoethanol and 400 ml. of 60 mM-triethylamine–60 mM-formic acid–60 mM-acetic acid–5 mM-mercaptoethanol, removed IMP (about 10 μmoles); thio-IMP started to emerge in the last 20 ml. of the linear gradient and was completely eluted by 300 ml. of 60 mM-triethylamine–60 mM-formic acid–60 mM-acetic acid–5 mM-mercaptoethanol. *Triethylammonium 6-thioinosine 5′-phosphate* (110 μmoles) was obtained by freeze-drying; the residue was freeze-dried with further portions of 5 mM-mercaptoethanol (3 × 50 ml.) to remove volatile salts and had $\lambda_{max.}$ 321·5 mμ and $\lambda_{min.}$ 253 mμ in 0·1 M-acetate (Na^+), pH 5·5 (mε 24·1 and 0·96 respectively, based on the sulphur content of a dried sample). The spectrum is shown in Fig. 1.

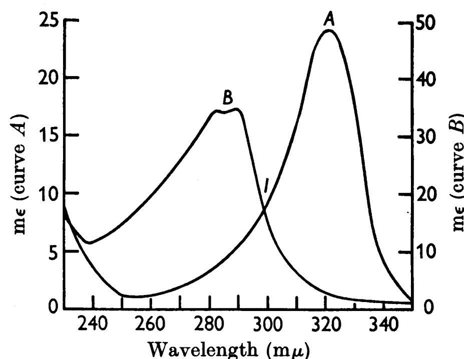

Fig. 1. Absorption spectra in 0·1 M-acetate (Na^+, pH 5·5) of 6-thio-IMP (*A*) and thio-IMP disulphide (*B*). The molecular-extinction-coefficient scale for *A* (left-hand ordinate) is twice that for *B* (right-hand ordinate). The isosbestic wavelength (*I*) for oxidation of 2 mol.prop. of thio-IMP to 1 mol.prop. of thio-IMP disulphide is 299 mμ.

Hampton & Maguire (1961) found that the corresponding barium salt had m$\epsilon_{max.}$ 23·1 at 322 mμ and m$\epsilon_{min.}$ 1·2 at 255 mμ. For use in enzymic studies the nucleotide was dissolved in 1 mM-mercaptoethanol. A 22 mM-thio-IMP solution in mercaptoethanol showed no spectroscopic evidence of hydrolysis to IMP or oxidation to thio-IMP disulphide during 14 days at −15°. To obtain material for analysis triethylammonium thio-IMP (420 μmoles) in 2 ml. of 5 mM-mercaptoethanol was mixed with 20 ml. of 95% ethanol and a trace of insoluble material was removed by centrifuging. The supernatant was dried at 20–30° and the resultant residue was washed with 20 ml. of dry ethanol and dissolved in 1 ml. of water. After filtration through a small sintered-glass plate (no. 4 porosity) the solution was concentrated to a syrup and mixed with 8 ml. of dry acetone. The residue was washed with acetone and dried for 16 hr. at 110°/< 1 mm. (Found: C, 38·2; H, 5·5; P, 7·2; S, 7·0. $C_6H_{15}N,2C_{10}H_{13}N_4O_7PS,C_2H_6O$ requires C, 38·4; H, 5·4; P, 7·1; S, 7·3%). No IMP (R_F 0·62) was detected in the thio-IMP (R_F 0·43) on chromatography in $(NH_4)_2SO_4$–0·1 M-phosphate (Na^+, pH 6·8)–propan-1-ol (60 : 100 : 2, w/v/v). On electrophoresis in 30 mM-triethylamine–30 mM-formic acid–30 mM-acetic acid (pH 4·15) the nucleotide migrated as a single component (0·30 cm.^{2} v^{-1} hr.$^{-1}$) and was free of thio-IMP disulphide (0·38 cm.^{2} v^{-1} hr.$^{-1}$).

