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THE EFFECT OF 6-MERCAPTOPURINE AND ITS DERIVATIVES ON PURINE NUCLEOTIDE INTERCONVERSIONS

by

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Being a thesis submitted in fulfilment of the requirements
for the degree of
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SUMMARY

1. Inosine 5'-phosphate dehydrogenase, adenylosuccinate synthetase and adenylosuccinate lyase were extracted from Ehrlich ascites-tumour cells and purified sufficiently for kinetic studies. Some properties of each of these enzymes were examined. 6-Thioinosine 5'-phosphate was purified and studied as an inhibitor of each of the above enzymes.

The dehydrogenase was competitively inhibited (K_i 3.6 μM at pH 8.0) and the synthetase was non-competitively inhibited (K_i 0.2 - 0.4 mM over the pH range 7 - 7.8). 6-Thioinosine 5'-phosphate was a competitive inhibitor of adenylosuccinate lyase; the inhibitor constant decreased from 0.8 mM at pH 5.64 to 0.1 mM at pH 8.38 (12 pH values). The Michaelis constant for adenylosuccinate increased from 6 μM to 70 μM in the same range. From a comparison of the activities and kinetic parameters of the dehydrogenase, synthetase and lyase in Ehrlich ascites-tumour cells, it was concluded that the first of these activities is most sensitive to inhibition by 6-thioinosine 5'-phosphate.

2. Extracts containing phosphoribosyltransferase activity with adenine, guanine, hypoxanthine and 6-mercaptopurine were isolated from Ehrlich ascites-tumour cells sensitive and resistant to 6-mercaptopurine. Techniques were developed to assay these activities using [8-^{14}C]-labelled purines.

At pH 7.8 and 25° Michaelis constants for adenine, guanine, hypoxanthine and 6-mercaptopurine were 0.9, 2.8, 11.0 and 7.5 μM respectively with extract from sensitive cells. Adenine phosphoribosyltransferase was not inhibited by 6-mercaptopurine and guanine and hypoxanthine phosphoribosyltransferases were competitively inhibited by this drug (inhibitor constants 4.7 and 8.3 μM). These kinetic parameters were not significantly different from those obtained with extracts from ascites-tumour cells resistant to 6-mercaptopurine. It was concluded that resistance to 6-mercaptopurine in these cells was not due to decreased 6-mercaptopurine phosphoribosyltransferase activity or to altered kinetic properties of the phosphoribosyltransferases studied.

3. Some experiments were carried out with intact Ehrlich ascites-tumour cells to test the effect of 6-mercaptopurine on the incorporation of [U-^{14}C]glycine, [8-^{14}C]adenine and [8-^{14}C]hypoxanthine into acid-soluble and nucleic acid purine nucleotides. Incorporation of [U-^{14}C]glycine into adenine and guanine nucleotides was inhibited to about the same extent by 6-mercaptopurine. Incorporation of adenine was unaffected by 6-mercaptopurine, but the drug markedly inhibited the incorporation of hypoxanthine into nucleotide material.

The experiments indicated that utilization of preformed purines is a more efficient process in ascites-tumour cells than synthesis of purines from non-cyclic precursors.

4. 6-Thioinosine 5'-triphosphate and nicotinamide-6-mercaptopurine dinucleotide were synthesised and purified. The triphosphate was shown to be a substrate of ATP:NMN adenylyltransferase from liver nuclei and to compete with ATP in the reaction catalysed by this enzyme (inhibitor constant 20 μM). Nicotinamide-6-mercaptopurine dinucleotide was a substrate of yeast alcohol dehydrogenase, potato pyrophosphatase and ox spleen NAD-ase.

5. It was concluded that part of the inhibitory activity of 6-mercaptopurine on Ehrlich ascites-tumour cells may be due to inhibition of guanine and hypoxanthine phosphoribosyltransferases by this drug. Chemotherapy with compounds that liberate free 6-mercaptopurine and 6-thioinosine 5'-phosphate inside cells was discussed.

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Declaration

I hereby declare that the work presented in this thesis has been performed by myself, except where otherwise stated in the text, and that it has not been submitted in any previous application for a degree.

Preface

Part of the work described in this thesis has been published or accepted for publication. The publications, in the order in which they were submitted, are listed below.

(i) Nicotinamide-6-mercaptopurine dinucleotide and related compounds : potential sources of 6-mercaptopurine nucleotide in chemotherapy.
by M. R. Atkinson, R. K. Morton, J. F. Jackson & A. W. Murray (1962). Nature, Lond., 196, 35.

(ii) Inhibition of inosine 5'-phosphate dehydrogenase from Ehrlich ascites-tumour cells by 6-thioinosine 5'-phosphate.
by M. R. Atkinson, R. K. Morton & A. W. Murray (1963). Biochem. J. 89, 167.

(iii) Inhibition of adenylosuccinate synthetase and adenylosuccinate lyase from Ehrlich ascites-tumour cells by 6-thioinosine 5'-phosphate.
by M. R. Atkinson, R. K. Morton & A. W. Murray (1964). Biochem. J. 92, 554.

(iv) Inhibition of purine phosphoribosyltransferases of Ehrlich ascites-tumour cells by 6-mercaptopurine.
by M. R. Atkinson & A. W. Murray (1964). Biochem. J. (accepted for publication).

(v) Inhibition by 6-mercaptopurine of purine phosphoribosyltransferases from Ehrlich ascites-tumour cells that are resistant to this drug.
by M. R. Atkinson & A. W. Murray (1964). *Biochem. J.* (accepted for publication).

Abbreviations

Where possible the abbreviations and other conventions of the Biochemical Journal have been used:

AMP	adenosine 5'-phosphate
ATP	adenosine 5'-triphosphate
DEAE-cellulose	diethylaminoethyl cellulose
EDTA	ethylene diamine tetra acetic acid
GMP	guanosine 5'-phosphate
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
IMP	inosine 5'-phosphate
ITP	inosine 5'-triphosphate
NAD	nicotinamide-adenine dinucleotide
$NADH_2$	reduced nicotinamide-adenine dinucleotide
NAD-ase	nicotinamide-adenine dinucleotidase
NADP	nicotinamide-adenine dinucleotide phosphate
$NADPH_2$	reduced nicotinamide-adenine dinucleotide phosphate
NMN	nicotinamide mononucleotide
PRPP	5-phosphoribosyl pyrophosphate
Thio-IMP	6-thioinosine 5'-phosphate
Thio-IMP disulphide	bis(9-β-D-5'-phosphoribofuranosyl-purin-6-yl)
Tris	tris (hydroxymethyl) amino methane
XMP	xanthosine 5'-phosphate

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INTRODUCTION

6-Mercaptopurine was first synthesised and tested as an inhibitor of growth as part of a general study of the relationship between the chemical structure of purines and pyrimidines and their influence on the biosynthesis of nucleic acids. During these studies the drug was shown to inhibit the growth of *Lactobacillus casei* (Elion, Hitchings & Vanderwerff, 1951). The inhibition could be reversed by any of the four naturally-occurring purine bases (adenine, xanthine, hypoxanthine and guanine), suggesting that 6-mercaptopurine was involved directly with purine metabolism. The potential importance of 6-mercaptopurine as a chemotherapeutic drug was indicated by Clarke *et al.* (1953) who showed that this compound effectively inhibited the growth of the Crocker mouse sarcoma and that treatment frequently resulted in loss of viability of the tumour cells. This work prompted the clinical evaluation of 6-mercaptopurine (Burchenal *et al.*, 1953) and the drug was found to give remissions in about 30% of children suffering from acute leukaemia. Toxic effects at the dosage levels used were limited to slight bone marrow depression in some patients. Since this initial work there have been numerous reports on the clinical use of 6-mercaptopurine and the drug is widely used for treatment of acute leukaemia, in children and adults, either alone or in combination with hormones or analogues of folic acid (for a review see Galton, 1957). Treatment with 6-mercaptopurine,

as with other anti-leukaemic drugs, invariably results in the development of resistance, and patients no longer respond to treatment. This relapse follows a period of remission during which the patient shows little or no clinical evidence of the disease.

Studies on the mechanism of action of 6-mercaptopurine may lead to the design of more effective chemotherapeutic drugs; in addition such studies would undoubtedly clarify many aspects of purine metabolism. The introduction which follows will deal with the metabolism of 6-mercaptopurine in relation to the mechanisms that have been proposed to explain the inhibition of cellular growth by this drug; the biochemical basis for the development of resistance to 6-mercaptopurine will also be discussed.

The metabolism of 6-mercaptopurine

Many enzymes that catalyse the anabolism and catabolism of naturally-occurring purine bases catalyse analogous reactions with 6-mercaptopurine. Thus in the presence of xanthine oxidase from milk (Silberman & Wyngaarden, 1961) or from bacteria (Carey & Mandel, 1960), 6-mercaptopurine is oxidized to 6-thiouric acid. 6-Thiouric acid is also a major metabolite of 6-mercaptopurine in mouse urine (Elion, Bieber & Hitchings, 1954) and human urine (Hamilton & Elion, 1954). The intermediate oxidation product appears to vary with the source of enzyme. Bergman & Ungar (1960), using milk xanthine oxidase, obtained evidence that the 8-hydroxy derivative (6-mercapto-8-hydroxypurine)

was an intermediate, and Carey & Mandel (1960) isolated the 2-hydroxy derivative (6-thioxanthine) after treatment of resting cells of *Bacillus cereus* with 6-mercaptopurine. It has been suggested that the same purine derivative can be oxidized along a different pathway by xanthine oxidase from bacteria and from mammalian sources (Bergman, Kwiety, Levin & Brown, 1960). Both 6-mercaptopurine and 6-thiouric acid are competitive inhibitors of oxidation of xanthine by milk xanthine oxidase (Silberman & Wyngaarden, 1961). It is significant that the final product of xanthine oxidase action, 6-thiouric acid, is ineffective as an inhibitor of the growth of bacterial (Carey & Mandel, 1960) and of tumour cells (Clarke, Elion, Hitchings & Stock, 1958), and hence oxidation protects the cells against the inhibitory action of 6-mercaptopurine.

In some microorganisms 6-mercaptopurine is converted into hypoxanthine, which is then incorporated into purine nucleotides and into adenine and guanine of nucleic acids (Balis & Hutchison, 1957; Brockman, Bennett & Skipper, 1957a; Balis, Hylin, Coultas & Hutchison, 1958a; Carey & Mandel, 1960). Removal of sulphur also occurs in mice (Elion *et al.*, 1954) and man (Hamilton & Elion, 1954), as indicated by the excretion of sulphate in the urine. In bacteria the released sulphate is rapidly incorporated into protein, and treatment with [^{35}S]6-mercaptopurine results in extensive labelling of the insoluble material remaining on treatment of *B. cereus* with hot acid (Carey & Mandel, 1960). Elion, Callahan, Rundles & Hitchings

(1963a) have recently made a detailed study of the urinary metabolites of 6-mercaptopurine in mice and man. The levels of 6-thiouric acid and sulphate in the urine of mice after administration of 6-mercaptopurine could be decreased by simultaneous treatment with 4-hydroxypyrazolo (3,4-d)pyrimidine, an inhibitor of xanthine oxidase. This decrease in 6-mercaptopurine catabolism resulted in an increase in the anti-tumour activity of the drug against Adenocarcinoma 755 in mice (see also Elion _et al._, 1963b). In the mouse 6-thiouric acid gave rise to sulphate after cleavage by uricase (Elion _et al._, 1963a). In man, however, there is no evidence of uricase action, and excretion of thiouric acid but not of sulphate was inhibited by 4-hydroxypyrazolo (3,4-d)pyrimidine. 6-Methylsulphinylpurin-8-ol and 6-methylthiopurin-2, 8-diol have been identified as urinary metabolites in man (Elion, Rundles & Hitchings, 1964) thus suggesting that a major route for the formation of sulphate from 6-mercaptopurine may be through an S-methyl derivative. 6-Methylmercaptopurine has been identified by Sarcione & Stutzman (1959) as an excretion product of 6-mercaptopurine; these workers found that this compound inhibited incorporation of formate into the nucleic acids of sarcoma-180 ascites-tumour cells _in vitro_. The 6-methyl derivative has been synthesised (Elion, Burgi & Hitchings, 1952) and shown to have a slight inhibitory effect on the Crocker mouse sarcoma, but to have no effect on the growth of _L. Casei_ (Clarke _et al._, 1958). Elion _et al._ (1963a) tested 6-methylmercaptopurine against experimental tumours and chronic

granulocytic leukaemia in adults and concluded that much of the effect of this compound may have been due to dealkylation to 6-mercaptopurine.

It has been suggested that increased chemotherapeutic activity may result if the sulfhydryl group of 6-mercaptopurine could be protected against oxidation; the protective group may also make the compound a poorer substrate for xanthine oxidase. A compound which has shown some promise in this respect is 6-mercaptopurine-S-glucoside (Bieber & Goodman, 1958; Clarke & Hitchings, 1958). The glucoside is split enzymically to 6-mercaptopurine and glucose, and the required thioglucosidase has been found in a number of mammalian tissues (including tumours), in plant tissues, and in microorganisms (Goodman _et al._, 1959). 6-Mercaptopurine-S-glucoside has anti-tumour activity, but it is rapidly cleared from the body, and has poor cellular penetration (Hitchings, Fouts, Philips & Sternberg, 1958). A more promising type of compound may be the S-heterocyclic thiopurines (see Elion _et al._, 1963a) one of which, 6-(1-methyl-4-nitro-5-imidazolyl)thiopurine, showed chemotherapeutic advantages over the parent compound (Elion, Bieber & Hitchings, 1960; Elion _et al._, 1961).

6-Mercaptopurine phosphoribosyltransferase catalyses the reaction between 6-mercaptopurine and 5-phosphoribosyl pyrophosphate to form the 5'-phosphoribosyl derivative (6-thioinosine 5'-phosphate). Purine phosphoribosyltransferases, which catalyse the general reaction:

base + 5-phosphoribosyl pyrophosphate ⇌ 5'-nucleotide + pyrophosphate

are widespread in bacterial and animal tissues, and catalyse the above reaction with the naturally-occurring purine bases (adenine, xanthine, guanine and hypoxanthine) and many structural analogues of these bases (for a recent review see Handschumacher & Welch, 1960). Lukens & Herrington (1957) described the enzymic synthesis of 6-thioinosine 5'-phosphate from 6-mercaptopurine and 5-phosphoribosyl pyrophosphate, with an enzyme partially purified from ox liver. The enzyme preparation was also active with hypoxanthine and guanine, but not with adenine. The phosphoribosyltransferase responsible for the conversion of adenine into adenosine 5'-phosphate and of 5-amino-4-imidazolecarboxamide into its 5'-nucleotide has been partially purified from ox liver (Flaks, Erwin & Buchanan, 1957) and from yeast (Kornberg, Lieberman & Simms, 1955a). The enzyme from liver was shown to be free of activity with guanine, hypoxanthine and 6-mercaptopurine. Carter (1959) partially purified an enzyme from _Escherichia coli_ that had activity with guanine, hypoxanthine, and 6-mercaptopurine. Thus although several distinct purine phosphoribosyltransferases exist, there is little information available on the specificity of these enzymes towards structural analogues of the purine bases. However, Way & Parks (1958) obtained pig liver extracts which formed nucleotides from guanine and hypoxanthine but which were inactive with 6-mercaptopurine. Many tumour cells have a high capacity for the synthesis of 6-thioinosige 5'-phosphate from 6-mercaptopurine, and this nucleotide has been shown to accumulate in intact cells _in vivo_ (Paterson, 1959;

Brockman, Roosa, Law & Stutts, 1962), *in vitro* (Paterson, 1959), and in cell culture (Brockman, Kelley, Stutts & Copeland, 1961a). 6-Thioinosine 5'-phosphate has also been isolated from bacterial cell cultures after treatment with 6-mercaptopurine (Carey & Mandel, 1960).

6-Thioinosine 5'-di- or triphosphate could not be detected in Ehrlich ascites-tumour cells (Paterson, 1959) or in L1210 ascites-tumour cells (Brockman, 1960) after administration of 6-mercaptopurine, and it is likely that 6-thioinosine 5'-phosphate is not phosphorylated further. The further phosphorylation of 6-thioinosine 5'-phosphate under some conditions can not be completely discounted as Way, Dahl & Parks (1959) have given indirect evidence for the formation of 6-thioinosine 5'- triphosphate from 6-thioinosine 5'-phosphate with an enzyme preparation from ox liver; a coupled assay with pyruvate kinase and lactate dehydrogenase was used. Any hypothesis for 6-mercaptopurine action based on its incorporation into nucleic acids would require formation of the di- and triphosphates of 6-thioinosine.

Bieber *et al*. (1961) reported that some radioactivity from [^{35}S] 6-mercaptopurine was incorporated into both RNA and DNA of several rodent tumours; however, a low level of incorporation was obtained and no 6-mercaptopurine derivatives were isolated from the nucleic acid material. Similar incorporation was obtained in tumours sensitive and resistant to 6-mercaptopurine. Recent studies indicate that much of the radioactivity associated with the nucleic acid fraction

may be unspecifically bound (Hansen & Nadler, 1961; Hansen, Bennett & Nadler, 1962). No evidence has been obtained for the incorporation of 6-mercaptopurine into the nucleic acids of _B. cereus_ (Carey & Mandel, 1960) or of mouse leukaemic cells _in vivo_ (Brockman, 1960) or in cell culture (Brockman _et al._, 1962). Carey & Mandel (1960) calculated that the upper limit for incorporation into _B. cereus_ was one molecule of 6-mercaptopurine in a molecule of RNA with a molecular weight of 3×10^6. Synthetic 6-thioinosine 5'-diphosphate was not a substrate for polynucleotide phosphorylase from _Micrococcus lysodeikticus_ (Carbon, 1962), and the diphosphate inhibited the polymerization of adenosine 5'-diphosphate. Škoda (1961) has suggested that the phosphorylase can catalyse the polymerization only of ribonucleoside diphosphates that are incorporated into microbial RNA.

The biological activity of 6-mercaptopurine and the biochemical basis of resistance to this drug.

Early studies on the mechanism of action of 6-mercaptopurine clearly indicated that purine metabolism was directly involved. These studies were carried out with a strain of _L. casei_ that could make purine nucleotides from non-cyclic precursors when grown in the presence of folic acids (Elion & Hitchings, 1950). In the absence of folic acids growth was supported, in the presence of thymine, by either adenine, xanthine, or hypoxanthine. 6-Mercaptopurine markedly inhibited the

growth of *L. casei* when growth was supported by *de novo* purine biosynthesis (Elion, Singer & Hitchings, 1953), and this inhibition was reversed when small amounts of adenine, xanthine, guanine or hypoxanthine were included in the medium (see also Balis *et al.*, 1957). A strain of *L. casei*, resistant to growth inhibition by 6-mercaptopurine, could not utilize hypoxanthine to support growth.

Since this work the development of resistance has been used as a major biochemical technique in studying the mechanism of action of 6-mercaptopurine. It has been shown that *Streptococcus faecalis* resistant to 6-mercaptopurine, unlike the sensitive parent strain, can not use free hypoxanthine, guanine or 6-mercaptopurine for nucleotide biosynthesis (Brockman, Sparks & Simpson, 1957b; Brockman *et al.*, 1957a). The sensitive strain converted [^{35}S]6-mercaptopurine into a compound with the properties of a nucleotide, and the resistant strain did not carry out this conversion. It was suggested that resistance in these cells may have been due to decreased formation of 6-mercaptopurine nucleotide, which was therefore identified as the actual inhibitory metabolite derived from 6-mercaptopurine (see also Balis *et al.*, 1958a, 1959b; Brockman, Sparks, Hutchison & Skipper, 1959a). Most available evidence implicating 6-thioinosine 5'-phosphate as the growth-inhibiting metabolite is based on the observation that, in many instances, the development of resistance has been associated with an apparent loss or decrease of 6-mercaptopurine phosphoribosyltransferase activity in soluble extracts from the resistant cells.

Thus Brockman (1960) found that leukaemia L1210 ascites-tumour cells that were sensitive to inhibition by 6-mercaptopurine could convert [8-^{14}C]hypoxanthine and [^{35}S]6-mercaptopurine into nucleotide derivatives _in vivo_. [8-^{14}C]Hypoxanthine extensively labelled the adenine and guanine moieties of nucleic acids; radioactive 6-thioinosine 5'-phosphate was isolated from the cells after treatment with [^{35}S]6-mercaptopurine. Similarly soluble enzyme preparations from sensitive cells catalysed the conversion of adenine, guanine, hypoxanthine, and 6-mercaptopurine into adenosine 5'-phosphate, guanosine 5'-phosphate, inosine 5'-phosphate, and 6-thioinosine 5'-phosphate respectively, in the presence of 5-phosphoribosyl pyrophosphate. However, a line of L1210 ascites-tumour cells resistant to 6-mercaptopurine did not metabolize hypoxanthine or 6-mercaptopurine to a significant extent. Soluble extracts from these resistant cells were deficient in guanine, hypoxanthine, and 6-mercaptopurine phosphoribosyltransferases but retained activity with adenine. Similarly extracts from strains of _S. faecalis_ resistant to 6-mercaptopurine (Brockman, Debavadi, Stutts & Hutchison, 1961b) had greatly reduced guanine, hypoxanthine and 6-mercaptopurine phosphoribosyltransferase activities but retained adenine and xanthine phosphoribosyltransferase activity. Strains were also developed that were resistant to 8-azaxanthine and that specifically lost the capacity to form xanthosine 5'-phosphate and 8-azaxanthosine 5'-phosphate; resistance to 8-azaadenine resulted in decreased conversion of 8-azaadenine into 8-azaadenosine 5'-phosphate and of adenine into

adenosine 5'-phosphate. On the basis of experiments such as these it has been postulated that adenine and xanthine phosphoribosyltransferases are separate enzymes and that a third enzyme is responsible for the conversion of guanine and hypoxanthine (and analogues of these compounds) into their corresponding nucleotides (for a recent review see Brockman, 1963). However, evidence has been obtained from mutants of *Salmonella typhimurium* that guanine and hypoxanthine phosphoribosyltransferases are separate enzymes (Kalle, Gots & Abramson, 1960).

A large number of investigators have found decreased phosphoribosyltransferase activity associated with resistance to 6-mercaptopurine in bacterial and tumour cells; references to these workers and to the cell type that was studied are listed in Table 1.

Some studies have been published showing decreased purine incorporation into cells that are resistant to 6-mercaptopurine *in vivo* (e.g. Brockman *et al.*, 1957b; Tomizawa & Aronow, 1960). The mechanism of resistance cannot be stated in these cases as factors other than a simple loss of an enzyme or enzymes may be operating. Thus a resistant line of Ehrlich ascites-tumour cells (Paterson, 1959, 1960a, 1960b & 1962; Paterson & Hori, 1962) did not convert 6-mercaptopurine into 6-thioinosine 5'-phosphate *in vivo*. However, extracts from the resistant cells contained as much 6-mercaptopurine phosphoribosyltransferase activity as extracts from sensitive cells, and it was suggested that resistance was due to a specific permeability barrier preventing the entry of 6-mercaptopurine into the cell; alternatively,

Table I. Examples of resistance to 6-mercaptopurine accompanied by a decrease in purine phosphoribosyltransferase activity

In each case soluble extracts from the resistant cells contained less apparent 6-mercaptopurine phosphoribosyltransferase activity.