Disulphide of 6-thioinosine 5'-phosphate. A solution of triethylammonium thio-IMP (124 μmoles) in 25 ml. of 0·2 mM-mercaptoethanol at 25° was kept at pH 7·0 by automatic addition of N-NaOH while N-iodine in 20% (w/v) NaI was added in 0·020 ml. portions. After addition of 0·060 ml. of iodine and consumption of 0·058 ml. of alkali the ratio $E_{321\cdot5\,m\mu}/E_{289\,m\mu}$ (measured on samples diluted in 0·1 M-acetate, Na^+, pH 5·5) had decreased from the initial value of 4·5 to 0·06. Reduction of iodine after each addition was complete within 3 sec., as indicated by the recorded consumption of alkali in the automatic titrator. After addition of 4 ml. of 0·1 M-barium acetate the solution (pH 6·5) was cooled to 0° and mixed with 75 ml. of 95% ethanol. After 2 hr. at −15° the barium salt was collected, washed with 40 ml. of 95% ethanol and dissolved in 10 ml. of 20 mM-acetate (Na^+, pH 5·5). The solution of *disulphide* (57 μmoles) was filtered. In 0·1 M-acetate (Na^+, pH 5·5) the compound (cf. Fig. 1) had $\lambda_{max.}$ 289 mμ (mϵ 34·7 with S.E.M. 0·5 from eight measurements on two separate preparations), $\lambda_{min.}$ 285 mμ (mϵ 34·1), $\lambda_{max.}$ 282 mμ (mϵ 34·2) and $\lambda_{min.}$ 239 mμ (mϵ 11·5). The molecular extinction coefficient was calculated from the mean value of N and P contents, which were in the atomic proportions 4·11 : 1·00. After precipitation with 4 vol. of 95% ethanol and washing with 95% ethanol (3 × 30 ml.) a sample of the barium salt was dried for 10 hr. at 110°/< 1 mm. (Found: C, 22·8; H, 3·2; N, 10·7; P, 5·9; S, 5·5. $C_{20}H_{26}Ba_2N_8O_{14}P_2S_2,4H_2O$ requires C, 22·4; H, 3·2; N, 10·4; P, 5·8; S, 5·9%). Nitrogen was determined by the Kjeldahl method; other elements were analysed by the Australian Microanalytical Service. The disulphide had the same electrophoretic mobility at pH 4·15 (see above) as the contaminant that was previously found in samples of thio-IMP (Atkinson, Jackson, Morton & Murray, 1962; see the Results and Discussion section).

Inosine 5'-phosphate. AMP (Sigma Chemical Co., St Louis, Mo., U.S.A.) was deaminated as described by Kaplan (1955). Barium IMP was converted into the potassium salt with Amberlite CG-120 (K^+ form). No contaminants that absorbed light at 254 mμ could be detected on chromatography in $(NH_4)_2SO_4$–phosphate–propanol (see above) or on electrophoresis at pH 4·15 (see above; mobility 0·29 cm.^{2} v^{-1} hr.$^{-1}$).

Xanthosine 5'-phosphate. GMP (C. F. Boehringer und Soehne, Mannheim, Germany) was deaminated and the XMP was purified by anion-exchange chromatography as described by Abrams & Bentley (1959). The product had R_F 0·49 in the $(NH_4)_2SO_4$–phosphate–propanol system (see above).

Nicotinamide–adenine dinucleotide. NAD^+ was obtained from C. F. Boehringer und Soehne.

Reduced nicotinamide–adenine dinucleotide. NADH was used as the sodium salt (Sigma Chemical Co.).

Inosine 5'-phosphate dehydrogenase

Preparation of dehydrogenase. Ascitic fluid and cells (about 10^8 cells/ml.) were collected from C3H mice 10 days after inoculation with about 2×10^7 Ehrlich ascites cells in 0·2 ml. The suspension was frozen and used within 2 weeks of collection. After thawing, the suspension was mixed with an equal volume of Hanks solution (Spector, 1956) and centrifuged at 2000g for 3 min. All stages of the purification were carried out at 2°. The supernatant was discarded and the ascites cells were freed of a few remaining erythrocytes by washing twice with a volume of Hanks solution equal to that of the original suspension. After dispersion in the same volume of 15 mM-phosphate (K^+, pH 7·4) the cells were shaken with no. 14 Ballotini beads (6 g. of beads and 10 ml. of suspension in each batch) for 30 sec. in a Nossal (1953) shaker. In a machine with different shaking characteristics the shaking time had to be decreased to 20 sec. to avoid extraction of interfering enzymes. After centrifuging for 30 min. at 20000g the supernatant fraction (about 11 ml./batch) was collected ('first extract'; Table 1). A portion (1 ml.) of 2% (w/v) protamine sulphate was added for each 300 mg. of apparent protein in the extract, as calculated from $E_{260\,m\mu}$ and $E_{280\,m\mu}$ (Warburg & Christian, 1942). After centrifuging at 20000g for 15 min., the supernatant fluid ('protamine supernatant'; Table 2) was passed through a column of DEAE-cellulose (5 cm. × 1·5 cm.^{2} for 25 ml. of protamine supernatant) that had been equilibrated with 15 mM-phosphate (K^+, pH 7·4). The column was washed with one bed-volume of 15 mM-phosphate. One bed-volume of effluent was discarded and the subsequent effluent was fractionated with $(NH_4)_2SO_4$. Material precipitated between 0·20 and 0·50 saturation (cf. Dixon, 1953) was dissolved in 15 mM-phosphate (K^+, pH 7·4; 1 ml./10 ml. of protamine supernatant) and dialysed with continuous rocking for 1 hr. against 1 l. of 5 mM-phosphate (K^+, pH 7·4) and for 1 hr. against another 1 l. portion of the same buffer. The supernatant solution ('dialysed enzyme'; Table 1) was centrifuged at 20000g for 15 min. and used for kinetic experiments and for studies on the products of the dehydrogenase reaction. Kinetic experiments were carried out within 3 hr. of the end of dialysis.

Assay of dehydrogenase. Enzyme (0·1–0·4 ml.) was added to blank and test cells containing KCl (100 μmoles), NAD^+ (1·5 μmoles) and tris (100 μmoles, adjusted to pH 8·0 with HCl). After equilibration for 4 min. in the thermostatic cell holder of a Beckman DK-2A spectrophotometer the reaction was started by addition of 0·02 ml. of 25 mM-IMP to the test cell (final volumes of cell contents, 2·5 ml.) and

changes in extinction at 290 mμ were recorded. The initial rate of increase was maintained for at least an hour, but the rates were usually measured for the first 15 min. The rate of oxidation of IMP to XMP was calculated by the use of $5{\cdot}4 \times 10^3$ as the net change of molecular extinction coefficients at 290 mμ. $\Delta m\epsilon$ was assumed to be 4·7 for IMP → XMP (Beaven, Holiday & Johnson, 1955; Pabst Laboratories, 1961*a*) and 0·7 for NAD^+ → NADH (Pabst Laboratories, 1961*b*). Dehydrogenase activity was expressed as μmoles of IMP oxidized/min. and the specific activity as μmoles of IMP oxidized/min./mg. of protein. The activity was also measured by recording the extinction at 340 mμ ($\Delta m\epsilon$ 6·2; Horecker & Kornberg, 1948). In studies of the inhibition of IMP dehydrogenase the final concentration of IMP was varied as indicated in Fig. 2, and thio-IMP was added to blank and test cells after the 4 min. equilibration period and about 15 sec. before the IMP.