	Cell type	References
A:	Microorganisms	
	S. faecalis	Brockman *et al.*, 1959b; Salser, Hutchison & Balis, 1960; Brockman *et al.*, 1961b.
	S. typhimurium	Kalle *et al.* 1960; Kalle & Gots, 1961.
B:	Mouse neoplasms in vivo	
	L1210 Leukaemia	Brockman *et al.*, 1959c; Brockman *et al.*, 1959b; Brockman, 1960.
	Adenocarcinoma 755	Brockman, R.W. & Kelley, G.G., cited by Brockman, 1963.
	Ehrlich ascites-tumour cells	Kimball & LePage, 1964
C:	Mammalian cells in culture	
	Fibroblasts (AMK-2)	Lieberman & Ove, 1960.
	P 388 Leukaemia	Brockman *et al.*, 1962; Davidson, Roosa & Law, 1962.
	Human bone-marrow cells (D98/AH)	Szybalski, Szybalska & Brockman, 1961.
D:	Human neoplasm in culture	
	KB cells	Brockman, R.W. & Kelley, G.G., cited by Brockman, 1963.
	Epidermal carcinoma cells	Brockman *et al.*, 1961a; Wheeler, Kelley & Montgomery, 1961.

since many compounds enter Ehrlich ascites-tumour cells by a process of active transport (Christensen, 1960) the apparent permeability barrier may be due to a change in such a transport system. In contrast Kimball & LePage (1964) reported isolation of a strain of Ehrlich ascites-tumour cells, resistant to 6-mercaptopurine, which had decreased purine phosphoribosyltransferase activity, indicating that different mechanisms of resistance may develop even within one particular cell type. This is also apparent in microorganisms, as Zimmerman & Magasanick (1964) isolated a strain of S. typhimurium which was resistant to 6-mercaptopurine and did not incorporate hypoxanthine and guanine efficiently into nucleotides in intact cells; extracts from these cells had the same hypoxanthine, adenine, and 6-mercaptopurine phosphoribosyltransferase activity as sensitive cells. However, Kalle & Gots (1961) had earlier isolated a separate resistant strain of S. typhimurium which had decreased hypoxanthine and 6-mercaptopurine phosphoribosyltransferase activity. Bieber et al. (1961) refer to experiments indicating that cell-free extracts from a line of Adenocarcinoma 755 resistant to 6-mercaptopurine had an increased activity for the conversion of 6-mercaptopurine and hypoxanthine into 6-thioinosine 5'-phosphate and inosine 5'-phosphate respectively. In a later communication (Bieber & Pomales, 1962) it was reported that this resistant tumour incorporated both hypoxanthine and guanine into nucleic acids in vivo. Davidson & Winter (1964)

found no loss of guanine, hypoxanthine or 6-mercaptopurine phosphoribosyltransferases in extracts of leukaemic cells from patients with chronic or acute leukaemia that had become resistant to 6-mercaptopurine. Balis *et al.* (1958a) considered that resistance to 6-mercaptopurine in a strain of *S. faecalis* was due to an increased capacity of this strain to synthesize purines from non-cyclic precursors. Scott & Marino (1962) reported that resistant human leukaemic leucocytes cleaved sulphur from [8-^{14}C]6-mercaptopurine with consequent labelling of nucleic acid adenine and guanine, and considered that this may have been important in clinical resistance to this drug.

It is of interest that 6-thioinosine has about the same inhibitory effect as 6-mercaptopurine on Adenocarcinoma 755 in mice and on *S. faecalis* (Skipper *et al.*, 1957). Resistance to 6-mercaptopurine was accompanied by cross-resistance to 6-thioinosine. Similarly the nucleoside was no more effective than 6-mercaptopurine in the clinical treatment of acute leukaemia that was sensitive or resistant to this drug (Pierce, Hall & Ozoa, 1961). A kinase for the conversion of 6-thioinosine into 6-thioinosine 5'-phosphate or of inosine into inosine 5'-phosphate has not been isolated, and it is likely that much of the formation of 6-thioinosine 5'-phosphate from 6-thioinosine requires the prior formation of 6-mercaptopurine. Paterson (1960b) has reported that extracts from Ehrlich ascites-tumour cells rapidly convert 6-thioinosine into 6-mercaptopurine. Indirect evidence for the existence of an inosine kinase was provided

by Brockman (1963) who found that 6-mercaptopurine inhibited the conversion of hypoxanthine but not of inosine into the purine nucleotides of _S. faecalis_.

9-Alkyl substitution of 6-mercaptopurine resulted in compounds that were active against Adenocarcinoma 755 _in vivo_ (Montgomery, 1959) and that inhibited 6-mercaptopurine-resistant human epidermoid carcinoma cells in culture (Kelley _et al._, 1962). Hansen, Giles & Nadler (1962) found no evidence for the dealkylation of 9-methyl or 9-ethyl-6-mercaptopurine in the rat, and it is probable that the 9-alkyl derivatives have a different mechanism of action not requiring prior formation of 6-thioinosine 5'-phosphate.

Whatever the actual mechanism of resistance to 6-mercaptopurine, in almost all cases the end result is decreased formation of 6-thioinosine 5'-phosphate within the resistant cells, and it has been suggested that resistance might be overcome if 6-thioinosine 5'-phosphate could be introduced into cells as such (Montgomery, Thomas & Schaeffer, 1961). Tomizawa & Aronow (1960) found that the growth of L cells in culture, under conditions such that an external supply of purines was required for growth, was supported by inosine 5'-phosphate but not by hypoxanthine or inosine; a 6-mercaptopurine resistant line of these cells was inhibited by 6-thioinosine 5'-phosphate (Tomizawa, 1962). However, 6-thioinosine 5'-phosphate did not inhibit a line of leukaemia L1210 that was resistant to 6-mercaptopurine (Montgomery, Schabel & Skipper, 1962) or a resistant line of human

epidermal carcinoma in cell culture (Kelley et al., 1962), and it has been reported that nucleotides are not incorporated intact into mammalian cells (Roll, Weinfeld, Carroll & Brown, 1956; Weinfeld et al., 1957). Montgomery et al. (1961) suggested that esters of nucleotides might penetrate the cell wall and then be metabolized to the nucleotide itself; these workers described the synthesis of several simple esters of 6-thioinosine 5'-phosphate. None of these esters had marked activity on resistant human epidermal carcinoma cells (Kelley, G.G., cited by Montgomery et al., 1963). However, a derivative of 6-thioinosine 5'-phosphate, bis (thioinosine) 5', 5"-phosphate, was found to markedly inhibit the line of cells that was resistant to 6-mercaptopurine (Montgomery et al., 1963). It was postulated that the compound was able to enter resistant cells, and was then cleaved to give 6-thioinosine 5'-phosphate; this was supported by the observation that venom phosphodiesterase cleaved bis (thioinosine) 5',5"-phosphate and that cleavage was also obtained with a soluble extract from disrupted cells (Brockman, R.W., Chumley, S. & Hays, F.; cited by Montgomery et al., 1963). Similar considerations led to the synthesis of nicotinamide-6-mercaptopurine dinucleotide (Atkinson, Jackson, Morton & Murray, 1962; cf. section IV of this thesis). This compound was converted into adenosine 5'-phosphate and 6-thioinosine 5'-phosphate by nucleotide pyrophosphatase.

Because of the close structural similarity between 6-thioinosine 5'-phosphate and inosine 5'-phosphate, it is logical to assume that 6-mercaptopurine (through its metabolite 6-thioinosine

5'-phosphate) might interfere with purine nucleotide interconversions (see Fig. 1). That this may be the case was first suggested by Elion et al., 1953 . These workers isolated a strain of L. casei that was resistant to 6-mercaptopurine; the mutant could not grow on hypoxanthine, and used adenine less efficiently than guanine or xanthine. It was postulated that if the main pathway for the conversion of adenine into guanine was through a hypoxanthine derivative, 6-mercaptopurine might interfere with the conversion of this derivative into a guanine metabolite. The hypothesis assumed that the conversion of guanine into adenine involved a separate route. This was a remarkably accurate prediction of the pathways involved, as it is now considered that the main pathway for the conversion of adenine into guanine involves deamination of adenosine 5'-phosphate to form inosine 5'-phosphate, followed by the conversion of inosine 5'-phosphate into guanosine 5'-phosphate. Inosine 5'-phosphate is regenerated from guanosine 5'-phosphate by a separate enzyme and can then be converted into adenosine 5'-phosphate (see Fig. 1.). It is probable that, at least in bacteria, adenosine 5'-phosphate can also be converted into guanosine 5'-phosphate through inosine 5'-phosphate by a series of enzymic reactions essential for histidine synthesis (Magasanik & Karibian, 1960; Moyed & Magasanik, 1960). There is little information available, however, concerning the inhibition of isolated enzyme systems by 6-thioinosine 5'-phosphate. Salser et al., (1960) showed that oxidation of inosine 5'-phosphate to xanthosine 5'-phosphate by

Fig. 1. Schematic representation of interconversion of purine nucleotides

(1) Inosine 5'-phosphate dehydrogenase. (Abrams & Bentley, 1955; Gehring & Magasanik, 1955; Lagerkvist, 1955, 1958; Magasanik, Moyed & Gehring, 1957; Turner & King, 1961).

(2) Xanthosine 5'-phosphate aminase. (Abrams & Bentley, 1955; Lagerkvist, 1955, 1958; Moyed & Magasanik, 1957).

(3) Guanosine 5'-phosphate reductase. (Mager & Magasanik, 1960).

(4) Adenylosuccinate synthetase. (Abrams & Bentley, 1955; Lieberman, 1956).

(5) Adenylosuccinate lyase. (Abrams & Bentley, 1955; Carter & Cohn, 1956).

(6) Adenylate deaminase. (Lee, 1960a).

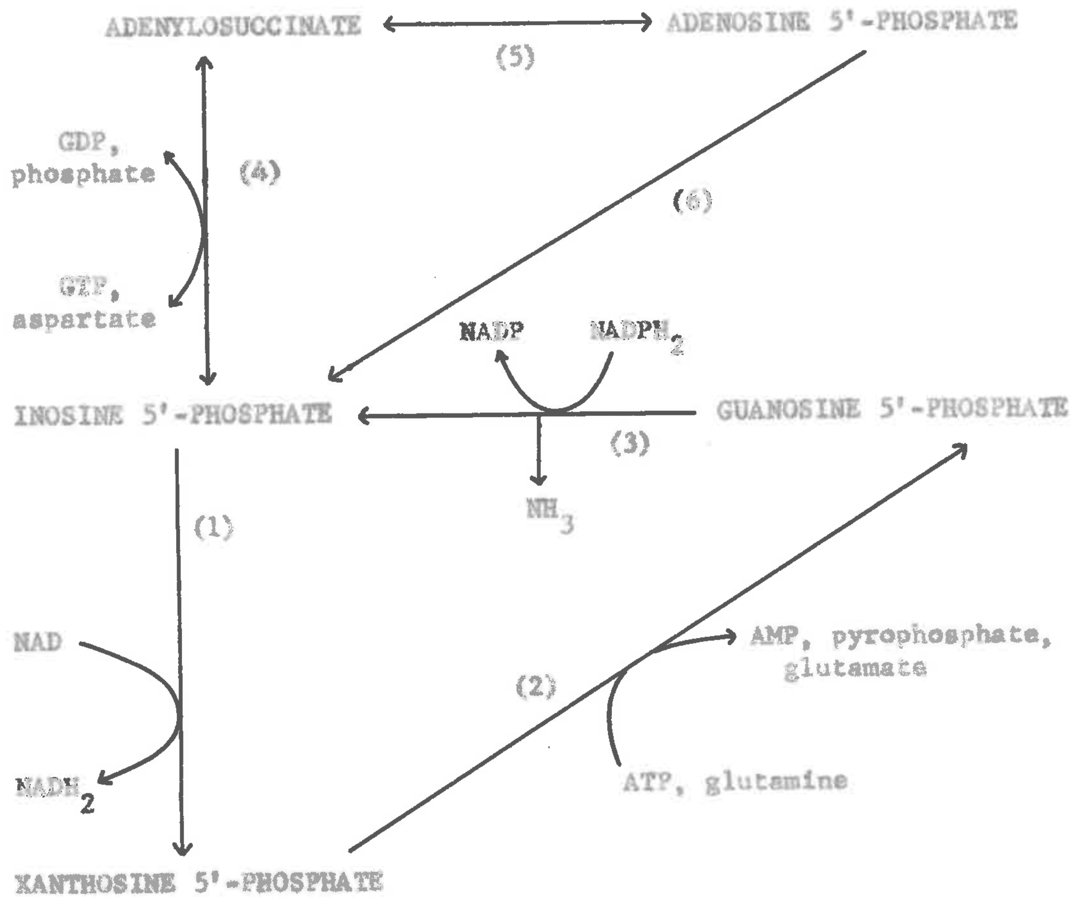


Fig. 1.

inosine 5'-phosphate dehydrogenase in extracts from pigeon liver was inhibited by 6-thioinosine 5'-phosphate but did not study the nature of the inhibition. Partially-purified inosine 5'-phosphate dehydrogenase from Aerobacter aerogenes was inhibited by 6-thioinosine 5'-phosphate (Hampton, 1963) and with this enzyme the inhibition could be partially overcome by including reduced glutathione in the assays. 6-Thioinosine 5'-phosphate was shown to compete for inosine 5'-phosphate and it was suggested that, in the absence of glutathione, 6-thioinosine 5'-phosphate formed a disulphide linkage with the enzyme after attachment to the reaction site of inosine 5'-phosphate. Under non-reducing conditions, 6-thioinosine 5'-phosphate was slowly oxidized by 6-thioxanthylic acid by the inosine 5'-phosphate dehydrogenase preparation; the formation of 6-thioxanthylic acid in cells after treatment with 6-mercaptopurine has not been reported, and it is unlikely that the reaction is important in the action of this drug.

The conversion of inosine 5'-phosphate and L-aspartate into adenylosuccinate by adenylosuccinate synthetase from S. faecalis was inhibited by 6-thioinosine 5'-phosphate (Salser et al., 1960); in the presence of equimolar inosine 5'-phosphate and 6-thioinosine 5'-phosphate, 50% inhibition was observed. Hampton (1960) reported inhibition of adenylosuccinate synthesis with the enzyme from E. coli and found that conversion of adenylosuccinate into adenosine 5'-phosphate by adenylosuccinate lyase from E. coli was also inhibited by

6-thioinosine 5'-phosphate. The 6-thio analogue of adenylosuccinate also inhibited adenylosuccinate lyase and was irreversibly cleaved to 6-thioinosine 5'-phosphate and either fumaric or malic acid by this enzyme (Hampton, 1962). There is, however, no evidence for the formation of this analogue from 6-thioinosine 5'-phosphate under physiological conditions. The overall conversion of inosine 5'-phosphate into adenosine 5'-phosphate by an extract from mouse Adenocarcinoma 755 was inhibited by 6-thioinosine 5'-phosphate (Barker & Bennett, 1964). Enzyme preparations from lines sensitive and resistant to 6-mercaptopurine showed the same degree of sensitivity to 6-thioinosine 5'-phosphate. Relatively high levels of the analogue were required to obtain significant inhibition, and it was suggested that the conversion of inosine 5'-phosphate into adenosine 5'-phosphate is not the site at which 6-thioinosine 5'-phosphate acts to inhibit growth.

It has been recognised for some time that various purines could decrease the incorporation of labelled, non-cyclic precursors such as formate (Balis et al., 1952; Goldthwait & Bendich, 1952), carbon dioxide (Bolton, Abelson & Aldous, 1952; Koch, Putnam & Evans, 1952), and glycine (Abrams, 1951b) into purine nucleotides. Later workers have studied this 'feed-back inhibition' by observing that natural purine bases inhibit the accumulation of formylglycinamide ribotide induced by azaserine (see Henderson, 1962). Azaserine, an analogue of glutamine, inhibits the conversion of formylglycinamide ribotide into formylglycinamidine ribotide in the presence of glutamine

and adenosine 5'-triphosphate (Levenberg, Melnik & Buchanan, 1957); the resultant accumulation of formylglycinamide ribotide has been used as a measure of purine biosynthesis up to this point (see Fig. 2). More specifically, Wyngaarden & Ashton (1959) have shown that 5-phosphoribosyl pyrophosphate amidotransferase from pigeon liver, which catalyses the first reaction specific for purine biosynthesis (see Fig. 2) was inhibited by adenine and guanine nucleotides and by inosine 5'-phosphate, but that the free bases and nucleosides were not inhibitory. These results tentatively defined the site of 'feed-back inhibition' and indicated that inhibition required the prior conversion of bases into nucleotides. Hartman (1963), however, was unable to show inhibition of a more highly purified enzyme from chicken liver by either adenosine 5'-phosphate or adenosine 5'-triphosphate.

Similar 'feed-back inhibition' was obtained with 6-mercaptopurine (Gots & Gollub, 1959), thus leading to the hypothesis that a part of 6-mercaptopurine action might be related to inhibition of _de novo_ purine biosynthesis. Many workers have shown that 6-mercaptopurine inhibits the incorporation of labelled non-cyclic precursors (such as glycine and formate) into the acid-soluble and nucleic acid adenine and guanine of microorganisms (Bolton & Mandel, 1957; Tomisek & Reid, 1962) and of tumours (Skipper, 1954; Jaffe & Mautner, 1958; Mautner, 1958; Davidson, 1960; LePage & Jones, 1961; Bieber & Pomales, 1962; Bennett, Simpson, Golden & Barker, 1963). In general, inhibition of incorporation into adenine and guanine nucleotides was

Fig. 2. Schematic representation of the synthesis of inosinic acid from non-cyclic precursors (cf. Buchanan & Standish, 1959).

THE SYNTHESIS OF INOSINIC ACID FROM NON-CYCLIC PRECURSORS

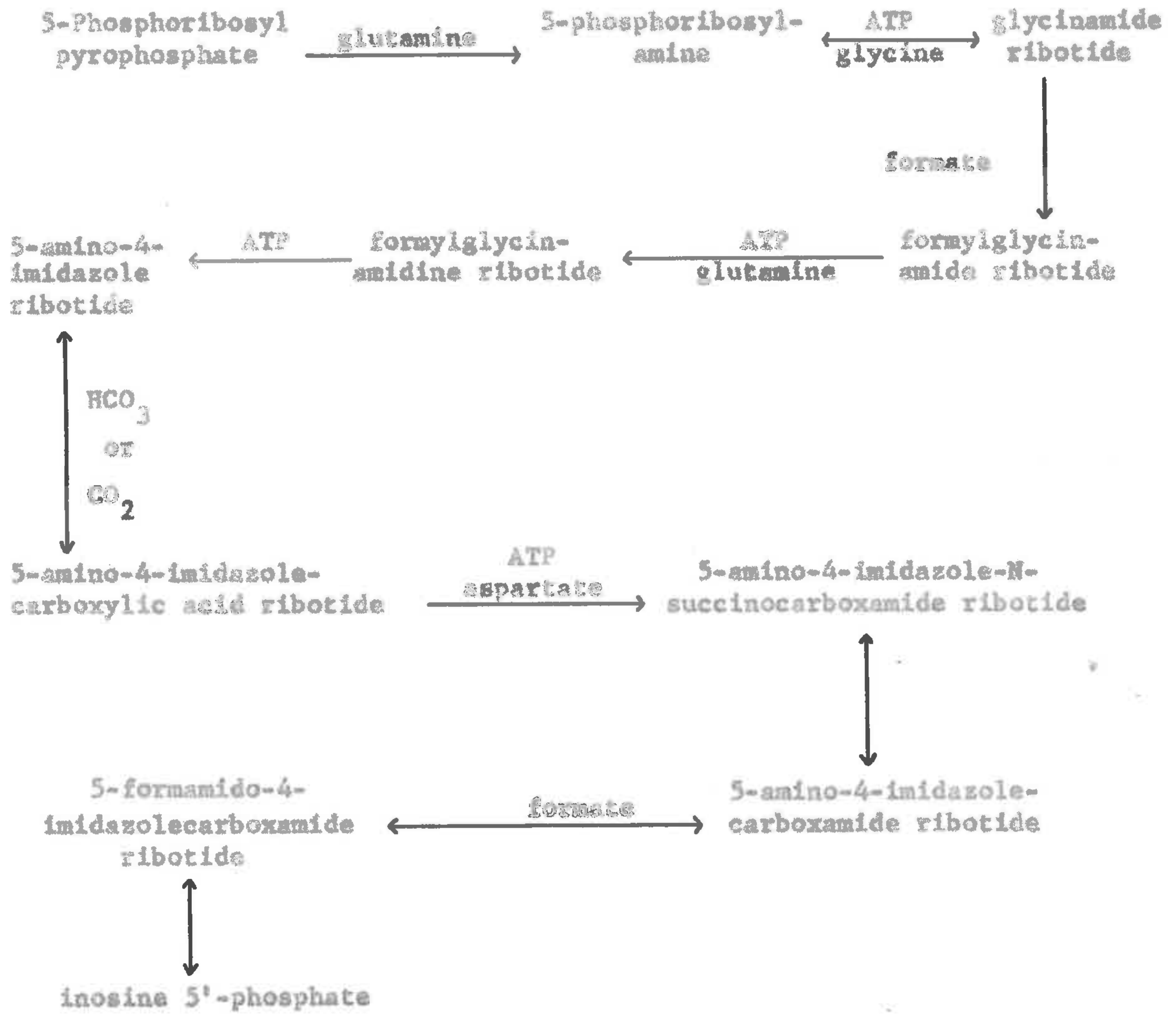


Fig. 2.

similar and, in addition, Bennett et al. (1963) showed that incorporation of [^{14}C]formate in vivo was inhibited under conditions that had little effect on incorporation of [^{14}C]5-amino-4-imidazolecarboxamide, thus suggesting a block prior to inosine 5'-phosphate formation (see Fig. 2). This was supported by the finding that 6-thioinosine 5'-phosphate was a powerful inhibitor of 5-phosphoribosyl pyrophosphate amidotransferase from pigeon liver (McCollister, Gilbert & Wyngaarden, 1962; McCollister, Gilbert, Ashton & Wyngaarden, 1964). 6-Mercaptopurine and 6-thioinosine had no inhibitory effect on the reaction catalysed by this enzyme.

Gots & Gollub (1959) found that in bacteria there was a correlation between 'feed-back inhibition' and the ability of a compound to inhibit growth. A relationship of this kind could not be shown in various lines of ascites-tumour cells (LePage & Jones, 1961); it was observed that strains of cells insensitive to inhibition of growth by 6-mercaptopurine still exhibited 'feed-back inhibition' by this purine, thus indicating that 'feed-back inhibition' was not related to the inhibition of growth. These workers found that only analogues that could be converted into nucleotides were inhibitory; in contrast to this Henderson (1963) tested 37 purine analogues and found no relation between 'feed-back inhibition' and the ability to form nucleotides. Brockman et al. (1957a) found that incorporation of [^{14}C] formate into purines was not inhibited by 6-mercaptopurine in S. faecalis that was resistant to this drug. In

addition Brockman & Anderson (1963) reported 'feed-back inhibition' in a tissue culture of human epidermoid carcinoma cells that were sensitive to 6-mercaptopurine, but not in resistant cells that lacked the capacity to form 6-thioinosine 5'-phosphate. It is of interest that bis (thioinosine)5',5''-phosphate, which inhibited the growth in culture of human epidermoid carcinoma cells that were resistant to 6-mercaptopurine (see above; Montgomery _et al._, 1963), inhibited the accumulation of formylglycinamide ribotide, induced in resistant cells by azaserine, to the same extent as did 6-mercaptopurine when acting on sensitive cells.