Analysis of the products of the IMP-dehydrogenase reaction. Dialysed enzyme (0·5 ml. containing 6·8 mg. of protein and 0·01 unit of activity) was added to 0·92 μmole of IMP, 1·5 μmoles of NAD^+, 100 μmoles of KCl and 100 μmoles of tris (adjusted to pH 8·0 with HCl) in a final volume of 2·7 ml. After 1 hr. at 37° the reaction was stopped with 2·5 ml. of 0·5M-trichloroacetic acid. A control tube was incubated in the same way, but the enzyme was added after the trichloroacetic acid. The suspensions were centrifuged at 2° and the precipitates were washed with 2·5 ml. of 0·5M-trichloroacetic acid. The combined supernatants from control and test respectively were extracted with ether (3 × 50 ml.) and the aqueous phases were evaporated at 20–30° in a rotary evaporator. The residues were transferred quantitatively in water to sheets of Whatman 3MM paper (20 cm. × 22·5 cm.) and chromatographed by the descending method at 25° in butan-1-ol–acetic acid–water (20 : 3 : 7, by vol.). After 4 hr. the papers were dried, without heating, in a stream of air. If hypoxanthine (R_F 0·41) and inosine (R_F 0·25) were present they were eluted with 5 ml. of water. The nucleotides, which remained at the origin, were eluted (cf. Atkinson, Burton & Morton, 1961) with 20 ml. of water. After evaporation, the residues were applied as bands (6 cm.) to a sheet (17·5 cm. × 35 cm.) of Whatman 3MM paper with a serrated lower edge; descending chromatography in the $(NH_4)_2SO_4$–phosphate–propanol solvent (see above) was carried out for 16 hr. at 25°. The regions from control and test corresponding to IMP (26–30 cm. migration) and XMP (20–23 cm. migration) were eluted with 5 ml. of water, as were blanks of the same areas. Absorption spectra of the eluates were recorded and their pH values were measured to permit calculation of the concentration of each nucleotide.

RESULTS AND DISCUSSION

Purification and assay of IMP dehydrogenase. The first extract from broken ascites cells was not suitable for studies of the inhibition of IMP dehydrogenase because it contained several interfering activities. The net rate of reduction of NAD^+, measured at 340 mμ, was only about one-fifth of the rate of oxidation of IMP measured at 290 mμ (Table 1) and sometimes less; the discrepancy was due to reoxidation of NADH. Thus a preparation that oxidized 2·6 μm-moles of IMP/min./ml. and catalysed the net reduction of 0·24 μm-mole of NAD^+/min./ml. was found, in a separate experiment, to oxidize 0·04 mM-NADH at a rate of 1·3 μm-moles/min./ml. under the conditions of the assay (for details see the Experimental section). This prevented accurate measurement of the rate of IMP oxidation from the rate of change of extinction at 290 mμ since 13 % of the extinction change in the reaction IMP + NAD^+ → XMP + NADH at pH 8·0 is due to the reduction of NAD^+. It was not possible to record the extinction at the isosbestic wavelength of NAD^+ and NADH (281·5 mμ) because of the high total extinction of enzyme and substrates at this wavelength. In the dialysed enzyme the ratio of the apparent rates of NAD^+ reduction and IMP oxidation, measured at 340 and 290 mμ respectively, was 0·99. Here the error, resulting from reoxidation of NADH, in calculating the rate of oxidation of IMP from the rate of change of extinction at 290 mμ was negligible.

A more serious objection to the use of the first extract in kinetic studies was the presence of enzymes that converted IMP into inosine and hypoxanthine. Thus on incubation of 0·49 μmole of IMP for 1 hr. with NAD^+ and a portion of the first extract (6 mg. of protein; for details see the Experimental section) formation of 0·29 μmole of XMP was accompanied by formation of 0·08 μmole of hypoxanthine and about 0·01–0·02 μmole of inosine; 0·033 μmole of unchanged IMP was recovered. Ascites cells that had not been frozen were not readily freed of erythrocytes and the first extracts then contained xanthine oxidase, which caused a

Table 1. *Properties of fractions obtained in the purification of inosine 5′-phosphate dehydrogenase from Ehrlich ascites cells*

Protein concentrations were calculated from $E_{260\,m\mu}$ and $E_{280\,m\mu}$ (Warburg & Christian, 1942).

Fraction	Volume (ml.)	$E_{280\,m\mu}/E_{260\,m\mu}$	Protein (mg.)	IMP oxidized (μm-moles/min.)	Net formation of NADH (μm-moles/min.)
First extract	31	0·67	297	45	9·75
Protamine supernatant	30	0·94	198	40	9·8
Column effluent	29	0·93	156	47	17·6
0·2–0·5 Saturated $(NH_4)_2SO_4$ fraction	2·2	1·16	50	42	35·5
Dialysed enzyme	2·6	1·16	48	28	27·7