It is perhaps of significance that neither 6-mercaptopurine nor 6-thioinosine 5'-phosphate inhibited the conversion of glycine into inosine 5'-phosphate by a soluble extract from pigeon liver (Lukens & Herrington, 1957), although details of the experimental methods were not given by these workers. Similarly Davidson (1960) found that 6-mercaptopurine had little effect on the incorporation of $[2-^{14}C]$ glycine into the guanine nucleotides of L1210 leukaemia cells _in vitro_. Davidson (1960) concluded that the conversion of inosine 5'-phosphate into adenosine 5'-phosphate was inhibited by 6-mercaptopurine.

Thus an effect on the biosynthesis of the purine ring from non-cyclic precursors may be of importance in the inhibition of growth by 6-mercaptopurine, but this has not been satisfactorily established.

Other effects of 6-mercaptopurine

A number of biochemical effects of 6-mercaptopurine that are

not obviously related to purine metabolism have been reported. These effects have received less attention from research workers and will only be briefly considered (for a recent review see Hitchings & Elion, 1963).

6-Mercaptopurine has an effect on the immune response, suppressing the inductive phase of antibody formation (Schwartz & Dameshek, 1959; Berenbaum, 1960; Šterzl, 1960). Treatment with 6-mercaptopurine results in delayed rejection of homografts but does not completely suppress antibody formation (Meeker et al., 1959; Hoyer, Condie & Good, 1960; Robinson & Christian, 1960). These findings have opened a new field of research which will undoubtedly have considerable practical importance.

6-Mercaptopurine causes a decrease in the respiration and anaerobic glycolysis of sarcoma-180 slices without affecting the metabolism of either liver or kidney (Mihich, Clarke & Philips, 1956). Respiration and glycolysis were also inhibited by 6-mercaptopurine in suspensions of leukaemic cells (Laszlo, Stengle, Wight & Burk, 1958; Laszlo, Ellis, Rundles & Elion, 1961). The drug caused an increase in the synthesis and storage of glycogen in Ehrlich ascites-tumour cells (Pecile, Tessari & Yamamoto, 1958) and a decrease in lactic dehydrogenase of these cells (Yamamoto, Riva & Tessari, 1958). Katchman, Zipf & Murphy (1962) found a disappearance of glucose when intact cancer cells were incubated with 6-mercaptopurine, but did not detect an inhibition of lactic dehydrogenase. 6-Mercaptopurine is reported to have some effects on protein synthesis and to interfere with

the formation of adaptive enzymes by microorganisms (Ottey, 1955), and mammalian liver (Lee, 1960b).

6-Mercaptopurine has effects on the metabolism of nucleotide coenzymes, but it is not known to what extent these are indirect. Thus the effect of this drug on the mitosis of cells in tissue culture is prevented by coenzyme A (Biesele, 1958). 6-Mercaptopurine inhibited the rise in liver nicotinamide-adenine dinucleotide observed after administration of nicotinamide to mice; the subsequent decrease in concentration of this coenzyme was also inhibited by 6-mercaptopurine (Kaplan *et al.*, 1956). Similarly synthesis of nicotinamide-adenine dinucleotide from nicotinamide by mouse liver slices was inhibited by 6-mercaptopurine (Bresnik & Hitchings, 1961). However, the drug had no effect on the level of nicotinamide-adenine dinucleotide or coenzyme A in *B. cereus* (Carey & Mandel, 1961).

More recently Frearson & Williams (1964) showed that 6-mercaptopurine inhibited 3α-hydroxy steroid-activated nicotinamide-adenine dinucleotide phosphate transhydrogenase from rat liver, but did not come to any conclusions concerning the possible physiological implications of this finding.

Outline of experimental problem

One way to obtain evidence concerning the specific site of 6-mercaptopurine action is to study the inhibition of individual enzymes, and this thesis deals mainly with the effects of 6-mercaptopurine and 6-thioinosine 5'-phosphate on enzymes isolated from Ehrlich

ascites-tumour cells.

The first section describes the preparation of pure 6-thioinosine 5'-phosphate and of its disulphide, a contaminant present in samples of this nucleotide that have been described previously. Inosine 5'-phosphate dehydrogenase, adenylosuccinate synthetase, and adenylosuccinate lyase of sufficient purity for kinetic studies were prepared from a strain of Ehrlich ascites-tumour cells that were sensitive to 6-mercaptopurine. 6-Thioinosine 5'-phosphate was shown to be a competitive inhibitor of the dehydrogenase and of the lyase and a non-competitive inhibitor of the synthetase. Michaelis and inhibitor constants and apparent activities of the enzymes were measured, and the biological significance of these inhibitions is discussed. Measurement of kinetic parameters of the lyase at a number of pH values has provided some evidence on the mechanism of action of this enzyme.

The second section describes the extraction, purification and assay of purine phosphoribosyltransferases from sensitive Ehrlich ascites-tumour cells and from cells that were resistant to the action of 6-mercaptopurine. Guanine and hypoxanthine phosphoribosyltransferases from both sources were inhibited competitively by 6-mercaptopurine; adenine phosphoribosyltransferase was not inhibited. Kinetic parameters of the enzymes from sensitive and resistant cells did not differ significantly. From the relative values of Michaelis and inhibitor constants it is clear that the competitive inhibition of formation of inosine 5'-phosphate and guanosine 5'-phosphate by free 6-mercapto-

purine is of considerable potential importance in the mechanism of action of this drug. No evidence was obtained that resistance involved changes in the activity or catalytic properties of any of these enzymes.

The third section describes studies of purine nucleotide metabolism in intact Ehrlich ascites-tumour cells and the effect of 6-mercaptopurine on these cells. Evidence was obtained that ascites-tumour cells use preformed purines much more effectively than the non-cyclic precursors for biosynthesis of purine nucleotides. 6-Mercaptopurine inhibited nucleotide biosynthesis from hypoxanthine but not that from adenine.

The fourth section describes the preparation and some biochemical properties of some derivatives of 6-mercaptopurine. The enzymic reduction and hydrolysis of nicotinamide-6-mercaptopurine dinucleotide is described and kinetic studies of 6-thioinosine 5'-triphosphate as an inhibitor of nicotinamide mononucleotide adenylyltransferase from liver nuclei is reported.

In the fifth section the relevance of these results to the mechanism of action of 6-mercaptopurine and the development of resistance is discussed and some proposals on the design of chemotherapeutic agents are made.

SECTION I

INHIBITION OF INTERCONVERSIONS OF PURINE NUCLEOTIDES IN EHRLICH ASCITES-TUMOUR CELLS BY 6-THIOINOSINE 5'-PHOSPHATE

Inosine 5'-phosphate formed from non-cyclic precursors or from preformed purines is the common intermediate for the synthesis of cellular adenosine 5'-phosphate and guanosine 5'-phosphate.

Inosine 5'-phosphate - nicotinamide-adenine dinucleotide oxidoreductase (inosine 5'-phosphate dehydrogenase; EC 1.2.1.14) catalyses the oxidation of inosine 5'-phosphate (IMP) to xanthosine 5'-phosphate (XMP) by nicotinamide-adenine dinucleotide (NAD) in the reaction:

$$IMP + NAD \longrightarrow XMP + NADH_2$$

Xanthosine 5'-phosphate is aminated in the presence of glutamine (ammonia can substitute in bacterial systems) and adenosine 5'-triphosphate to form guanosine 5'-phosphate in a reaction catalysed by xanthosine 5'-phosphate aminase.

IMP-L-aspartate ligase (GDP) [adenylosuccinate synthetase; EC 6.3.4.4.] catalyses the conversion of inosine 5'-phosphate and L-aspartate into adenylosuccinate in the reaction:

$$IMP + L\text{-aspartate} + GTP \longleftrightarrow \text{adenylosuccinate} + GDP + \text{inorganic phosphate.}$$

The adenylosuccinate formed from inosine 5'-phosphate is converted into adenosine 5'-phosphate and fumarate by the action of adenylosuccinate AMP-lyase (EC 4.3.2.2.).

These pathways are considered to be obligatory in the formation of adenine and guanine nucleotides from non-cyclic precursors; these compounds are then utilized for nucleic acid biosynthesis and other biochemical reactions. Inhibition of any one of these reactions would therefore be expected to have a profound effect on cellular metabolism in systems that are not using preformed purines. This section describes the partial purification and some properties of inosine 5'-phosphate dehydrogenase, adenylosuccinate synthetase, and adenylosuccinate lyase from Ehrlich ascites-tumour cells. The enzyme extracts have been used to study the inhibitory effects of purified 6-thioinosine 5'-phosphate on each of these reactions.

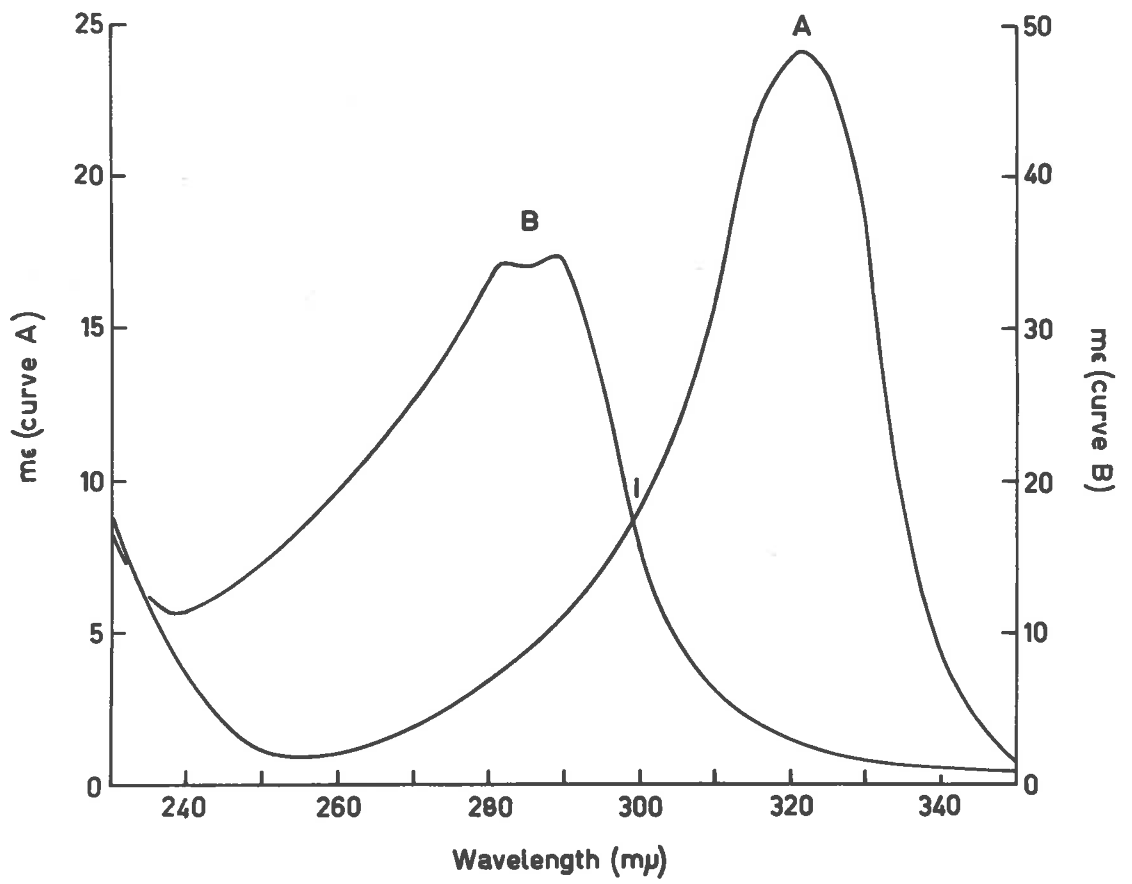
A. MATERIALS AND METHODS

1. Preparation of nucleotides

(a) 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 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. x 1 cm.^{2}) of Amberlite CG-120 (K^+ form) and the combined resins were washed with water (3 x 5 ml.). The combined effluent (pH 4) was passed through a column (15 cm. x 3.5 cm.^{2}) of ECTEOLA (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 x 50 ml.) to remove volatile salts and had $\lambda_{max.}$ 321.5 mμ and $\lambda_{min.}$ 255 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. I.1. 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 3 months 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 $cm.^2\ v^{-1}hr.^{-1}$).

Fig. I.1. Absorption spectra in 0.1 M-acetate (Na^{+}, pH 5.5 of 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.



A
B
mε (curve A)
mε (curve B)
Wavelength (mμ)
25
20
15
10
5
0
50
40
30
20
10
0
240
260
280
300
320
340

(b) Disulphide of 6-thioinosine 5'-phosphate. A solution of triethylammonium thio-IMP (124 µmoles) in 25 ml. of 0.2 mM-mercapto-ethanol at 25° was kept at pH 7.0 by automatic addition of N-NaOH while N-iodine in 20% (w/v) sodium iodide was added in 0.20 ml. portions. After addition of 0.60 ml. of iodine and consumption of 0.058 ml. of alkali the extinction ratio $E_{321.5\ m\mu}/E_{289\ m\mu}$ (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. I.1.) had $\lambda_{max.}$ 289 mµ (mε, 34.7 ± 0.5; mean ± S.E. of 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 nitrogen and phosphorus 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 x 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 measured 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 (see the Results and Discussion section).

(c) Guanosine 5'-triphosphate. Most of the GDP in a commercial sample of GTP (Pabst Laboratories, Milwaukee, U.S.A.) was removed by chromatography on DEAE-cellulose with a linear gradient (0.05 - 0.4 M) of NH_4HCO_3. GTP was eluted between 0.21 M and 0.28 M-NH_4HCO_3 and freed of bicarbonate by evaporation. On electrophoresis in 0.04 M-citrate (Na^+, pH 4.2) the product was found to contain 0.02 mol. prop. of GDP.

(d) Adenylosuccinate. The nucleotide was prepared from adenylate and fumarate with adenylosuccinate lyase from yeast and was purified by anion-exchange chromatography as described by Carter & Cohen (1956). Only one component that absorbed light at 254 mμ could be detected on chromatography in $(NH_4)_2SO_4$ - 0.1 M-phosphate (Na^+, pH 6.8) - propan-1-ol (60:100:2, w/v/v; R_F 0.52) and in butan-1-ol - acetic acid - water (2:1:1, by vol.; R_F 0.40). The spectrum at pH 7 was the same as that described by Carter & Cohen (1956); the ratio of nucleotide : phosphate, based on the molecular extinction coefficient reported by these authors (ε, 1.96 x 10^4 at 268 mμ) was 0.92 : 1.00.

(e) Other nucleotides.

Inosine 5'-phosphate. AMP (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was deaminated as described by Kaplan (1957). 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 ammonium sulphate - 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 ammonium sulphate - phosphate - propanol system (see above).

Nicotinamide-adenine dinucleotide. NAD was obtained from C.F. Boehringer und Soehne.

Reduced nicotinamide-adenine dinucleotide. $NADH_2$ was used as the sodium salt (Sigma Chemical Co.).

2. Inosine 5'-phosphate dehydrogenase

(a) Preparation of dehydrogenase. Ascitic fluid and cells (about 10^8 cells/ml.) were collected from C_3H mice 10 days after inoculation with about 2 x 10^7 Ehrlich ascites-tumour cells in 0.2 ml. The suspension

was frozen and used within two weeks of collection. After thawing, the suspension was mixed with an equal volume of Hanks (1948) solution and centrifuged at 2 000 g 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 (1948) 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 shaker (Nossal, 1953). In a machine with different shaking characteristics the shaking time had to be decreased to 20 sec. to avoid extraction of interfering enzymes (see the Results and Discussion section). After centrifuging for 30 min. at 20 000 g. the supernatant (about 11 ml./batch) was collected ('first extract'; Table I.1). One ml. of 5% (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 20 000 g. for 15 min. the supernatant ('protamine supernatant'; Table I.1.) was passed through a column of DEAE-cellulose (5 cm. x 1.5 cm.^{2} for 25 ml. of 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 ammonium sulphate.

Material precipitating 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 ('dialysed enzyme'; Table I.1) after centrifuging at 20 000 g. for 15 min. was 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.

(b) 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.). The extinction at 290 mμ was recorded; its 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 5.4×10^3 as the net change of molecular extinction coefficients at 290 mμ. Δme was assumed to be 4.7 for IMP ⟶ XMP (Beaven, Holiday & Johnson, 1955; Pabst Laboratories, 1961a) and 0.7 for NAD ⟶ $NADH_2$ (Pabst Laboratories, 1961b). Dehydrogenase activity was expressed as μm-moles of IMP oxidised/min. in this assay and the specific actiity as μm-moles of IMP oxidised/min./

Table I.1. Properties of fractions obtained in the purification of IMP dehydrogenase from Ehrlich ascites-tumour cells.

Details are given in the text. Protein content was calculated from $E_{260\ m\mu}$ and $E_{280\ m\mu}$ (Warburg & Christian, 1942)

Fraction	Volume (ml.)	$\frac{E_{280\ m\mu}}{E_{260\ m\mu}}$	Protein (mg.)	μm-Moles of IMP oxidized/min.	Net formation of $NADH_2$ (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
$(NH_4)_2SO_4$ fraction	2.2	1.16	50	42	35.5
Dialysed enzyme	2.6	1.16	48	28	27.7

mg. of protein. The activity was also measured by recording the extinction at 340 mμ (Δ mε, 6.22; Horecker & Kornberg, 1948). In studies of the inhibition of IMP dehydrogenase the final concentration of IMP was varied from 15 μM to 120 μM, and thio-IMP was added to blank and test cells after the 4 min. equilibration period and about 15 sec. before the IMP.

(c) 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 0.5 M-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 0.5 M-trichloroacetic acid. The combined supernatants from control and test respectively were extracted with ether (3 x 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. x 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 x 35 cm.) of Whatman 3MM paper with a serrated lower edge; descending chromatography in the ammonium sulphate - 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 was measured to permit calculation of the concentration of each nucleotide.

3. Adenylosuccinate synthetase

(a) Preparation of the synthetase. The apparent specific activity of this enzyme in extracts of Ehrlich ascites-tumour cells depends on the time for which the cells have been stored at -15° (Fig. I.10., see the Results and Discussion section); low apparent activities in extracts of fresh cells are due to interference in the assay system by the lyase (see the Results and Discussion section). The specific activities reported in this purification procedure were those obtained with cells that had been kept at -15° for 84 days. Cells were collected and broken as described for IMP dehydrogenase. The supernatant obtained by centrifuging at 20 000 g for 15 min. ('first extract', Table I.2.) was treated with 1 ml. of 5% streptomycin sulphate/40 mg. of protein (final vol. about 4 ml.). After 5 min. at 1° the suspension

Table I.2. Properties of fractions obtained in the purification of adenylosuccinate synthetase from Ehrlich ascites-tumour cells

Details are given in the text. Protein content was calculated from $E_{260\ m\mu}$ and $E_{280\ m\mu}$ (Warburg & Christian, 1942).

Fraction	Volume (ml.)	$\frac{E_{280\ m\mu}}{E_{260\ m\mu}}$	Protein (mg.)	µm-Moles of adenylo-succinate formed/min.
First extract	44	0.63	343	50
Streptomycin supernatant	54	0.93	226	110
Heated enzyme	64	0.94	172	170
$(NH_4)_2SO_4$ fraction	7.4	1.20	47	54.5
Dialysed enzyme	8.5	1.21	30	47.5

was centrifuged at 10 000 g for 10 min. The supernatant ('streptomycin supernatant', Table I.2; pH 7.4) was adjusted to pH 5.4 with 0.1 M-acetic acid, placed in a thermostat, and stirred while the bath temperature was raised from 23° to 31° during 10 min. The suspension was cooled to 1° and centrifuged at 10 000 g for 10 min. The supernatant was brought to pH 7 with N-KOH ('heated enzyme', Table I.2.) and fractionated with $(NH_4)_2SO_4$. Material which precipitated between 0.45 and 0.75 saturation was dissolved in 0.1 M-glycine (Na^+), pH 8 ('$(NH_4)_2SO_4$ fraction', Table I.2.). After dialysis for 16 hr. at 2° against 5 mM-glycine (Na^+, pH 8) insoluble material was removed by centrifuging at 20 000 g for 15 min. The supernatant ('dialysed enzyme', Table I.2.) was used for kinetic studies; its activity was unchanged after storage for 2 days at -15°.

(b) Assay of the synthetase. For routine assays used in following the purification of the enzyme the extinction of a solution containing synthetase, 0.5 μmole of IMP, 0.5 μmole of GTP, 2 μmoles of L-aspartate, 8 μmoles of $MgCl_2$ and 125 μmoles of glycine (final vol. 2.8 ml; adjusted to pH 7.5 with NaOH) was recorded at 280 mμ. The test cell and a reference cell without aspartate were kept at 25° in the thermostatic cell-holder of a Beckman DK-2A spectrophotometer. Rates were constant for about 10 min. after the reaction had been started by addition of aspartate. Specific activities were expressed as μm-moles of adenylosuccinate formed/min./mg. of protein; the increase

of molecular extinction coefficient for conversion of IMP into adenylosuccinate is 1.17×10^{4} at 280 mμ in the pH range 7 - 8 (cf. Carter & Cohen, 1956; Pabst Laboratories, 1961a). In studies of Michaelis constants and of inhibition by thio-IMP this assay was modified by variation of any one of the reactants and by inclusion of thio-IMP in both reference and test cells. Test solutions containing thio-IMP but no IMP showed no change of extinction at 280 mμ. For comparison of kinetic parameters at pH 7.01 and pH 7.76 the glycine was replaced by phosphate (75 μmoles) and tris (30 μmoles) and solutions were adjusted to the required pH with NaOH.

(c) Analysis of the products of the adenylosuccinate synthetase reaction. Synthetase (1.8 mg. of protein; 'dialysed enzyme', Table I.2) was kept at 30° for 2 hr. in a solution containing IMP (0.5 μmole), GTP (0.75 μmole), L-aspartate (4 μmoles), $MgCl_2$ (16 μmoles) and glycine (125 μmoles) in 5.65 ml. of solution adjusted to pH 7.5 with NaOH. This solution and a control without enzyme were mixed with 5 ml. of 0.5 M-trichloroacetic acid and centrifuged at 2°. The precipitate was washed with 5 ml. of 0.2 M-trichloroacetic acid and the combined supernatants were extracted three times with 50 ml. portions of ether and evaporated to dryness at 30°. The residues from test and control experiments were applied as bands (10 cm. long) to sheets of Whatman 3MM paper and chromatographed in butan-1-ol - acetic acid - water (2:1:1, by vol.) for 21 hr. (descending). Solvent was allowed to drip from the serrated lower edge of the paper.