spurious increase of extinction at 290 and 340 mμ due to the oxidation of hypoxanthine by NAD^+. This reaction showed a lag period of several minutes after addition of IMP and with some preparations (e.g. extracts of acetone-dried powder of chicken liver) completely obscured the presence of IMP dehydrogenase. Interference by xanthine oxidase was easily recognized by tests in which hypoxanthine (0·4 μmole) replaced the IMP in the standard assay, or in which 0·2 mM-2-amino-6-formyl-4-hydroxypteridine (Lowry, Bessey & Crawford, 1949) was used to inhibit this enzyme. When the first extract was made from cells that had been frozen and thawed the washed ascites cells were almost free of erythrocytes, and xanthine oxidase was usually undetectable. The dialysed enzyme obtained after ammonium sulphate fractionation was always free of xanthine oxidase and almost free of enzymes that convert IMP into hypoxanthine and inosine. During incubation for 1 hr. (for details see the Experimental section) with dialysed enzyme and NAD^+ 0·535 μmole of IMP gave a mixture of 0·427 μmole of XMP and 0·073 μmole of unoxidized IMP. The total recovery of the two nucleotides (0·500 μmole) was 94% of that in the control (0·530 μmole of IMP).

Stability of IMP dehydrogenase. The enzyme usually lost about 30% of its activity during dialysis for 2 hr. Further dialysis for 16 hr. resulted in the loss of more than half of the original activity. The activity was restored on mixing diffusate and dialysis residue (Fig. 2) but only 2% of the diffusate was needed to restore full activity. The dialysis residue was not reactivated by addition of 2 mM-magnesium chloride or of reduced glutathione (2 mM) to the assay mixture; the nature of this activation is not known. Enzyme used for studies of inhibition has not been reactivated with diffusate. The presence of 0·1 mM-EDTA in all solutions did not prevent loss of activity. In the experiment shown in Fig. 3 no loss of activity was detected between the first and last kinetic measurements.

Purity of thio-IMP used as an inhibitor. Samples of thio-IMP obtained by treatment of cyanoethyl 2′,3′-isopropylidene thio-IMP with acid and alkali to remove protecting groups (Montgomery & Thomas, 1961) had a ratio $E_{255\,m\mu}/E_{321\cdot5\,m\mu}$ 0·21 and contained 7–10% of IMP. The presence of this amount of substrate in a material added to both blank and test cells would cause serious errors in calculating K_i of the thio-IMP. Removal of IMP by anion exchange gave a product containing no detectable contaminants. From its $E_{255\,m\mu}/E_{321\,m\mu}$ ratio (0·045) this product is slightly purer than thio-IMP prepared by Hampton & Maguire (1961) from a 6-chloropurine nucleotide ($E_{255\,m\mu}/E_{322\cdot5\,m\mu}$ 0·056) and has a similar spectroscopic purity to 6-thioinosine (Fox, Wempen, Hampton & Doerr, 1958; cf. Hampton & Maguire, 1961), which had a ratio 0·045, and which had not been heated with alkali during preparation.

The spectrum of the crude barium salt of IMP,

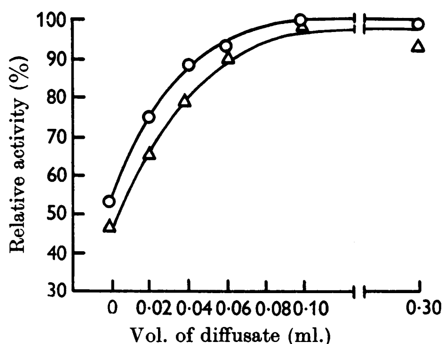

Fig. 2. Restoration of the activity of the dialysis residue of IMP dehydrogenase when assayed in the presence of diffusate. Activities are shown as percentages of the activity before dialysis when measured at 290 mμ (○) and 340 mμ (△). In this experiment the volume of diffusate, after dialysis for 18 hr., was 250 ml. and the dialysis residue contained 66 mg. of protein in 4·8 ml. Portions of the dialysed enzyme (0·1 ml.) were assayed in the usual way (for details see the Experimental section). The volumes of the diffusate shown in the Figure were added to the assay mixtures.