Adenylosuccinate (17.5 cm. movement) and IMP (12 cm. movement) were extracted from the paper with 10 ml. portions of water and the spectra of the extracts were measured with the use of suitable blanks from corresponding regions of the paper. The control contained 0.48 μmole of IMP and the test contained 0.24 μmole of IMP and 0.22 μmole of adenylosuccinate. Chromatography in the $(NH_4)_2SO_4$ - phosphate - propanol system (see above) showed no AMP (R_F 0.26) as a contaminant in the IMP (R_F 0.55) which had been eluted from the first chromatogram. These nucleotides have the same mobility in the butanol - acetic acid - water system.

4. Adenylosuccinate lyase

(a) Purification of lyase from ascites-tumour cells. The streptomycin supernatant, prepared as described for the synthetase preparation, but from cells that had been kept at -15° for 28 days, was heated at 55° for 10 min., cooled and centrifuged at 10 000 g for 10 min. at 1°. The supernatant ('heated enzyme', Table I.3.) was fractionated with $(NH_4)_2SO_4$ at 2°; material that precipitated between 0.2 and 0.6 saturation of $(NH_4)_2SO_4$ was dissolved in 10 mM-phosphate (Na^+), pH 7.4 ('$(NH_4)_2SO_4$ fraction', Table I.3.) and dialysed for 16 hr. at 2° against 5 mM-phosphate (Na^+, pH 7.4). After removal of insoluble material at 20 000 g for 15 min. the supernatant ('dialysed enzyme', Table I.3) was used for kinetic studies

Table I.3. Properties of fractions obtained in the purification of adenylosuccinate lyase from Ehrlich ascites-tumour cells

Details are given in the text. Protein content was calculated from $E_{260\ m\mu}$ and $E_{280\ m\mu}$ (Warburg & Christian, 1942).

Fraction	Volume (ml.)	$\frac{E_{280\ m\mu}}{E_{260\ m\mu}}$	Protein (mg.)	μMoles of adenylosuccinate cleaved/min.
First extract	86	0.62	1135	2.15
Streptomycin supernatant	108	1.00	518	2.8
Heated enzyme	108	1.12	340	3.4
$(NH_4)_2SO_4$ fraction	6.6	1.20	170	2.8
Dialysed enzyme	7.5	1.20	168	2.75

and did not lose activity during storage for 7 days at -15°.

(b) Adenylosuccinate lyase from yeast. This enzyme was purified as described by Carter & Cohen (1956). For kinetic studies and for preparation of adenylosuccinate a solution of the final ammonium sulphate fraction in water was used. The specific activity of this preparation was 3.1 μmoles of adenylosuccinate formed/min./mg. of protein (for details of assay, see below).

(c) Assay of adenylosuccinate lyase. For routine assays the extinction of a solution containing the lyase (from ascites-tumour cells or from yeast), 125 μmoles of phosphate (Na^+, pH 7.0) and 0.25 μmole of adenylosuccinate (final vol. 2.5 ml.) was recorded at 280 mμ and 25° against a reference solution containing no adenylosuccinate. Initial rates of hydrolysis were maintained for at least 10 min.

Specific activity was expressed as μm-moles of adenylosuccinate converted into AMP/min./mg. of protein. The decrease of molecular extinction coefficient on cleavage of adenylosuccinate to AMP and fumarate is 1.07×10^4 at pH 8 (Carter & Cohen, 1956). In experiments where K_m, K_p, V, V_p and K_i of thio-IMP were measured, 4 or 5 concentrations of adenylosuccinate were used in the presence or absence of thio-IMP. For experiments at other pH values the phosphate concentration was kept at 50 mM with suitable mixtures of NaH_2PO_4 and Na_2HPO_4 and the actual pH of each reaction mixture was measured with a glass electrode after the spectrophotometric assay. A second

reaction mixture was prepared in each case and the pH was measured as soon as the adenylosuccinate had been added. In no case was the change of pH during the assay period more than 0.02.

In the pH range 5.6 - 8.4 there is no significant change in the molecular extinction decrement at 280 mμ accompanying the conversion of adenylosuccinate into AMP and fumarate. If all the fumarate formed in the reaction had been converted into malate by fumarase the molecular extinction decrement would have been 1.10×10^4; however, no fumarase activity was detected in the samples of lyase used for kinetic studies.

For assay of adenylosuccinate lyase from yeast during the preliminary purification to obtain enzyme for the synthesis of adenylosuccinate, formation of this nucleotide from 0.1 mM-AMP and 4.3 mM-fumarate was measured spectrophotometrically at 25° in 50 mM-phosphate (Na^+), pH 7.0 (Carter & Cohen, 1956).

(d) Analysis of the products of cleavage of adenylosuccinate by adenylosuccinate lyase from ascites-tumour cells. The lyase (1.8 mg. of a dialysed preparation with specific activity 5.8) was kept for 1 hr. at 30° with 0.369 μmole of adenylosuccinate and 100 μmoles of phosphate (Na^+, pH 7) in a final volume of 0.6 ml. After chromatography in the butanol - acetic acid - water system and spectrophotometric analysis the product was found to contain 0.24 μmole of AMP (R_F 0.34), 0.096 μmole of adenylosuccinate (R_F 0.41); fumarate (R_F 0.82) was detected but the concentration was not measured.

5. Preparation of acetone-dried powder from chicken liver

Chickens were killed by decapitation and the livers removed and stored in crushed ice until used (about 45 min.). Liver (200 g.) was minced twice with a mechanical mincer at -15° and the tissue was then stirred with 10 volumes of redistilled acetone (precooled to -15°) for 30 min. The suspension was rapidly filtered at 2° and the residue was washed with a further 10 volumes of acetone (-15°). The powder was dried at 2° for 45 min. and then at room temperature for 30 min. Approximately 45 g. of acetone-dried powder was obtained from 200 g. of liver.

6. Calculation of kinetic parameters

Values of Michaelis constant (K_m), and the apparent K_m in the presence of inhibitor (K_p), of the maximum velocity (V), and of the maximum velocity in the presence of inhibitor (V_p), were calculated by Wilkinson's (1961) statistical method programmed for use with the IBM 1620 computer (The programme used for these calculations is shown in an Appendix at the end of this thesis). Where appropriate the results have been plotted by the reciprocal method of Lineweaver & Burk (1934). With this method plots of 1/velocity against 1/[substrate] result in straight lines of slope K_m/V when Michaelis-Menten kinetics are observed. The graphs cut the vertical axis at points giving $1/V$ and the base line at points giving $-1/K_m$. Where such reciprocal plots

are shown the lines have been drawn by fixing the points on the base line and vertical axis with values calculated by Wilkinson's (1961) statistical method.

Values of the inhibitor constants (K_i) for competitive inhibition were calculated from the expression

$$K_p = K_m (1 + [\text{thio-IMP}]/K_i)$$

and for non-competitive inhibition from the expression:

$$V_p = V (1 + [\text{thio-IMP}]/K_i).$$

The method of calculating gave the standard errors of K_m, K_p, V, and V_p, and coefficients of variation (C V) were calculated by dividing each parameter into its standard error (the coefficient of variation was therefore less than one until the standard error became larger than the corresponding kinetic parameter). The coefficient of variation of K_i for competitive inhibition was given by the expression

$$CV_{K_i} = K_p/(K_p - K_m) \sqrt{(CV_{K_m})^2 + (CV_{K_p})^2}$$

and for non-competitive inhibition by the expression:

$$CV_{K_i} = V/(V - V_p) \sqrt{(CV_V)^2 + (CV_{V_p})^2}.$$

B. RESULTS AND DISCUSSION

1. The effect of disintegration time on the extraction of enzymically-active proteins from Ehrlich ascites-tumour cells

Experiments were carried out to find the time of shaking with a Nossal (1953) shaker which gave the highest specific activity of initial extracts for each of the enzyme activities studied.

Ascites-tumour cells were collected and washed as described in the Materials and Methods section. The cells were suspended in an equal volume of 15 mM-phosphate (K^+, pH 7.4) and shaken with No. 14 Ballotini beads (6 g. of beads and 10 ml. of suspension in each batch) for 5, 10, 20, 30, 35, 40 and 45 sec. in a Nossal shaker. After centrifuging at 20 000 g for 30 min. the supernatant solutions were assayed for IMP dehydrogenase and adenylosuccinate lyase activity (see the Materials and Methods section). Assays were also carried out for 6-mercaptopurine phosphoribosyltransferase activity by the spectrophotometric method (see Section II, p. 91). Portions of each supernatant were also taken for protein determination. The results are given in Table I.4. and shown graphically in Fig. I.2. It is clear that the amount of protein extracted from the cells rises only slowly with shaking times of from 5 to 30 sec., while the enzyme activity/ml. increases rapidly. With shaking times of greater than 30 sec. the protein concentration increases more rapidly but there is little increase in any of the enzyme activities. Based on these

Fig. I.2. The extraction of enzymes from Ehrlich ascites-tumour cells by increasing disintegration times with a Nossal (1953) shaker.

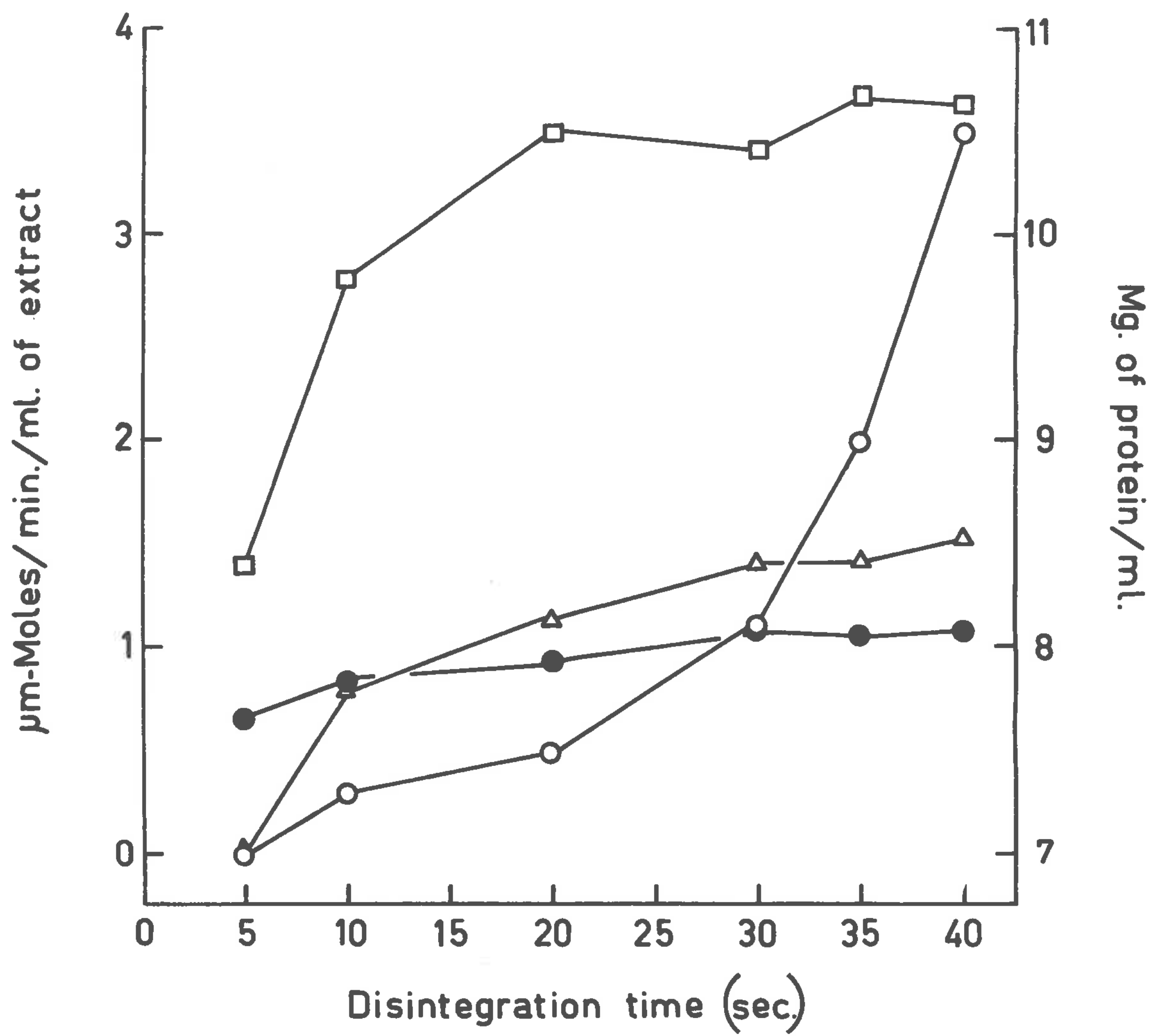
See the text for descriptions of the enzymic assays. Activities of adenylosuccinate lyase have been divided by 100.

○ protein (mg./ml.)

△ IMP dehydrogenase

● adenylosuccinate lyase

□ 6-mercaptopurine phosphoribosyltransferase



µm-Moles/min./ml. of extract
Mg. of protein/ml.
Disintegration time (sec.)
0
5
10
15
20
25
30
35
40
1
2
3
4
7
8
9
10
11

Table I.4. The effect of disintegration time on the extraction of enzymes from Ehrlich ascites-tumour cells

Details of the assay systems for measurement of enzymic activity are given in the text. Protein content was calculated from $E_{260\ m\mu}$ and $E_{280\ m\mu}$ (Warburg & Christian, 1942).

Disintegration time (sec.)	Protein (mg./ml.)	$\frac{E_{280\ m\mu}}{E_{260\ m\mu}}$	μm-moles/min./ml. of extract		
			Adenylosuccinate lyase	IMP dehydrogenase	6-mercaptopurine phosphoribosyltransferase
5	7.0	0.66	65.4	0.02	1.40
10	7.3	0.65	82.0	0.80	2.78
20	7.5	0.65	93.0	1.14	3.50
30	8.1	0.65	107.0	1.40	3.42
35	9.0	0.65	106.0	1.41	3.66
40	10.5	0.66	108.0	1.52	3.62

results a disintegration time of 30 sec. was used to prepare initial extracts for each of the enzymic activities studied.

2. 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 and Thomas, 1961) had a ratio $E_{255\ m\mu}/E_{321.5\ m\mu}$ of 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 the K_i of thio-IMP. Removal of IMP by anion-exchange chromatography gave a product containing no detectable contaminants. From its $E_{255\ m\mu}/E_{321\ m\mu}$ ratio (0.045) this product was slightly purer than thio-IMP prepared by Hampton & Maguire (1961) from a 6-chloropurine nucleotide ($E_{255\ m\mu}/E_{322.5\ m\mu}$ 0.056) and has a similar spectroscopic purity to 6-thioinosine (Fox, Wempen, Hampton & Doerr 1958; cf. Hampton & Maguire, 1961), which had a ratio of 0.045, and which had not been heated with alkali during preparation.

The spectrum of the crude barium salt of thio-IMP, like published spectra of thio-IMP of synthetic (Hampton, 1962) or biological (Paterson, 1959) origin, showed inflections near 280 mμ and 290 mμ. The compound responsible for these inflections was partially purified by electrophoresis (for details of the buffer see the Materials and Methods section) and was found to have two

absorption maxima near 280 mμ and 290 mμ. The compound was found to have the same spectrum (Fig.I.1.), 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 were converted into thio-IMP in 0.5% mercaptoethanol or in alkaline solution. The spectroscopic and chemical properties of the disulphide closely resemble those of the disulphide of 6-thioinosine (Doerr, Wempen, Clarke and Fox, 1961). Addition of mercaptoethanol to all aqueous solutions of thio-IMP permitted the isolation of this nucleotide free of detectable disulphide.

3. IMP dehydrogenase

(a) Purification and assay

The first extract from broken ascites-tumour cells was not suitable for kinetic studies with IMP dehydrogenase because of several interfering enzymic activities. With these extracts 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; the discrepancy was due to reoxidation of $NADH_2$. 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-$NADH_2$ at a rate of 1.3 μm-moles/min./ml. under the conditions of the assay (for details see the Materials and Methods 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 this reaction 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 $NADH_2$ (281.5 mμ) because of the high total extinction of enzyme and substrates at this wavelength. In preparations of dialysed enzyme (see Table I.1.), the ratio of the apparent rates of NAD reduction and IMP oxidation, measured at 340 mμ and 290 mμ respectively, was 0.99. Here the error, resulting from reoxidation of $NADH_2$, in calculating the rate of oxidation of IMP from the rate of change of extinction at 290 mμ was negligible.

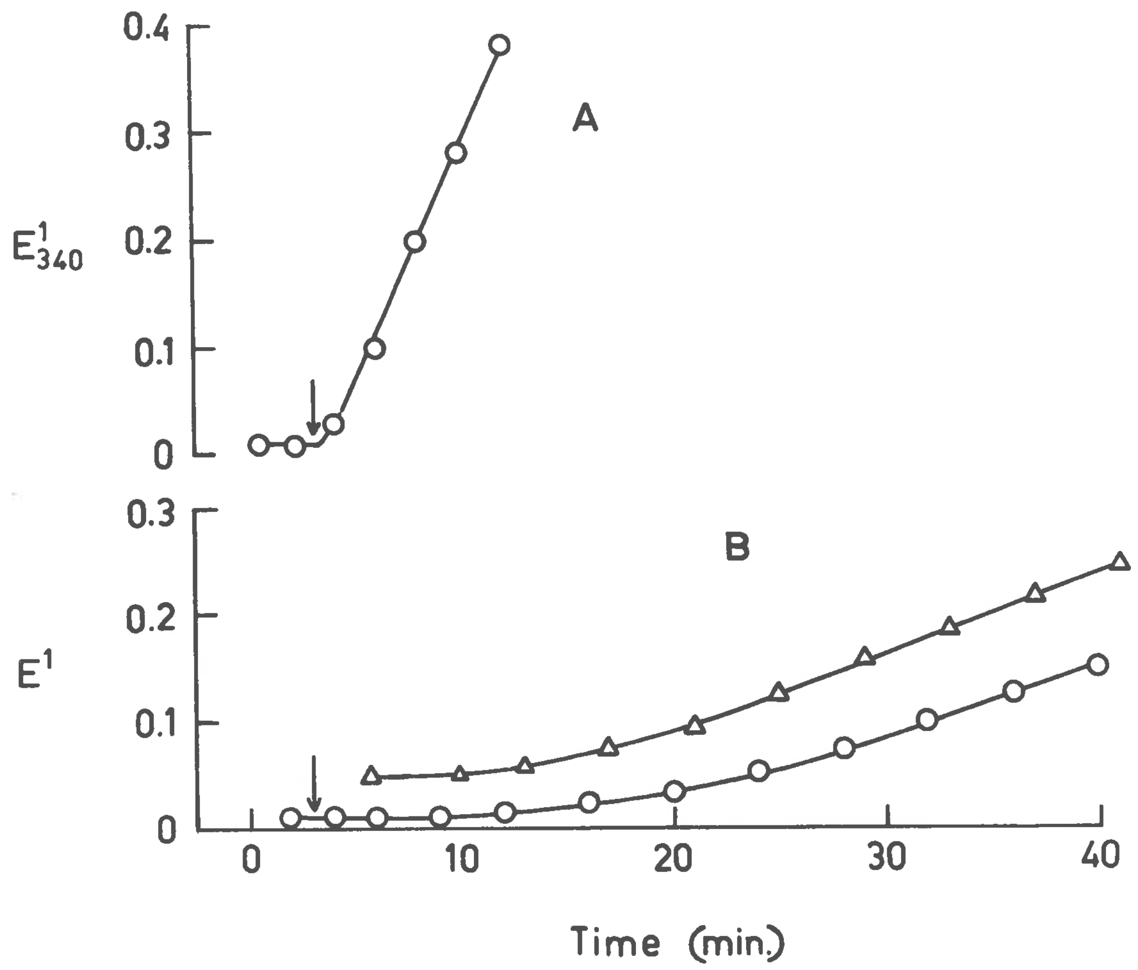
First extracts also contained enzymes that converted IMP into inosine and hypoxanthine. Thus on incubation of 0.49 μmole of IMP for 1 hr. with NAD and a portion of the first extract (6 mg. of protein; for details see the Materials and Methods section) formation of 0.29 μmole of XMP was accompanied by 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-tumour cells that had not been frozen were not readily freed of erythrocytes and the first extracts then contained xanthine oxidase, which caused a spurious increase of extinction at 290 mμ and 340 mμ due to the oxidation of hypoxanthine by NAD and the consequent formation of xanthine and uric acid (both of these compounds absorb light at 290 mμ). The importance of obtaining

preparations free of xanthine oxidase was emphasised in the following preliminary experiments with extracts from chicken liver. Crude extracts were made by stirring 5 g. of acetone-dried powder of chicken liver with 50 ml. of 10 mM-tris (Cl^-, pH 8.0) for 30 min. at 2°. The supernatant obtained after centrifuging at 20 000 g for 15 min. was assayed for IMP dehydrogenase activity as described in the Materials and Methods section. There was a lag period of 4-6 min. followed by a linear increase at both 290 mμ and 340 mμ (see Fig. I.3.B.). In assays in which 0.23 μmole of hypoxanthine replaced IMP there was no lag period (Fig. I.3.A.) suggesting that extracts converted IMP into hypoxanthine which was then oxidised by xanthine oxidase. This was supported by the results of assays in systems containing 0.2 mM-2-amino-6-formyl-4-hydroxypteridine, a powerful inhibitor of xanthine oxidase (Lowry, Bessy and Crawford, 1949) and either IMP or hypoxanthine. The reaction with IMP was completely abolished in the presence of inhibitor, and that with hypoxanthine reduced by 75%. Initial extracts from ascites-tumour cells were tested for interference by xanthine oxidase by assays 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 was used to inhibit this enzyme. When the initial extract was made from ascites-tumour cells that had been frozen and thawed the washed cells were almost free of erythrocytes, and xanthine oxidase was usually undetectable. The dialysed enzyme obtained after ammonium

Fig.I.3. The reaction of IMP and hypoxanthine with an extract from chicken liver acetone-dried powder in the presence of NAD.

Assays were carried out as described in the text and hypoxanthine (A) and IMP (B) were added as indicated by the arrows. The reaction with IMP (curve B) was followed at 340 mμ (○) and 290 mμ (△).



0.4
0.3
0.2
0.1
0
E^1_{340}
A
0.3
0.2
0.1
0
E^1
B
0
10
20
30
40
Time (min.)

sulphate fractionation was always free of xanthine oxidase and almost free of enzymes that convert IMP into hypoxanthine and inosine. During incubation (for details see the Materials and Methods 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 (0.53 μmole of IMP).

(b) Some properties of the partially purified IMP dehydrogenase

(1) Passage of an extract from ascites-tumour cells through a Sephadex column.

Sephadex, which separates materials on the basis of molecular size, is often useful in enzyme purification. Use of Sephadex only resulted in a small increase of specific activity of IMP dehydrogenase, but gave some information on the apparent particle size of this enzyme. A column of Sephadex G-50 (8 cm. x 1.3 cm.^{3}) was prepared and equilibrated by allowing 15 mM-phosphate (K^+, pH 7.4) to pass through the gel until the pH of the effluent was constant at 7.4 (about 3 ml.). The column was enclosed in a jacket through which water (4°) was circulated during the experiment. A portion (2.5 ml.; 16 mg. of protein; 0.08 units of activity) of 'Protamine supernatant' (Table I.1; see p. 36) was layered onto the top of the column and allowed to enter the gel under gravity. Elution was carried out with 15 mM-phosphate (K^+, pH 7.4) and fractions of 1.1 - 3.6 ml. were collected by hand. Each fraction was assayed for IMP dehydrogenase activity and portions taken for the spectrophotometric determination of protein

(Warburg & Christian, 1942). When all the protein had been eluted from the gel, the column was standardized with a mixture of 10 mg. of cytochrome C (Molec. Wt. 13,200) and 10 mg. of serum albumin (Molec. Wt. 70,000) dissolved in 4 ml. of water. Elution was carried out with 15 mM-phosphate (K^+, pH 7.4) and cytochrome c and serum albumin detected by reading extinctions at 418 mμ and 280 mμ respectively. The elution patterns of protein, IMP dehydrogenase activity, cytochrome c and serum albumin are shown in Fig. I.4. There was no loss in total activity during passage through the Sephadex column; 101% of the original activity was recovered in the collected fractions.

(ii) 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 by mixing diffusate and dialysis residue (Fig. I.5.) but only 2% of the diffusate was needed to restore full activity. This suggested that dialysis removed a compound or compounds required in catalytic amounts for maximum enzymic activity. The dialysis residue was not reactivated by addition of 2 mM-$MgCl_2$ or of reduced glutathione (2 mM) to the assay mixture; the loss of activity was not prevented by the presence of 0.1 mM-EDTA in all solutions.

It is of interest that reduced glutathione did not stimulate

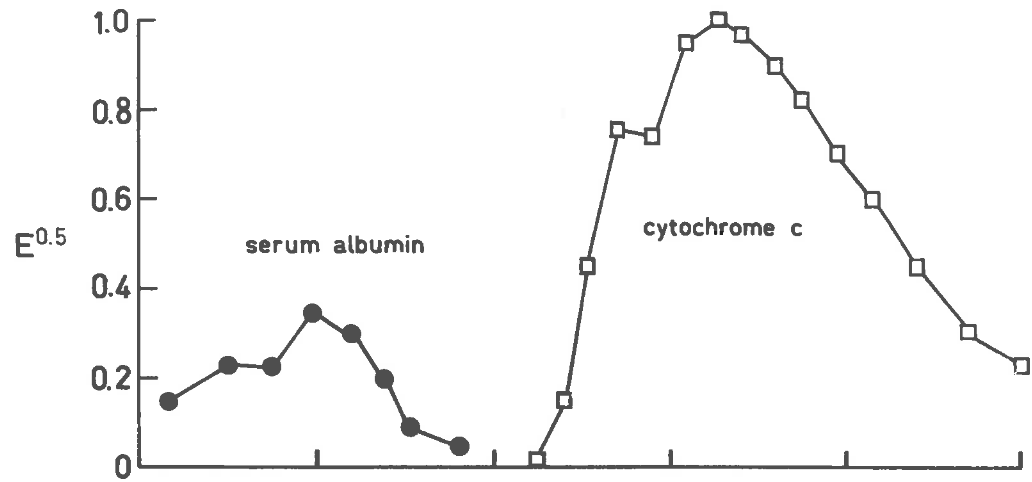
Fig. I.4. The passage of IMP dehydrogenase through Sephadex G-50. See the text for details

○ protein (mg./ml.)

△ IMP dehydrogenase activity (μm-moles/min./mg. of protein)

● serum albumin ($E^{0.5}_{280\ m\mu}$)

□ cytochrome c ($E^{0.5}_{418\ m\mu}$)


1.0
0.8
0.6
0.4
0.2
0
$E^{0.5}$
serum albumin
cytochrome c

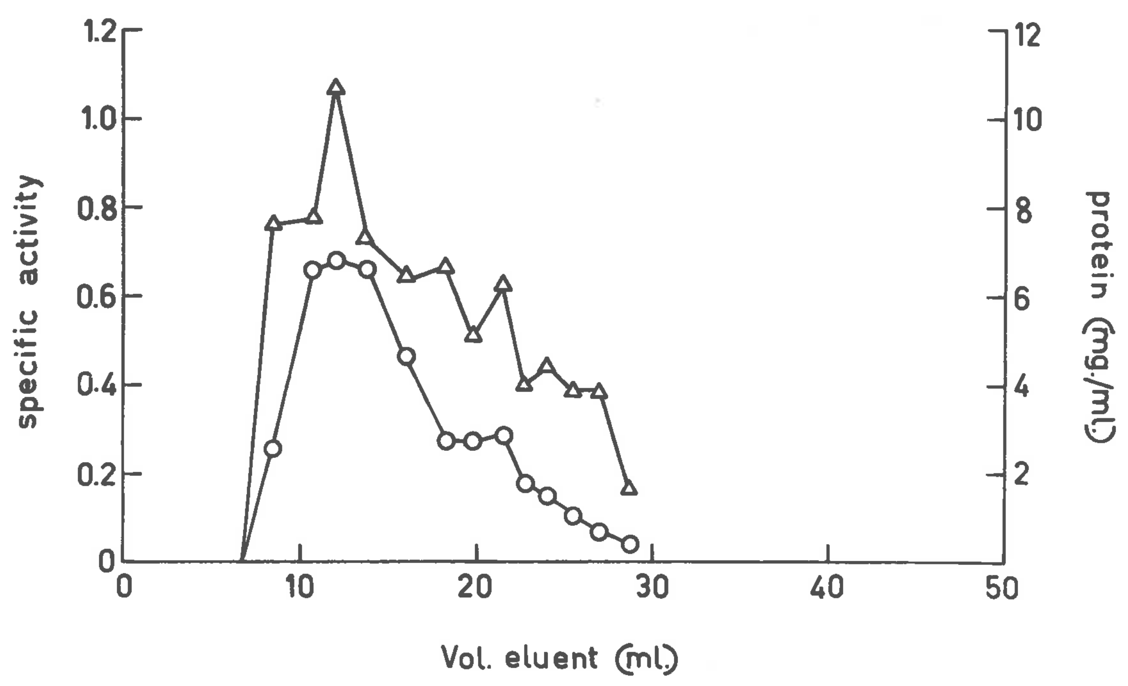
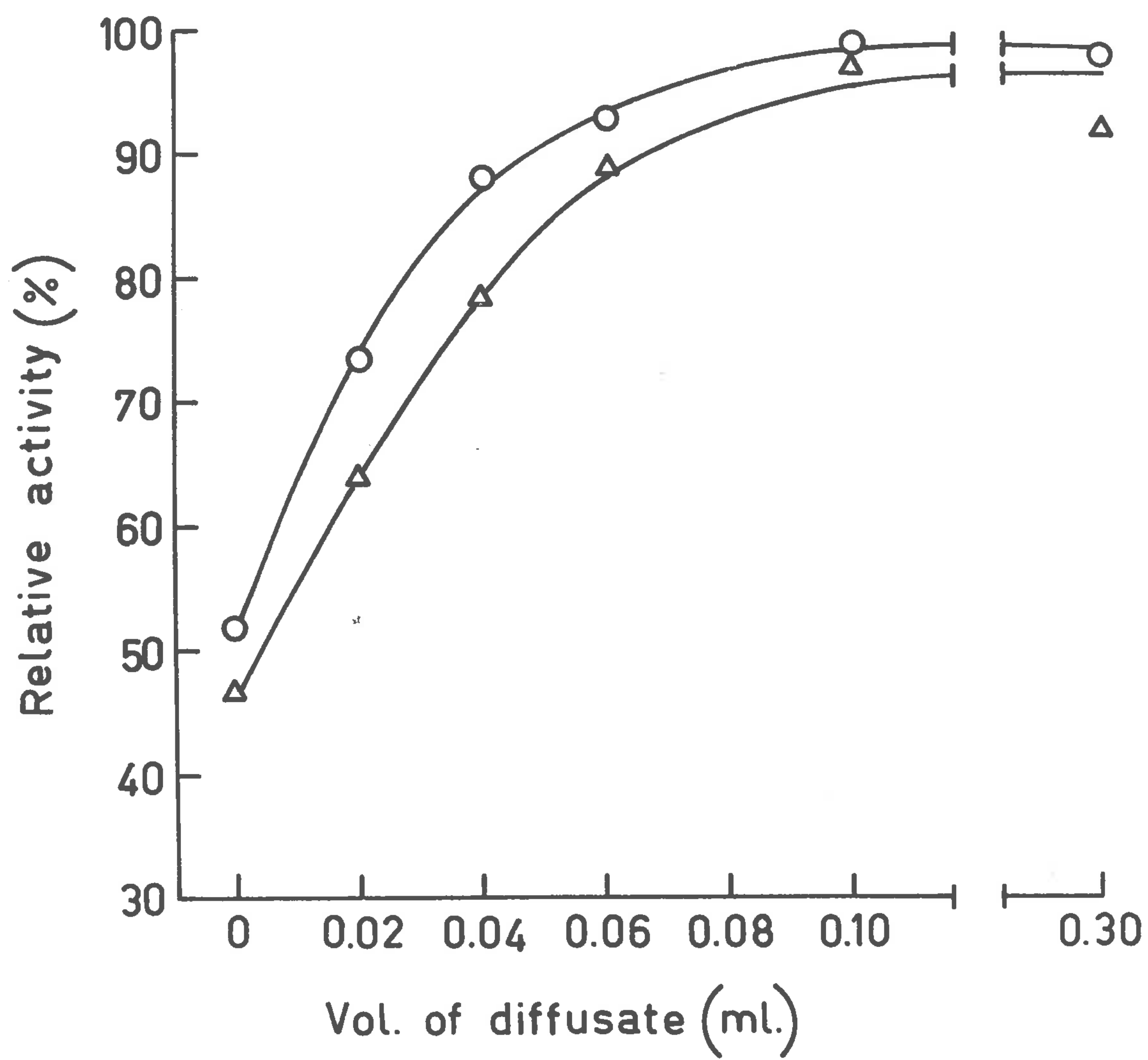

1.2
1.0
0.8
0.6
0.4
0.2
0
specific activity
12
10
8
6
4
2
protein (mg./ml.)
0
10
20
30
40
50
Vol. eluent (ml.)

Fig. I.5. 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 Materials and Methods section). The volumes of the diffusate shown in the Figure were added to the assay mixtures.



100
90
80
70
60
50
40
30
Relative activity (%)
0
0.02
0.04
0.06
0.08
0.10
0.30
Vol. of diffusate (ml.)

the IMP dehydrogenase from ascites-tumour cells. Magasanik _et al._ (1957) reported that the enzyme from _A. aerogenes_ was usually stimulated three-fold by glutathione and that some preparations were completely inactive in its absence. This was confirmed by Hampton (1963) with enzyme from the same source. However, Turner and King (1961) found no requirement for glutathione with IMP dehydrogenase from pea seeds.

The nature of the activation by diffusate in these experiments is not known and enzyme used for kinetic studies had not been reactivated with diffusate. In all experiments described here, no loss of activity through storage was detected between the first and last kinetic experiments.

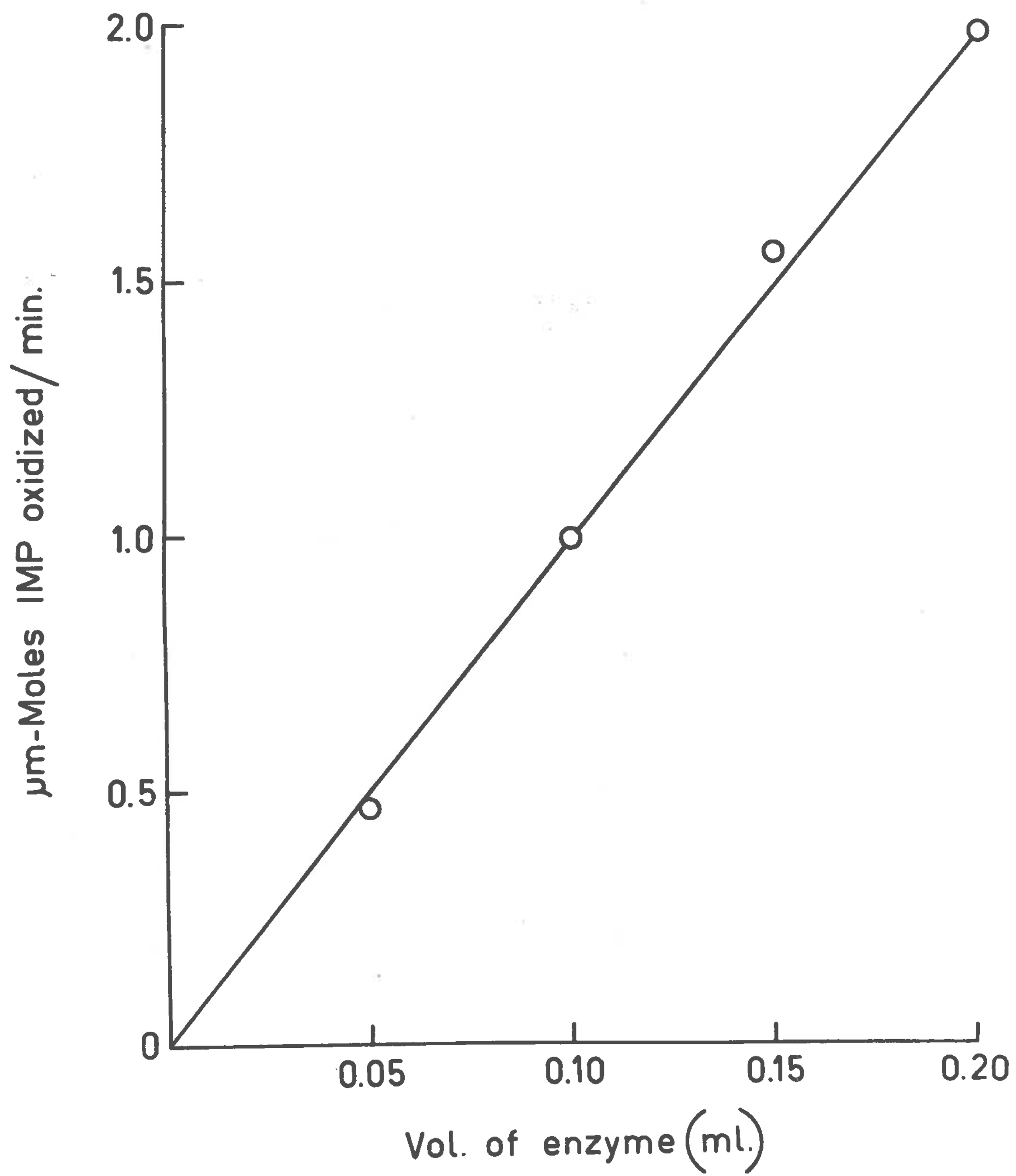
(iii) The effect of protein concentration on the reaction catalysed by IMP dehydrogenase

Using the standard assay system for IMP dehydrogenase described in the Materials and Methods section, the initial velocity was found to be directly proportional to the amount of enzyme ('dialysed fraction'; Table I.1.; see p. 36) added in each assay (see Fig. I.6.)

(iv) The effect of pH on the reaction catalysed by IMP dehydrogenase

Activities of IMP dehydrogenase at pH values between 7 and 9 were measured with the standard assay system adjusted to the appropriate pH with HCl. A dialysed enzyme preparation was used (0.1 ml.;

Fig. I.6. The effect of protein concentration on the reaction catalysed by IMP dehydrogenase.
The indicated amounts of 'dialysed enzyme' (see Table I.1.; p. 36) were assayed as described in the Materials and Methods section.


2.0
1.5
1.0
0.5
0
μm-Moles IMP oxidized/ min.
0.05
0.10
0.15
0.20
Vol. of enzyme (ml.)

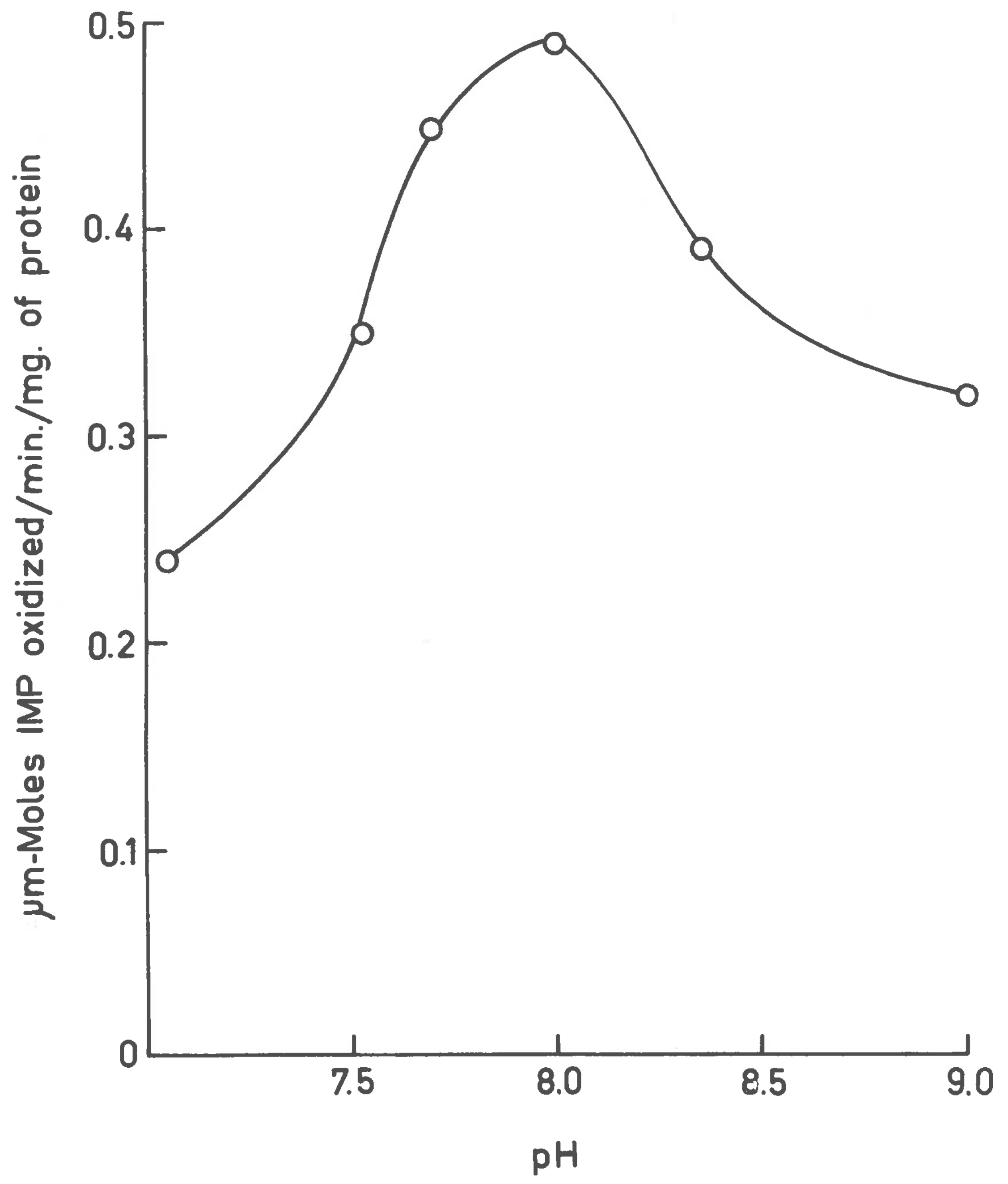
1.6 mg. of protein, see p. 36). As shown in Fig. I.7. there was a sharp maximum near pH 8.0. A similar relationship between initial velocity and pH has been reported with enzyme isolated from *A. aerogenes* (Magasanik *et al*., 1957) and from pea seeds (Turner & King, 1961).

(v) The effect of temperature on the reaction catalysed by IMP dehydrogenase

To study the effect of temperature on the initial velocity of the IMP dehydrogenase reaction, routine assays were carried out with dialysed enzyme (1.4 mg. of protein), and curvettes were kept at the required temperature in the thermostatic cell holder of a Beckman DK-2A spectrophotometer for 4 min. before the reaction was started by the addition of IMP. The results (see Fig. I.8.A.) show an increase in initial velocity with increased temperature up to 45°, but at 50° the rate has started to decrease rapidly. An estimate of the activation energy for IMP dehydrogenase may be obtained by plotting log V against 1/T (see Fig. I.8.B. cf. Dixon & Webb, 1958, p. 150). The energy of activation for the reaction was calculated to be 20.8×10^{3} calories per mole and the Q_{10} for IMP oxidation to be 1.4. The results are not conclusive as the value of K_m will also alter with temperature; in these experiments the same concentrations of substrates were used at each temperature and it was assumed these were sufficient to give rates approaching the maximum velocity in each case. A more complete interpretation requires

Fig. I.7. The effect of pH on the reaction catalysed by IMP dehydrogenase.

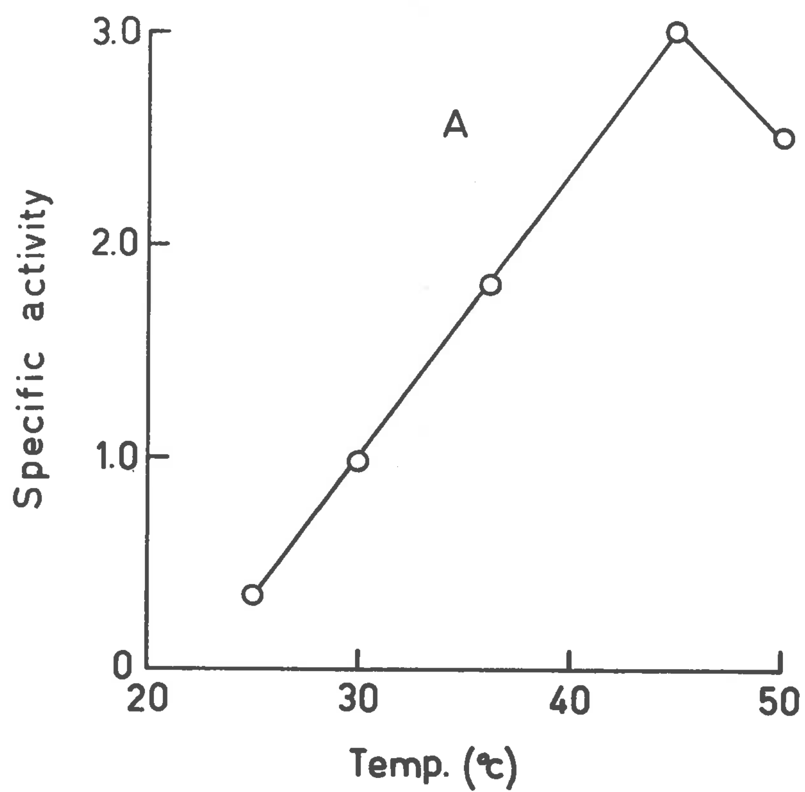
Standard assay systems (see the Materials and Methods section) were adjusted to the appropriate pH with HCl. A preparation of 'dialysed enzyme' was used (0.1 ml.; 1.6 mg. of protein; cf. Table I.1., p. 36).