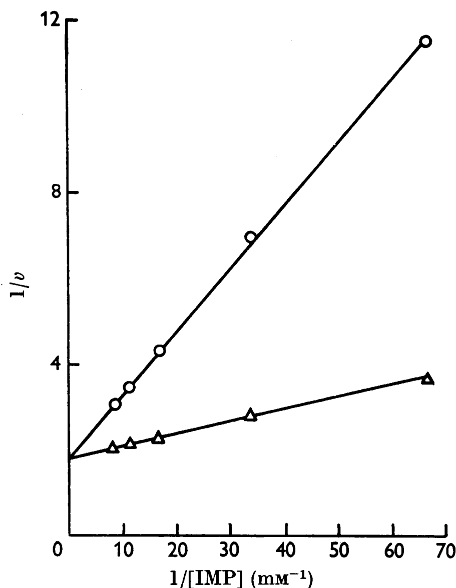

Fig. 3. Plots of v^{-1} against $[IMP]^{-1}$ for the oxidation of IMP to XMP in the presence of IMP dehydrogenase (1·7 mg. of protein/ml.) with (○) and without (△) 0·016 mM-thio-IMP; for details see the text. v, μm-moles/min./mg. of protein.

like published spectra of thio-IMP of synthetic (Hampton, 1962) or biological (Paterson, 1959) origin, showed inflexions near 280 mμ and 290 mμ. The compound responsible for these inflexions was partially purified by electrophoresis (for details of the buffer see the Experimental section) and was found to have two absorption maxima near 280 mμ and 290 mμ. From its rapid conversion into thio-IMP in alkaline solution it was suggested (Atkinson *et al.* 1962) that this might be 5′-phosphoribosyl formylaminoimidazole thiocarboxyamide. This suggestion is unacceptable since the compound has the same spectrum, electrophoretic properties and behaviour in acid and alkali as thio-IMP disulphide that was prepared from thio-IMP by oxidation with iodine. Both the synthetic disulphide and the contaminant are reduced to thio-IMP in 0·5% mercaptoethanol. The spectroscopic and chemical properties of the disulphide closely resemble those of the disulphide of 6-thioinosine (Doerr, Wempen, Clarke & Fox, 1961). Addition of mercaptoethanol to all aqueous solutions of thio-IMP (see the Experimental section) has permitted the isolation of this nucleotide free of detectable disulphide. The presence of 1 mM-triethylamine–1 mM-acetic acid–1 mM-formic acid or of 5 mM-mercaptoethanol in the assay did not inhibit the dehydrogenase. The dialysed enzyme preparation caused no detectable change of $E_{290\,m\mu}$ or $E_{340\,m\mu}$ in the reference cell containing thio-IMP during the assay period.

Kinetic studies with IMP dehydrogenase. The reciprocal (v^{-1}) of dehydrogenase activity was a linear function of the reciprocal of IMP concentration (Fig. 3). K_m for IMP at pH 8·0 and 25° in the presence of 0·6 mM-NAD$^+$ was 1·43 (±0·08) × 10^{-5}M. Values of parameters were calculated by Wilkinson's (1961) method. With a different batch of enzyme a value 1·51 (±0·08) × 10^{-5}M was found. Magasanik *et al.* (1957) reported that K_m for IMP was 1·4 × 10^{-5}M with dehydrogenase from *Aerobacter aerogenes* at pH 7·8 and 25° in the presence of 0·66 mM-NAD$^+$. With an enzyme from pea seeds Turner & King (1961) found that K_m for IMP was 2·6 × 10^{-5}M at pH 8·0 and 25° in the presence of 0·14 mM-NAD$^+$. In the presence of 0·016 mM-thio-IMP the reciprocal of dehydrogenase activity was again a linear function of the reciprocal of IMP concentration (Fig. 3). The extrapolated maximum rate was 0·525 ± 0·018 μm-mole of IMP oxidized/min./mg. of protein, which did not differ significantly ($P < 0{\cdot}001$) from the maximum rate (V 0·528 ± 0·007) in the absence of inhibitor. Thio-IMP is thus a competitive inhibitor of IMP oxidation by NAD$^+$ with this enzyme. K_i for thio-IMP, calculated from the expression