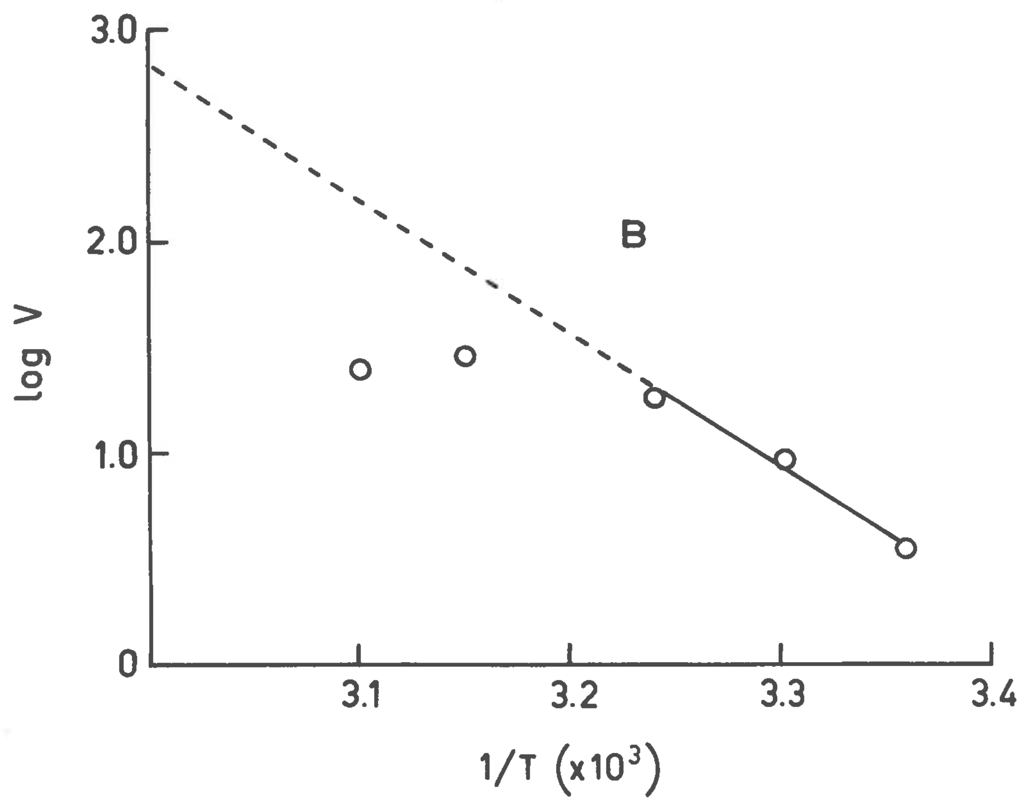


μm-Moles IMP oxidized/min./mg. of protein
0.5
0.4
0.3
0.2
0.1
0
7.5
8.0
8.5
9.0
pH

Fig. I.8. The effect of temperature on the reaction catalysed by IMP dehydrogenase.

Details of the assay systems are given in the text. Curve A shows the change in initial velocity of IMP dehydrogenase with increasing temperature (specific activity is µm-moles of IMP oxidized/min./mg. of protein). In curve B the results are shown as an Arrhenius plot (cf. Dixon & Webb, 1958, p. 157). As only the slope of the line is important, values of the specific activity (see curve A) were multiplied by ten before logarithms were taken.


A
Specific activity
3.0
2.0
1.0
0
20
30
40
50
Temp. (°c)


B
log V
3.0
2.0
1.0
0
3.1
3.2
3.3
3.4
1/T (x10³)

determination of the maximum velocity by extrapolation, after determination of initial velocities at a number of substrate concentrations, for each temperature.

(c) Kinetic studies with IMP dehydrogenase.

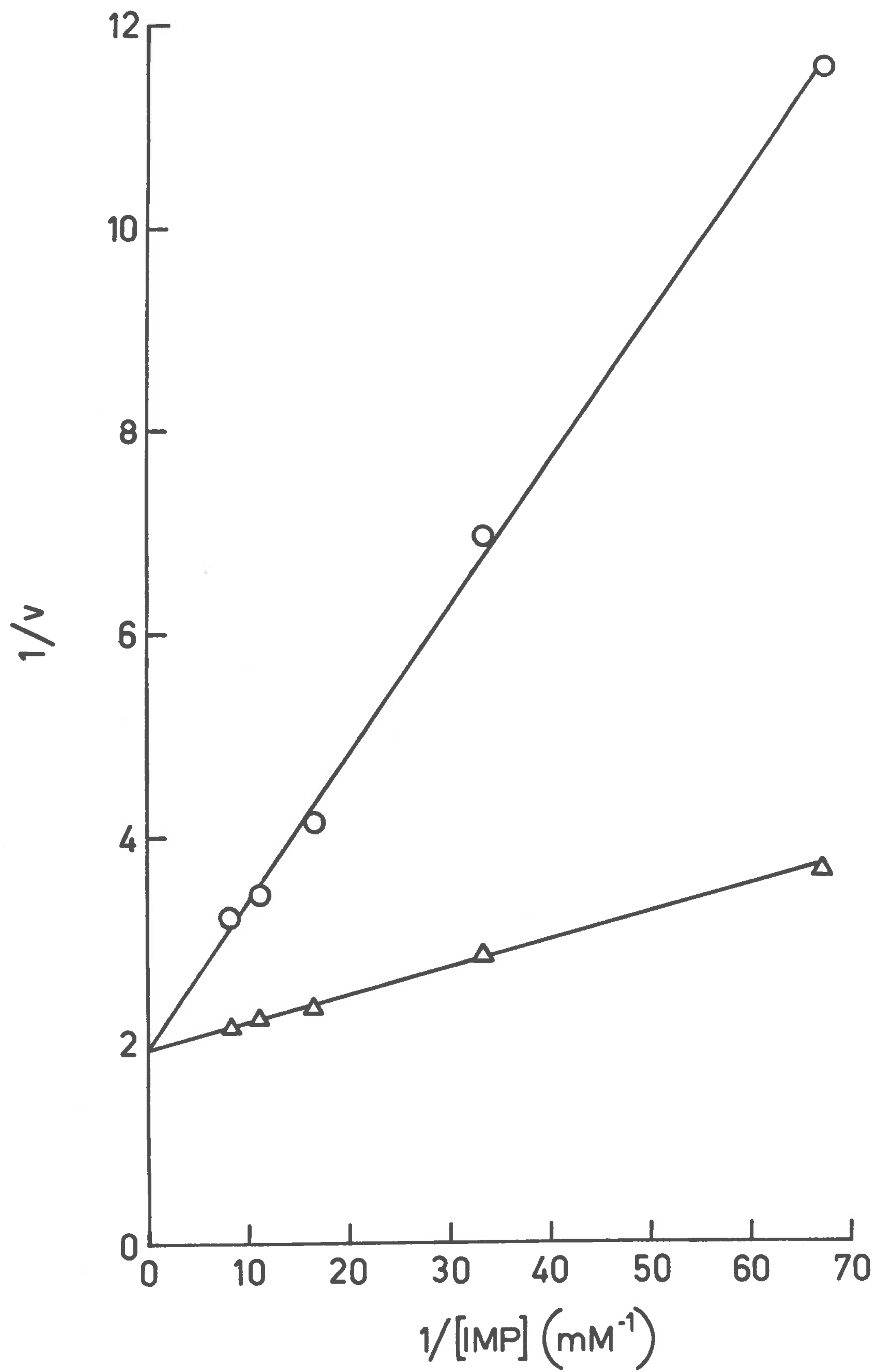
The reciprocal (V^{-1}) of dehydrogenase activity was a linear function of the reciprocal of IMP concentration (Fig. I.9.). K_m for IMP at pH 8.0 and 25° in the presence of 0.6 mM-NAD was 14.3 ± 0.8 μM. With a different batch of enzyme a value of 15.1 ± 0.8 μM was found.

Magasanik *et al*. (1957) reported that K_m for IMP was 14 μM with dehydrogenase from *A. 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 26 μM at pH 8.0 and 25° in the presence of 0.14 mM-NAD.

In the presence of 16 μM-thio-IMP the reciprocal of dehydrogenase activity was again a linear function of the reciprocal of IMP concentration (Fig. I.9.). The extrapolated maximum rate (V_p) was 0.525 ± 0.018 μm-mole of IMP oxidized/min./mg. of protein, which did not differ significantly 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. In the presence of thio-IMP the apparent value of K_m was increased from 14.3 ± 0.8 μM to 77.4 ± 5.3 μM and K_i for thio-IMP, calculated from the expression for competitive inhibition (see the Materials and Methods section) was 3.6 ± 0.2 μM. The kinetic parameters and the initial velocities

Fig. I.9. The inhibition of IMP dehydrogenase by thio-IMP.

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 (△) 16 μM-thio-IMP; for details see the text. v, μm-moles/min./mg. of protein.


12
10
8
6
4
2
0
1/v
0
10
20
30
40
50
60
70
1/[IMP] (mM-1)

used in their calculation are listed in Table I.5.

The inclusion of 30 µM-6-mercaptopurine in assays containing 15 µM-IMP caused no decrease in the rate of reaction. The thio-IMP used in these studies was dissolved in 1 mM-mercaptoethanol and may have contained traces of volatile salts not completely removed by the purification procedure (see the Materials and Methods section); however, 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 or 6-mercaptopurine during the assay period. Prolonged incubations were not carried out to detect a possible slow formation of 6-thioxanthylic acid from thio-IMP as reported by Hampton (1963).

Salser *et al*. (1960) found that catalysis of IMP oxidation by an enzyme extract from pigeon liver was inhibited by thio-IMP but the nature of the inhibition was not studied. Quantitatively it is not possible to compare the results obtained here with those obtained by Salser et al. (1960) as their method was rather indirect and did not give initial rates of oxidation.

Some approximate estimations can be made concerning the potential physiological importance of this inhibition in ascites-tumour cells that have been treated with 6-mercaptopurine. The ascites-tumour cells used here have been shown to accumulate about 0.3 µmole of thio-IMP/ml. of cells that had been exposed to

Table I.5. Kinetic parameters for the inhibition of IMP dehydrogenase by thio-IMP

IMP concentration (μM)	v (μm-moles IMP oxidized /min./mg. of protein)	v_p* (μm-moles IMP oxidized /min./mg. of protein)
15	0.27	0.086
30	0.35	0.143
60	0.43	0.240
90	0.45	0.290
120	0.46	0.310

* Measured in the presence of 16 μM-thio-IMP.

K_m (IMP) 14.7 μM.

K_p (IMP + thio-IMP) 77.4 μM.

V 0.528 μm-moles/min./mg. of protein.

V_p 0.525 μm-moles/min./mg. of protein.

K_i (thio-IMP) 3.6 μM.

6-mercaptopurine (see Section II p. 109). 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; Schmitz, Hart & Reid, 1955; Section III of this thesis) that the concentration of IMP in ascites-tumour 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. 6-Mercaptopurine had no effect on the IMP dehydrogenase from Ehrlich ascites-tumour cells, indicating that conversion to thio-IMP is necessary for the inhibition of this enzyme.

4. Adenylosuccinate synthetase

(a) The effect of storage at -15° on the apparent activity of adenylosuccinate synthetase

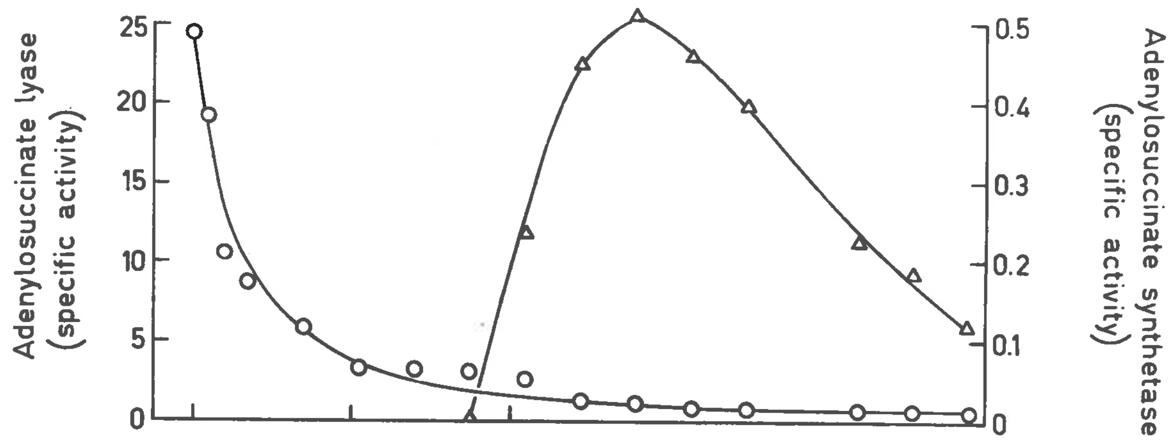
As shown in Fig. I.10, there are marked changes in the apparent activities of adenylosuccinate lyase, adenylosuccinate synthetase, IMP dehydrogenase, and 6-mercaptopurine phosphoribosyltransferase in extracts of Ehrlich ascites-tumour cells that have been stored at -15°. The last of these enzymes, which catalyses the formation of thio-IMP from PRPP and 6-mercaptopurine, was assayed spectrophotometrically as described in Section II of this thesis (see p. 91). No adenylosuccinate synthetase could be shown by the spectrophotometric assay in the first extracts from cells which

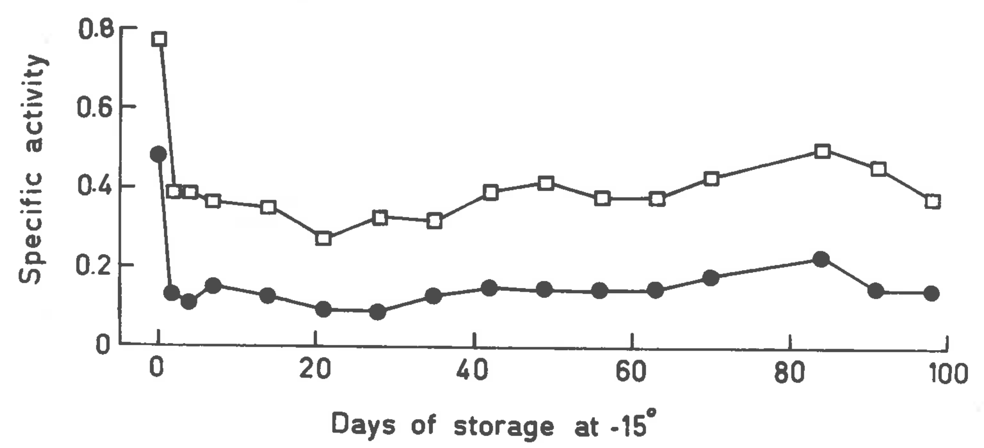
had been stored for less than 28 days; this failure to detect the synthetase was due to cleavage of the adenylosuccinate by adenylosuccinate lyase. After cells had been stored for 35 days at -15° traces of adenylosuccinate synthetase activity (i.e. net conversion of IMP into adenylosuccinate) could be detected in the extract (Fig. I.10) and the real activity of the synthetase was therefore greater than that of the adenylosuccinate lyase (3μm-moles of adenylosuccinate cleaved/min./mg. of protein) at this stage of storage. Even after fractionation as described in the Materials and Methods section the synthetase from fresh cells could not be sufficiently freed of lyase for use in kinetic studies; the small residual activity of lyase in cells that had beem kept at -15° for 84 days was easily removed.

(b) Assay of adenylosuccinate synthetase

Using the assay for synthetase activity as described in the Materials and Methods section, the rate was constant for about 10 min. after the reaction had been started by the addition of aspartate. After this time the rate began to decrease, probably due to inhibition by GDP formed during the reaction. 'Product inhibition' by GDP has been reported with adenylosuccinate synthetase from *E. coli* by Lieberman (1956), who found that the inhibition could be overcome by the inclusion of ATP, phosphoenolpyruvate and pyruvate phosphokinase in the assay to constantly regenerate GTP from the GDP formed. However, as linear initial rates could be obtained in the absence of a regenerating system, it was thought better not

Fig. I.10. Changes in the apparent activities of adenylosuccinate lyase (○), adenylosuccinate synthetase (△), IMP dehydrogenase (●), and 6-mercaptopurine phosphoribosyltransferase (□) in extracts of Ehrlich ascites-tumour cells after various periods of storage at -15°. For details of assays see the Materials and Methods section. Individual samples were frozen and thawed once for each assay.


Adenylosuccinate lyase (specific activity)
Adenylosuccinate synthetase (specific activity)
25
20
15
10
5
0
0.5
0.4
0.3
0.2
0.1
0


Specific activity
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Days of storage at -15°

to introduce further components into the assay system when studying the kinetics of this reaction. The purified samples of GTP used for assays contained 0.02 mol. prop. of GDP (see the Materials and Methods section), and the resultant concentration of GDP in the assay solution was 3.5 µM. Addition of GDP to increase the concentration of this compound in the assay solution to 7.0 µM caused no inhibition of the initial rate of formation of adenylosuccinate. However, in agreement with Lieberman (1956), who observed 50% inhibition of the synthetase from E. coli in similar assays with 100 µM-GDP, the presence of 70 µM-GDP was found to inhibit partially-purified enzyme from ascites cells (see the Materials and Methods section) by 29%.

No extinction change could be detected at 280 mµ in assays in which either GTP, $MgCl_2$, L-aspartate or IMP were omitted, or in which 0.5 µmole of ATP replaced GTP in the complete assay.

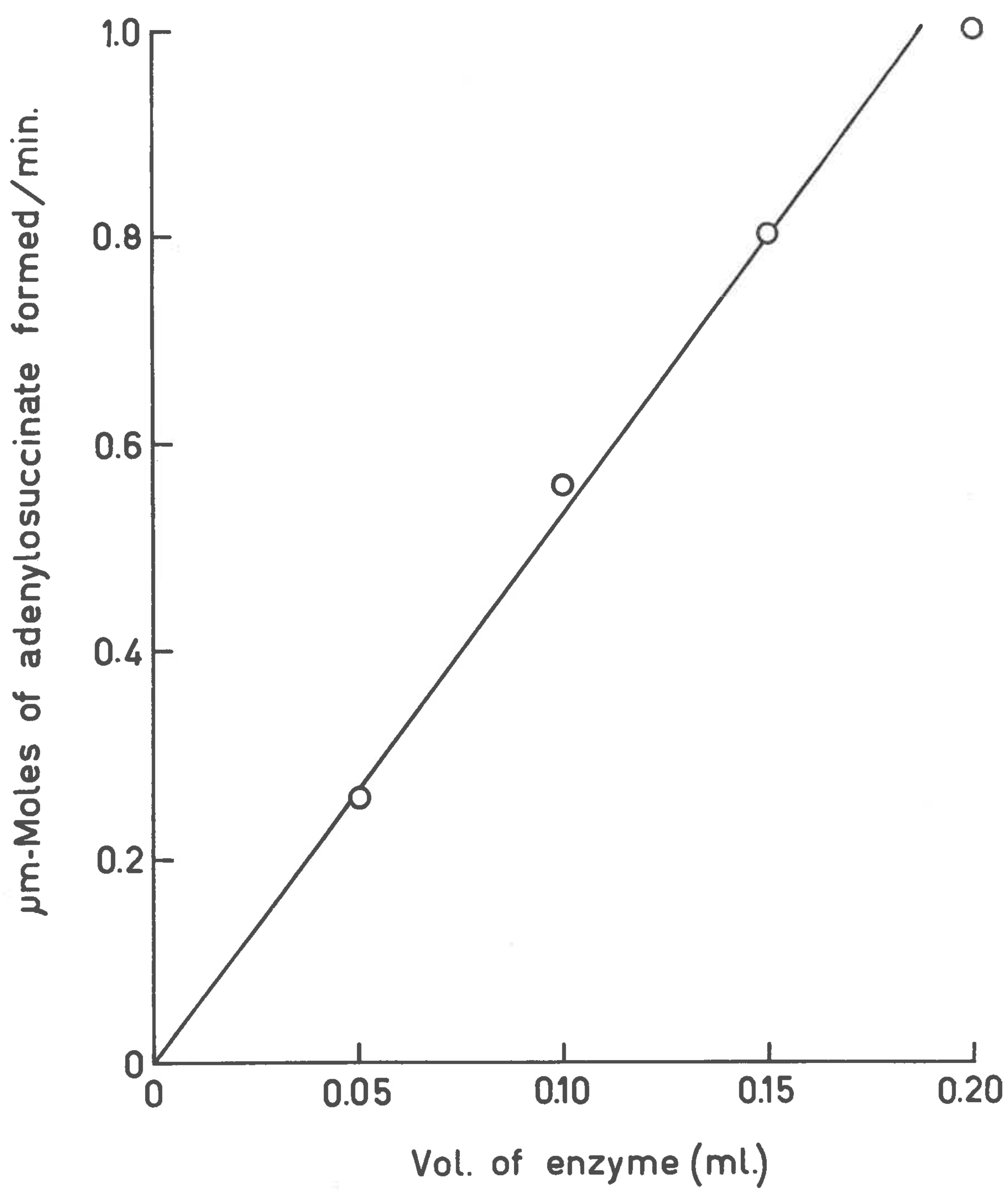
(c) The effect of protein concentration on the reaction rate

As shown in Fig. I.11 the initial velocity of adenylosuccinate synthetase was directly proportional to the amount of protein ('dialysed enzyme', Table I.2.; see p. 39) which was present in the assays (for details of the assay system see the Materials and Methods section).

(d) Kinetics of the reaction catalysed by adenylosuccinate synthetase; inhibition by thio-IMP

The assay conditions and the purity of the synthetase used

Fig. I.11. The effect of protein concentration on the reaction catalysed by adenylosuccinate synthetase.
The indicated amounts of 'dialysed enzyme' (see Table I.2.; p. 39) were assayed as described in the Materials and Methods section.



μm-Moles of adenylosuccinate formed/min.
1.0
0.8
0.6
0.4
0.2
0
0
0.05
0.10
0.15
0.20
Vol. of enzyme (ml.)

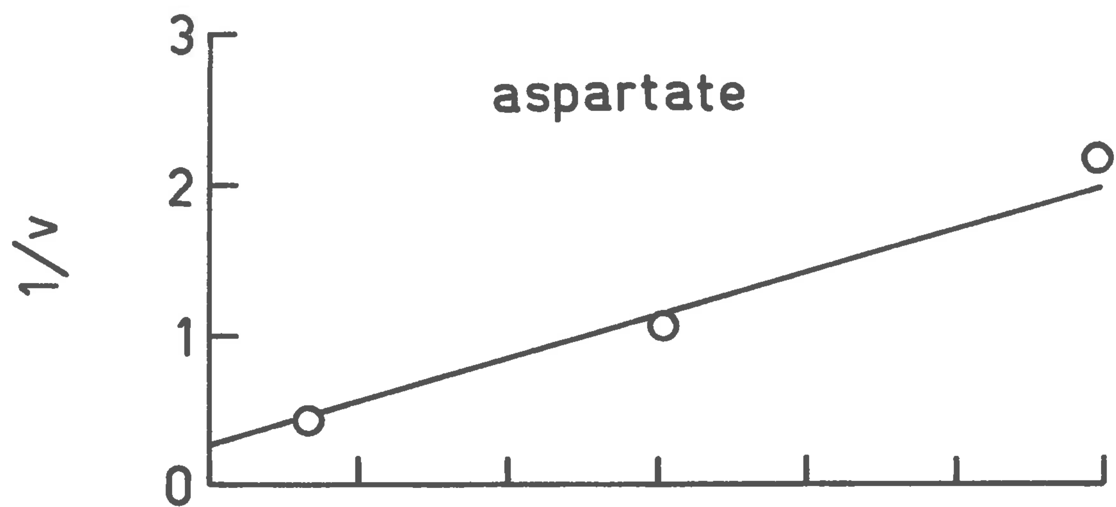
in these studies seemed adequate for the measurement of K_m and the K_i of thio-IMP since the reaction was zero order with respect to substrates during the assay period and since 96% of the IMP added to the reaction mixture (see Materials and Methods section) was recovered unchanged or as adenylosuccinate. In the assay system with glycine buffer (final pH 7.5) K_m for GTP was 96 ± 16 μM and K_m for L-aspartate was 58 ± 8 μM (see Table I.6.). Lineweaver & Burk (1934) plots for GTP and L-aspartate are shown in Fig. I.12. In three separate determinations of the K_m for IMP, values of 37 ± 3.3, 26 ± 4.4 and 33 ± 3.6 μM were obtained (see Table I.7.). With adenylosuccinate synthetase from E. coli, Lieberman (1956) obtained K_m values of 40 μM, 100 μM and 30 μM for GTP, L-aspartate and IMP respectively.

In the presence of thio-IMP there appeared to be non-competitive inhibition of the synthetase. As shown in Table I.7. V_p was always less than V ($P < 0.05$), and with glycine buffer and with the tris-phosphate system at pH 7.76 there was no significant difference between K_m and K_p ($P > 0.2$). With tris-phosphate at pH 7.01 the difference between K_m and K_p was only significant at the 10% level. Values of K_i, calculated from the expression for non-competitive inhibition (see the Materials and Methods section) were in the range 170 - 390 mM-thio-IMP. As with IMP dehydrogenase, adenylosuccinate synthetase was not inhibited by 6-mercaptopurine. Thus assays with 6 μM-IMP had the same initial velocity in the presence or absence of 200 μM-6-mercaptopurine (assays were carried out at pH 7.5). Kinetic parameters are summarized in Table I.7. and

Fig. I.12. The reaction of L-aspartate and GTP with adenylosuccinate synthetase.

○ L-aspartate

△ GTP


aspartate
1/v
3
2
1
0

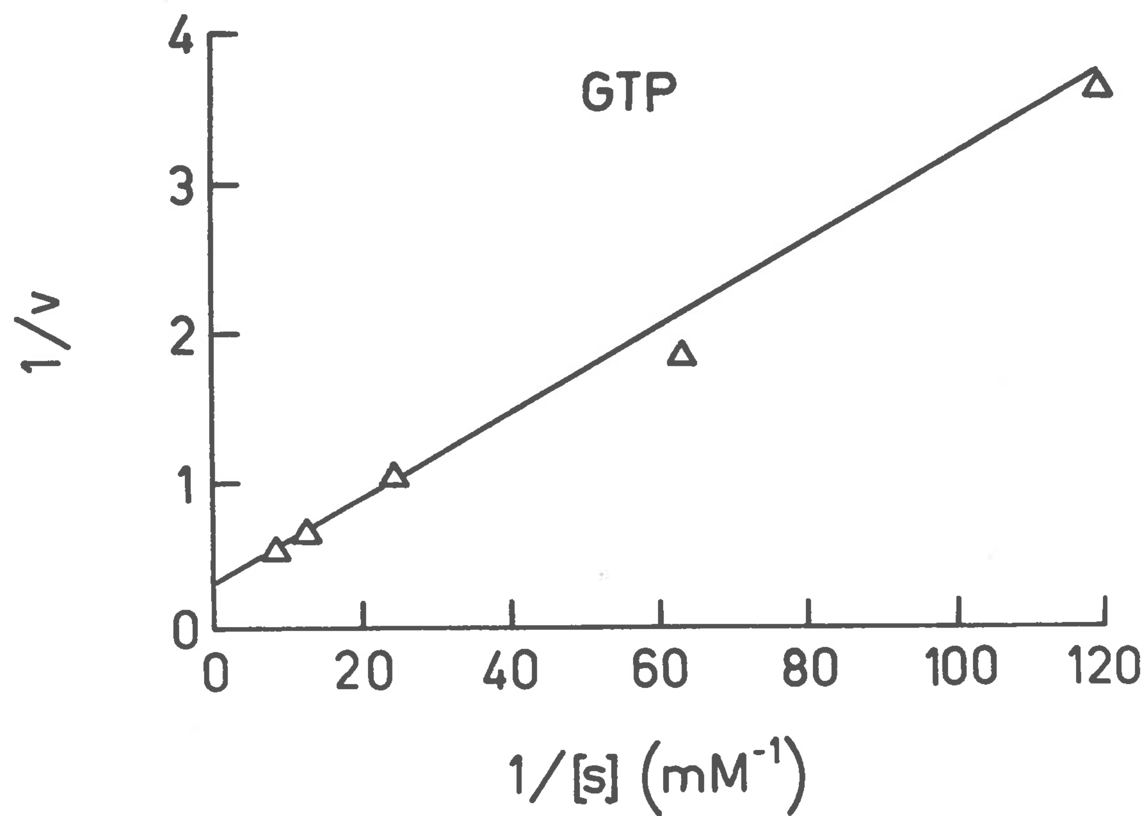

GTP
1/v
4
3
2
1
0
0
20
40
60
80
100
120
1/[s] (mM^{-1})

Table I.6. Kinetic parameters of GTP and L-aspartate in the reaction catalysed by adenylosuccinate synthetase

GTP concentration (µM)	v (µm-moles/min./mg. of protein)	L-aspartate concentration (µM)	v (µm-moles/min./mg. of protein)
8.4	0.276	8.3	0.456
16.0	0.539	16.6	0.918
41.0	0.954	83.0	2.270
80.0	1.580		
119.0	1.870		

K_m (GTP) 96 $\pm$ 16 µM

V (GTP) 3.1 $\pm$ 0.3 µm-moles/min./mg. of protein

K_m (L-aspartate) 58 $\pm$ 8 µM

V (L-aspartate) 3.4 $\pm$ 0.25 µm-moles/min./mg. of protein

Table 1.7. Inhibition of adenylosuccinate synthetase from Ehrlich ascites-tumour cells by thio-IMP.

Expts. A-E were carried out with different batches of synthetase; values of V and V_p for different bracketed groups are not comparable. Expts. A-C were carried out with the glycine buffer system, and Expts. D and E with the tris-phosphate buffer (for details see the Materials and Methods section). Values in parentheses are the coefficients of variation of the means (for details of calculations see the Materials and Methods section).

Expt.	pH	[thio-IMP] (μM)	K_m of IMP (μM)	K_p (μM)	μm-moles/min./mg. of protein: V	μm-moles/min./mg. of protein: V_p	K_i (μM)
A	7.5	0	37 (0.09)	-	2.5 (0.04)	-	-
B	7.5	0	26 (0.17)	-	1.5 (0.08)	-	-
B	7.5	228	-	32 (0.17)	-	0.95 (0.08)	394 (0.30)
C	7.5	0	33 (0.11)	-	1.98 (0.04)	-	-
C	7.5	92	-	29 (0.13)	-	1.29 (0.05)	172 (0.18)
C	7.5	240	-	26 (0.05)	-	0.89 (0.02)	196 (0.08)
D	7.01	0	22 (0.06)	-	0.208 (0.02)	-	-
D	7.01	195	-	34 (0.11)	-	0.131 (0.04)	332 (0.12)
E	7.76	0	54 (0.17)	-	0.291 (0.01)	-	-
E	7.76	195	-	74 (0.04)	-	0.177 (0.02)	303 (0.05)

values of the initial velocities used in calculating these parameters in Table I.8. Lineweaver & Burk (1934) plots for IMP, alone or in the presence of either 92 or 240 μM-thio-IMP are shown in Fig. I.13.

5. Adenylosuccinate lyase

(a) The effect of protein concentration on the rate of adenylosuccinate cleavage.

Assays with 0.05, 0.1, 0.15 and 0.2 ml. of lyase from ascites-tumour cells ('dialysed enzyme'; 22 mg. of protein/ml.) indicated a linear relationship between the initial rate of adenylosuccinate cleavage and the protein concentration (see Fig. I.14).

(b) The kinetics of adenylosuccinate cleavage, in the presence and absence of thio-IMP, at different pH values.

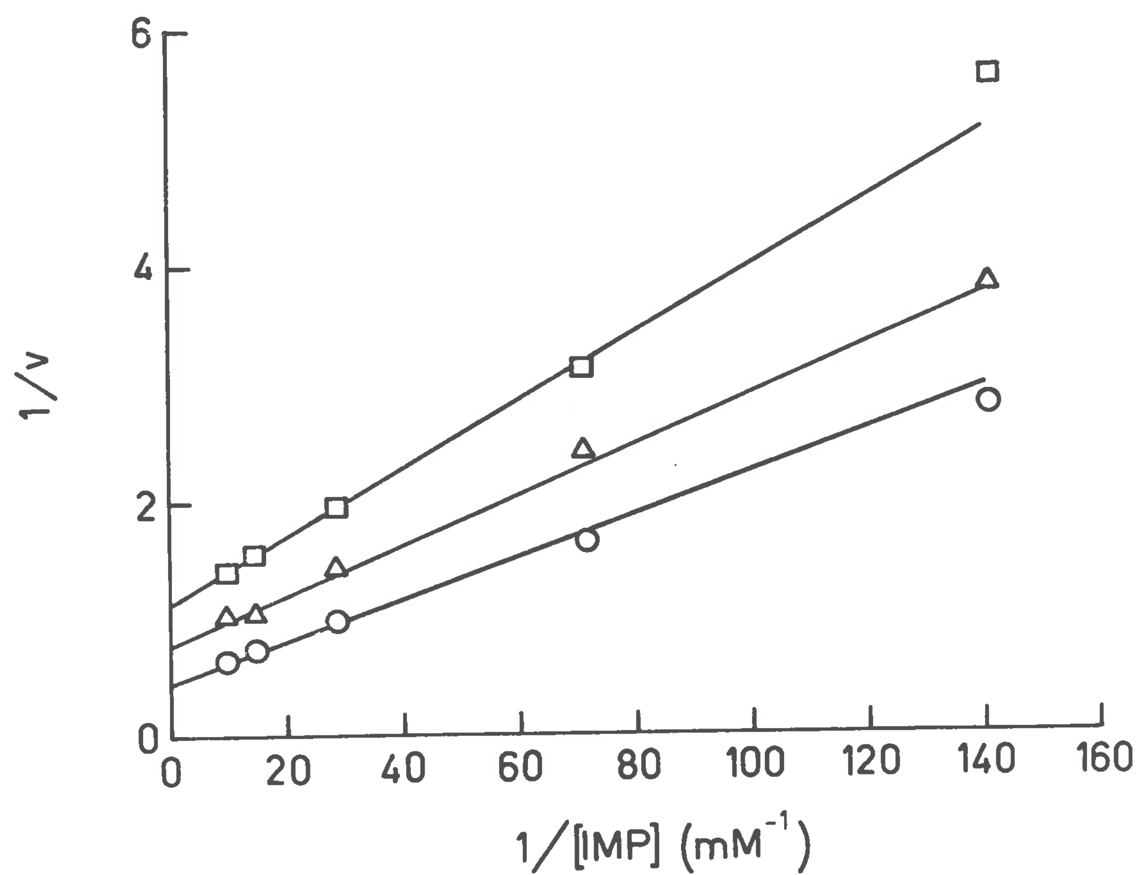
Values of K_m, K_p, V and V_p for cleavage of adenylosuccinate by the lyase from ascites-tumour cells at 12 pH values from 5.6 to 8.4 in the presence and absence of thio-IMP are listed in Table I.9. There was no evidence of a significant difference between V and V_p at any pH value. The weighted mean value of $(V - V_p)$, with weights inversely proportional to variance, did not differ significantly from zero within the 95% fiducial limits. Values of K_i, calculated from the expression for competitive inhibition are also listed in Table I.9. The initial velocities used in calculating these parameters are listed

Fig. I.13. Inhibition of adenylosuccinate synthetase from Ehrlich ascites-tumour cells by thio-IMP.

○ no thio-IMP

△ 92 μM-thio-IMP

□ 240 μM-thio-IMP



6
4
2
0
1/v
0
20
40
60
80
100
120
140
160
1/[IMP] (mM^{-1})

Fig. I.14. The effect of protein concentration on the reaction catalysed by adenylosuccinate lyase.

The indicated amounts of 'dialysed enzyme' (see Table I.3.) were assayed as described in the Materials and Methods section.

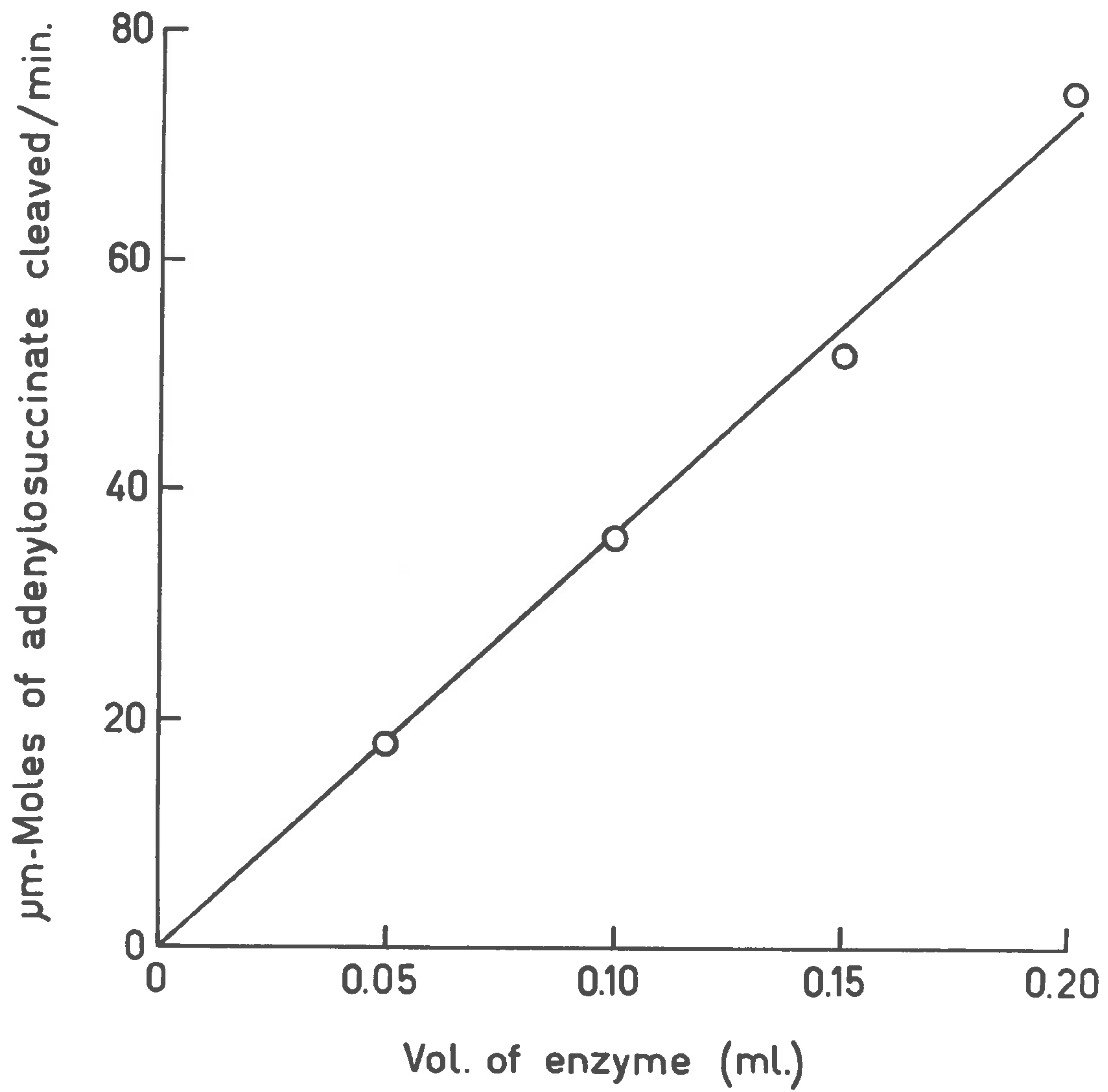

µm-Moles of adenylosuccinate cleaved/min.
80
60
40
20
0
0
0.05
0.10
0.15
0.20
Vol. of enzyme (ml.)

Table I.8. Initial velocities of adenylosuccinate synthetase from Ehrlich ascites-tumour cells, in the presence and absence of thio-IMP, used in the calculation of K_m, V, K_p, and V_p (see Table I.7.)

Values in parentheses are the concentrations of thio-IMP (μM) used in inhibitor studies. All assays were carried out at pH 7.5, and rates are given as μm-moles of adenylosuccinate formed min./mg. of protein at 25°.

IMP concentration (μM)	v	v_p (228)
5.8	0.264	0.157
11.6	0.499	0.260
29.0	0.760	0.429
58.0	1.051	0.621

IMP concentration (μM)	v	v_p (92)	v_p (240
7.1	0.355	0.261	0.178
14.0	0.605	0.415	0.320
34.7	1.000	0.688	0.509
67.0	1.300	0.949	0.639
99.0	1.520	0.971	0.710

IMP concentration (μM)	v
7.6	0.508
15.0	0.849
37.0	1.343
73.0	1.853
108.0	2.110

Table I.8 (continued)

pH*	IMP concentration (μM)	v	v_p (195)
7.01	15.0	0.085	0.0425
	37.0	0.129	0.0656
	72.0	0.162	0.0890
	140.0	0.179	0.1060
7.76	15.0	0.0598	0.0290
	37.0	0.116	0.0598
	72.0	0.175	0.0871
	140.0	0.206	0.1160

* Tris-phosphate buffer system (for details see the Materials and Methods section).

Table I.9. Kinetic parameters of the conversion of adenylosuccinate into adenylate by adenylosuccinate lyase from Ehrlich ascites-tumour cells.

Details are given in the text. Values in parentheses are the coefficients of variation of the means.

pH	K_m (µM)		K_p (µM)		V (µm-moles/ min./mg. of protein)		V_p (µm-moles/ min./mg. of protein)		K_i (µM)	
5.64	5.8	(0.17)	7.5	(0.14)*	1.99	(0.05)	1.87	(0.05)*	750	(0.97)
5.85	4.5	(0.08)	5.7	(0.20)*	2.86	(0.02)	2.62	(0.06)*	825	(1.05)
6.08	4.8	(0.09)	6.6	(0.09)*	4.45	(0.03)	3.90	(0.03)*	587	(0.47)
6.30	4.9	(0.09)	9.8	(0.05)*	5.2	(0.03)	5.4	(0.02)*	333	(0.25)
6.54	8.4	(0.14)	14	(0.02)*	6.9	(0.04)	7.2	(0.01)*	330	(0.38)
6.77	17.5	(0.15)	29	(0.25)*	8.9	(0.05)	9.3	(0.10)*	335	(0.72)
6.94	28	(0.15)	53	(0.12)*	10.5	(0.06)	10.6	(0.06)*	246	(0.39)
7.16	33	(0.04)	55	(0.04)†	10.3	(0.02)	10.2	(0.21)†	270	(0.14)
7.45	57	(0.06)	106	(0.08)†	11.0	(0.03)	10.9	(0.05)†	209	(0.22)
7.75	79	(0.02)	184	(0.13)†	11.8	(0.01)	14.0	(0.08)†	135	(0.23)
7.99	76	(0.19)	159	(0.55)†	10.1	(0.11)	8.6	(0.40)†	165	(1.26)
8.38	71	(0.22)	205	(0.80)†	7.1	(0.11)	7.0	(0.57)†	95	(1.26)

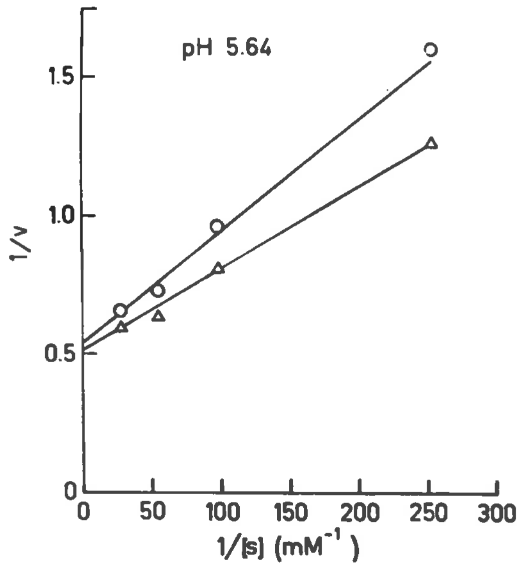
* Measured in the presence of 0.22 mM-thio-IMP.

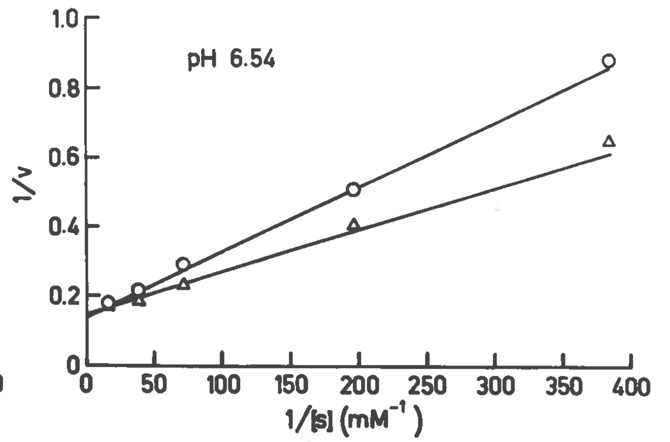
† Measured in the presence of 0.18 mM-thio-IMP.