$$\frac{V}{v} = 1 + K_m\left(\frac{1+[\text{thio-IMP}]/K_i}{[\text{IMP}]}\right)$$

was 3·6 (±0·2) × 10^{-6}M. It is not possible to compare these results with those obtained with enzyme from pigeon liver by Salser *et al.* (1960) as their method is rather indirect and does not give initial rates of oxidation. Wilkinson's (1961) method for calculation of K_m and V, together with their standard errors, is readily extended to the calculation of K_i and provides an objective measure of the difference of extrapolated maximum rates in the presence and absence of inhibitor. It is therefore useful in experiments to determine if inhibition is competitive.

Until more is known of the intracellular concentration and distribution of IMP dehydrogenase, IMP and thio-IMP in cells exposed to 6-mercaptopurine it will not be possible to decide if the inhibitory activity described here is the only effect leading to decreased XMP formation in treated cells. However, some approximate estimates can be made. Paterson (1959) found about 0·4 μmole of thio-IMP in 1 ml. of packed ascites cells that had been exposed to 6-mercaptopurine. If this corresponds to an intracellular concentration of about 0·5–1 mM-thio-IMP (i.e. 140–280 times K_i), the concentration of IMP in the cell would have to rise to 2–4 mM to maintain half the uninhibited rate of formation of XMP. There are indications (Klouven, 1962; cf. Schmitz, Hart & Ried, 1955) that the concentration of IMP in ascites cells is much less than this and it is evident that thio-IMP is potentially a powerful inhibitor of the formation of XMP under physiological conditions.

SUMMARY

1. 6-Thioinosine 5′-phosphate was freed of its disulphide and of inosine 5′-phosphate by anion-exchange chromatography in the presence of mercaptoethanol. The disulphide was prepared from 6-thioinosine 5′-phosphate by oxidation with iodine.

2. Inosine 5′-phosphate–nicotinamide–adenine dinucleotide oxidoreductase was prepared from Ehrlich ascites cells in a sufficiently pure state for kinetic studies of xanthosine 5′-phosphate formation. The validity of a spectrophotometric assay of the dehydrogenase was confirmed by chromatographic methods.

3. At pH 8·0 and 25° in the presence of 40 mM-potassium chloride and 0·6 mM-nicotinamide–adenine dinucleotide K_m for inosine 5′-phosphate was 1·4 × 10^{-5}M. 6-Thioinosine 5′-phosphate was shown to be a competitive inhibitor of the dehydrogenase. K_i under these conditions was 3·6 × 10^{-6}M.

4. The significance of this inhibition in the biological action of 6-mercaptopurine is discussed.

We are grateful to Mr H. R. Lovelock who made the barium thio-IMP for this work.

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The Isolation of *myo*Inositol Pentaphosphates from Hydrolysates of Phytic Acid

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(*Received* 11 *March* 1963)

The mixture of inositol phosphoesters produced by the partial dephosphorylation of phytic acid by the enzyme 'phytase' was first investigated by Posternak & Posternak (1929), and later by Courtois (1951), by fractional-precipitation techniques. Subsequently Desjobert & Petek (1956) showed that *myo*inositol polyphosphate intermediates from the phytase reaction, as isolated by the methods of Posternak & Posternak (1929), gave discrete phosphate zones on paper chromatograms. They claimed to be able to separate the hydrolysate into seven zones, corresponding to hexa-, penta-, tetra-, tri-, di- and mono-esters, together with orthophosphate. However, their claim to be able to separate pentaphosphate from hexaphosphate was later questioned by Preece, Grav & Wadham (1960), who were unable to achieve such a separation on paper chromatograms. The latter workers stated that it was uncertain whether a pentaphosphate had ever been isolated.

The application of ion-exchange chromatography to the separation of inositol phosphates was first attempted by Smith & Clark (1952). Stepwise elution with hydrochloric acid of increasing concentration separated the hydrolysate into a number of components whose phosphorus : inositol ratios were determined. The identity of nine separated compounds, including two pentaphosphates, was tentatively established, but no attempt was made to isolate specimens of any of them for further