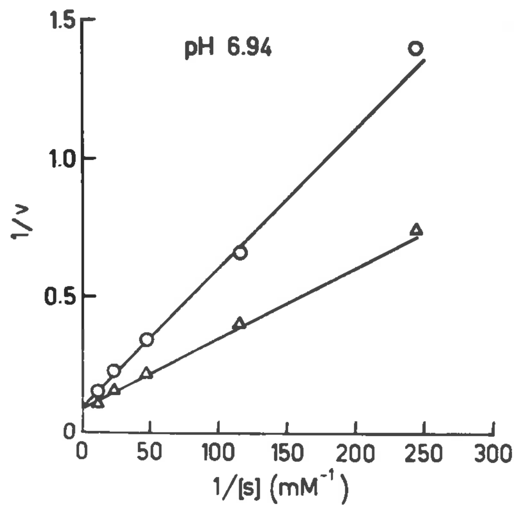
in Table I.10. Representative Lineweaver & Burk (1934) plots in the absence and presence of thio-IMP are shown in Fig. I.15., and illustrate the relatively large changes observed in K_m and V over the range studied. The variation of pK_m (i.e. $-\log K_m$), pK_i (i.e. $-\log K_i$) and log V with pH is shown in Fig. I.16. In the case of log V the solid line describes the relationship of log V and pH where $V = \tilde{V}/(1 + [H^+]/K_1)$ where $\tilde{V}$ is 11.5 μm-moles of adenylosuccinate cleaved/min./mg. of protein and pK_1 (i.e. $-\log K_1$) is 6.3 (cf. Wilkinson, 1961). Dixon & Webb (1958) have discussed the variation of log V with pH and have shown that a relationship of the type found here results from increasing conversion of an active form of the enzyme-substrate complex into an inactive form by protonation at lower pH values.

The unit change of slope about pH 6.3 (Fig. I.16) may be due to protonation of an essential imidazole nitrogen in the enzyme-adenylosuccinate complex, giving an inactive species. If the action of the lyase involved nucleophilic displacement of AMP from bound adenylosuccinate by the imidazole nitrogen of a histidine residue in the active centre a relationship of this kind would be expected, as the imidazolium ion predominating at lower pH values would be a much weaker nucleophile than the uncharged species predominating above the pK of the histidyl residue. However, there is no direct evidence for such a displacement, and other groups such as the phosphoryl group of the bound nucleotide might be involved in the change of V with pH.

Fig. I.15. Inhibition of adenylosuccinate lyase from ascites-tumour cells by thio-IMP at various pH values. Rates at pH 5.64, 6.54, and 6.94 were assayed in the presence (○) or absence (△) of 0.22 mM-thio-IMP, and at pH 7.75 in the presence (○) or absence (△) of 0.18 mM-thio-IMP.


pH 5.64
1/v
1.5
1.0
0.5
0
0 50 100 150 200 250 300
1/[s] (mM^{-1})


pH 6.54
1/v
1.0
0.8
0.6
0.4
0.2
0
0 50 100 150 200 250 300 350 400
1/[s] (mM^{-1})


pH 6.94
1/v
1.5
1.0
0.5
0
0 50 100 150 200 250 300
1/[s] (mM^{-1})

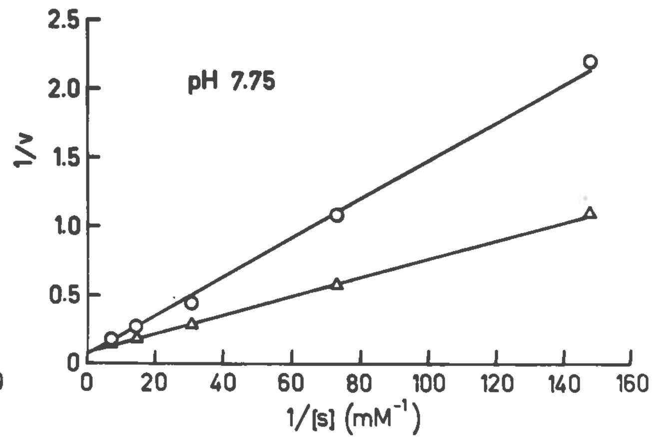
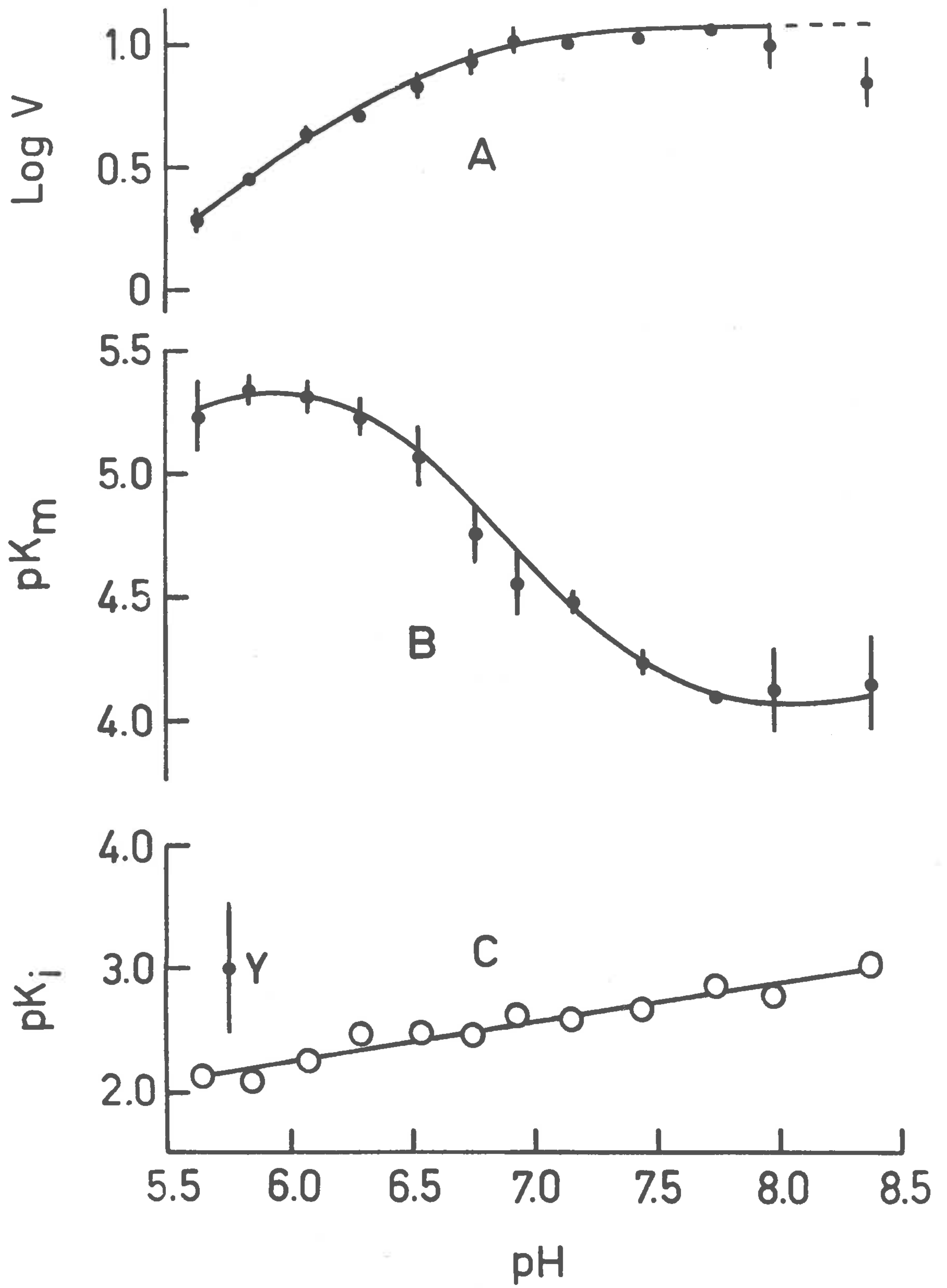

pH 7.75
1/v
2.5
2.0
1.5
1.0
0.5
0
0 20 40 60 80 100 120 140 160
1/[s] (mM^{-1})

Fig. I.16. Plots of log V(A), pK_m (B), and pK_i (C) against pH for the conversion of adenylosuccinate into AMP by adenylosuccinate lyase from Ehrlich ascites-tumour cells. For details see Table I.9. The solid line in A was derived from the relationship $V = \tilde{V}/(1 + [H^+]/K_1)$ where $pK_1 = 6.3$ (see the text). The 95% fiducial limits of individual points are shown in A and B. The line at Y in C indicates the mean 95% fiducial limit of the individual values of K_i.



Log V
1.0
0.5
0
A
pK m
5.5
5.0
4.5
4.0
B
pK i
4.0
3.0
2.0
Y
C
5.5
6.0
6.5
7.0
7.5
8.0
8.5
pH

Table I.10. Inhibition of adenylosuccinate lyase from Ehrlich ascites-tumour cells by thio-IMP.

Initial velocities in the presence and absence of thio-IMP used in the calculation of K_m, V, K_p and V_p at different pH values (see Table I.9.). Values of v_p were measured in the presence of 220 μm-thio-IMP.

pH	μm-moles/min./mg. of protein		adenylo-succinate concentration (μM)
	v	v_p	
5.64	0.795	0.625	3.94
	1.24	1.07	10.20
	1.58	1.38	18.20
	1.68	1.52	36.70
5.85	0.97	0.69	2.51
	1.45	1.12	4.30
	2.10	1.92	11.70
	2.29	2.05	20.10
	2.58	2.21	39.40
6.08	1.52	1.07	2.51
	2.07	1.60	4.40
	3.24	2.35	11.00
	3.52	3.07	21.20
	4.02	3.35	42.60
6.30	1.60	1.14	2.80
	2.30	1.82	4.78
	3.61	2.93	11.70
	4.18	3.83	22.50
	4.55	4.44	46.20
6.54	1.53	1.12	2.62
	2.48	1.96	5.12
	4.36	3.46	13.10
	5.46	4.61	25.20
	5.92	5.89	64.00
6.77	1.60	1.02	3.54
	2.83	2.05	7.73
	5.10	4.16	27.90
	6.10	5.56	36.50
	7.25	6.60	73.60
6.94	1.34	0.692	4.1
	2.48	1.51	8.7
	4.55	2.93	21.6
	6.41	4.80	41.2
	7.83	6.40	83.3

Table I.10 (continued)

pH	μm-moles/min./mg. of protein		adenylo-succinate concentration	pH	μm-moles/min./mg. of protein		adenylo-succinate concentration
	v	v_p*	(μM)		v	v_p*	(μM)
7.16	1.30	0.735	4.66	7.99	1.17	0.345	7.92
	2.21	1.380	8.41		1.83	0.705	17.80
	4.23	2.050	23.40		3.56	1.609	41.60
	5.90	4.550	44.0		5.50	3.06	89.00
	7.68	6.400	94.5				
7.45	0.901	0.501	5.23	8.38	1.17	0.314	12.50
	1.670	0.962	9.75		1.89	0.571	24.30
	3.300	2.205	25.60		2.98	1.74	57.20
	5.160	3.520	49.5		4.45	2.44	113.00
	7.110	5.420	106.0				
7.75	0.905	0.451	6.72	8.50	0.941	0.227	10.4
	1.760	0.930	13.60		1.670	0.470	22.1
	3.450	2.250	33.10		3.220	0.132	69.2
	5.480	3.720	69.0		4.450	0.238	137.0
	7.400	5.910	134.0				

* Velocity in the presence of 0.18 mM-thio-IMP.

The variation of pK_m and pK_i with pH is not described by any of the simple relationships discussed by Dixon & Webb (1958). The increase of K_m from 4.8 µM at pH 6.08 to 79 µM at pH 7.75 may be the result of a number of dissociations from essential groups in adenylosuccinate, lyase or enzyme-substrate complex and there is no change of slope corresponding to that in the log V - pH curve. Dixon & Webb (1958) have pointed out that dissociations, at essential groups with similar pK values, in both enzyme-substrate complex and in enzyme or substrate will have counteracting effects, resulting in no net change in the slope in the pK_m - pH line.

Similar results were obtained with adenylosuccinate lyase from yeast at 5 pH values from 6.2 to 8.0 (Table I.11); the relevant rate data are listed in Table I.12.

As with IMP dehydrogenase and adenylosuccinate synthetase, adenylosuccinate lyase from ascites-tumour cells was not inhibited by 6-mercaptopurine. The rate (with 4.2 µM-adenylosuccinate) was the same in the absence or presence of 200 µm-6-mercaptopurine in assays carried out at pH 6.77 or at pH 7.75.

(c) The effect of copper on adenylosuccinate lyase

It has recently been claimed that the apparent inhibition of adenylosuccinate lyase from yeast by thio-IMP requires the presence of copper (Bridger & Cohen, 1963). A decrease was observed in the

Table I.11. Kinetic parameters of the conversion of adenylosuccinate into adenylate by adenylosuccinate lyase from yeast.

Details are given in the text. Values in parentheses are the coefficients of variation of the means.

pH	K_m (μM)	K_p (μM)	V (μm-moles/ min./mg. of protein)	V_p (μm-moles/ min./mg. of protein)	K_i (μM)
6.20	8.8 (0.07)	14.2 (0.08)*	61 (0.02)	58 (0.03)*	636 (0.26)
6.46	6.9 (0.04)	12.3 (0.06)*	134 (0.01)	122 (0.02)*	498 (0.09)
7.05	15.7 (0.05)	22.7 (0.12)†	216 (0.02)	215 (0.05)†	404 (0.42)
7.40	28 (0.24)	60 (0.29)*	265 (0.09)	203 (0.15)*	340 (0.69)
7.94	39 (0.16)	88 (0.07)†	248 (0.07)	203 (0.04)†	143 (0.30)

* Measured in the presence of 0.39 mM-thio-IMP.

† Measured in the presence of 0.18 mM-thio-IMP.

Table I.12. Inhibition of adenylosuccinate lyase from yeast by thio-IMP.

Initial velocities in the presence and absence of thio-IMP used in the calculation of K_m, V, K_p and V_p at different pH values (see Table I.11)

pH	v*	v_p †	Adenylosuccinate concentration (μM)
6.20	22.60	13.65	4.85
	30.20	22.60	8.80
	44.10	36.40	23.00
	51.90	-	46.00
	48.70	47.80	69.00
6.46	56.70	34.20	4.85
	73.80	51.10	8.80
	101.80	79.00	23.00
	116.50	93.80	46.00
	122.00	104.90	69.00
7.05	53.8	37.3	4.85
	77.9	57.8	8.80
	126.2	113.3	23.00
	160.8	139.5	46.00
	177.8	164.0	69.00
7.40	52.1	-	6.0
	84.3	31.1	12.0
	132.2	72.7	30.0
	167.2	95.8	660.0
	213.0	124.0	90.0
7.94	31.1	11.4	4.85
	50.5	18.8	8.80
	86.6	42.2	23.00
	136.1	69.0	46.00
	160.0	89.9	69.00

* Velocity (μm-moles/min./mg. of protein)

† Velocity in the presence of 0.39 mM-thio-IMP (pH 6.2, 6.46 and 7.4) or 0.18 mM-thio-IMP (pH 7.05 and 7.94) [μm-moles/min./mg. of protein].

inhibition of yeast adenylosuccinate lyase with thio-IMP by low pH or by the addition of 25 mM-cysteine, 25 mM-mercaptoethanol, or 10 mM-EDTA to the assays. These workers found that inhibition by thio-IMP was greatly decreased if this compound was first passed through a chelating resin, and could be increased by the addition of copper to the reaction mixture (assays were carried out at pH 7.6). In a typical experiment with the preparation from ascites-tumour cells used here the presence of 10.0 μM-thio-IMP inhibited the lyase by 35.1%; the lyase from yeast was inhibited by 33.4% (16.4 μM-adenylosuccinate; pH 7.67). In the presence of 20 mM-EDTA the inhibitions were 34.2% and 29.6% respectively. This concentration of EDTA appeared to give a small stimulation of activity (1 - 3%) when included in the above assays without thio-IMP. Similarly there was a small decrease in inhibition after the thio-IMP had been passed through a column (1 cm. x 1.2 $cm.^2$ for 2.26 μmoles of thio-IMP) of Chelex-100 resin (cf. Bridger & Cohen, 1963). Table I.13. lists the kinetic parameters obtained for inhibition of yeast adenylosuccinate lyase by thio-IMP at pH 7.67 both before and after its passage through the chelating column, calculated from the data listed in Table I.14. Passage of the thio-IMP through Chelex-100 resin caused less than 8% increase in the apparent value of K_i.

The thio-IMP used in the experiments described here contained 0.001 atom of copper/molecule of thio-IMP and the final concentrations of copper in assay solutions for lyase from tumour or from yeast were

Table I.13. <u>The effect of passage of thio-IMP through a chelating resin on its inhibition of yeast adenylosuccinate lyase.</u>

Values in parentheses are coefficients of variation.

Kinetic parameter	With 0.217 mM-thio-IMP	With 0.113 mM-thio-IMP (after Chelex-100)
K_p (μM)	100 (0.10)	66.7 (0.08)
V_p^*	101 (0.06)	102 (0.07)
K_i (μM)	120 (0.24)	129 (0.18)

* μm-moles/min./mg. of protein.

K_m (adenylosuccinate) = 35.6 (0.08) μM

V (adenylosuccinate split) = 111.0 (0.04) μm-moles/min./mg. of protein.

Table I.14. Initial velocities of yeast adenylosuccinate lyase used in calculating the kinetic parameters given in Table I.13.

Adenylosuccinate concentration (µM)	v*	Adenylosuccinate concentration (µM)	v_p †	Adenylosuccinate concentration (µM)	$v_{p(R)}$ ≠
3.2	9.1	3.2	3.3	2.9	4.4
7.9	19.1	7.8	7.5	7.1	10.1
15.7	29.1	15.5	11.8	14.1	18.3
30.7	49.5	30.5	23.0	27.6	32.2

* Initial velocity (µm-moles/min./mg. of protein)

† Initial velocity in the presence of 0.217 mM-thio-IMP (µm-moles/min./mg. of protein)

≠ Initial velocity in the presence of 0.113 mM-thio-IMP that had been passed through a chelating resin (µm-moles/min./mg. of protein).

0.55 μM and 0.4 μM respectively. More than half of this copper was introduced with the enzyme in each case (copper analyses were carried out by Mr. P. Monk). Addition of 0.55 μM-$CuSO_4$ to assays with enzyme from yeast caused small variable inhibitions of the reaction rate (1 - 5%; 10.4 μM-adenylosuccinate; pH 7.67). The presence of 2.5 μM-$CuSO_4$ caused 27.5% inhibition of the lyase; 0.09 mM-thio-IMP in similar conditions also caused 27.5% inhibition. An assay with 2.5 μM-$CuSO_4$ and 0.096 μM-thio-IMP resulted in 53.2% inhibition of adenylosuccinate lyase, showing the effects of copper and thio-IMP to be additive under these conditions.

C. CONCLUSIONS

From the results in Fig. I.10. it is likely that fresh Ehrlich ascites-tumour cells contain less IMP dehydrogenase than adenylosuccinate synthetase and less synthetase than adenylosuccinate lyase. When the assay of the synthetase is corrected for interference by the lyase the activities of dehydrogenase, synthetase, and lyase in extracts of fresh cells are in the approximate ratio 1:6:50. The ratios of K_m/K_i are 3.9 for the dehydrogenase at pH 8.0, 0.18 for the synthetase at pH 7.76 (Table I.7.) and 0.47 for the lyase from ascites-tumour cells at pH 7.99 (Table I.9.). These results suggest that, in Ehrlich ascites-tumour cells, the conversion of IMP into xanthosine 5'-phosphate might be more readily inhibited by 6-mercapto-

purine (through its metabolite thio-IMP) than the conversion of IMP into adenylosuccinate and of adenylosuccinate into AMP. The inhibition of IMP dehydrogenase would only be of importance in the physiological action of 6-mercaptopurine if the reaction was essential for providing cellular guanine nucleotides for nucleic acid synthesis and other reactions. Alternative pathways would be the direct reaction of xanthine or of guanine with PRPP in the presence of xanthine or guanine phosphoribosyltransferase to form XMP or GMP (see Section II). The generally accepted route for the conversion of adenine nucleotides into guanine nucleotides first requires the deamination of AMP by adenylate deaminase, followed by oxidation of the IMP formed to XMP by IMP dehydrogenase. XMP is then converted into GMP by amination in the presence of XMP aminase (Moyed & Magasanik, 1957; Abrams & Bentley, 1959). However if AMP could be converted into guanine nucleotides by a pathway not involving IMP as an intermediate, inhibition of IMP dehydrogenase would not upset cellular metabolism. This possibility has been considered by Sartorelli & Booth (1962). These workers found that 6-chloropurine inhibited the conversion of IMP into GMP in Sarcoma-180 in vivo, but had no effect on the conversion of IMP into AMP. However, labelling of guanine by exogenously added [8 - ^{14}C]adenine was not inhibited by 6-chloropurine, indicating a pathway from adenine to guanine nucleotides not involving IMP as an intermediate.

SECTION II

INHIBITION OF PURINE PHOSPHORIBOSYLTRANSFERASES FROM NORMAL AND RESISTANT EHRLICH ASCITES-TUMOUR CELLS BY 6-MERCAPTOPURINE

Purine phosphoribosyltransferases catalyse the direct reaction between free purines and 5-phosphoribosyl pyrophosphate to form the corresponding 5'-phosphoribosyl derivatives. The reactions would be of obvious importance in Ehrlich ascites-tumour cells if these cells depend to any extent on a supply of preformed purines from the host tissues. 6-Mercaptopurine is converted into 6-thioinosine 5'-phosphate in Ehrlich ascites-tumour cells by such a reaction (Paterson, 1962) and it might be expected that free 6-mercaptopurine would inhibit transferase activity. This section describes the preparation of extracts from Ehrlich ascites-tumour cells containing AMP-pyrophosphate phosphoribosyltransferase (adenine phosphoribosyltransferase; EC 2.4.2.7), GMP-pyrophosphate phosphoribosyltransferase (guanine phosphoribosyltransferase), IMP-pyrophosphate phosphoribosyltransferase (hypoxanthine phosphoribosyltransferase; EC 2.4.2.8) and 6-mercaptopurine-pyrophosphate phosphoribosyltransferase (6-mercaptopurine phosphoribosyltransferase) activity. The extracts were used in studies of the inhibition of the conversion of adenine, guanine, and hypoxanthine into adenosine 5'-phosphate, guanosine 5'-phosphate, and inosine 5'-phosphate,

respectively, by 6-mercaptopurine. Similar studies were carried out with extracts from ascites-tumour cells that were resistant to growth inhibition by 6-mercaptopurine.

A. MATERIALS AND METHODS

1. Development of a strain of Ehrlich ascites-tumour cells that are resistant to the action of 6-mercaptopurine.

After 20 successive inoculations in the presence of 6-mercaptopurine, as described by Paterson (1960a), a strain of ascites-tumour cells which resisted the action of the antimetabolite was obtained. Growth of these resistant cells during 7 days in animals injected daily with 38 mg. of 6-mercaptopurine/Kg. of body weight was 84 ± 4% (in 20 animals) of the growth in control animals injected with 0.9% NaCl (for conditions of the test see Paterson, 1960a). In the same conditions growth of sensitive cells was 11 ± 2% (in 20 animals) of that in controls.

2. Tests for conversion of 6-mercaptopurine into 6-thioinosine 5'-phosphate in vivo with sensitive and resistant cells.

(a) Analysis by electrophoresis. Groups of 3 mice (body weights about 25 g.) were inoculated with about 2×10^7 sensitive or resistant cells and after 7 days the mice were injected intra-

peritoneally with 5 μmoles of 6-mercaptopurine in 0.5 ml. of 0.9% NaCl at 40°. The injection was repeated twice at 24 hr. intervals, and 1 hr. after the last injection the ascitic fluid was collected and centrifuged for 5 min. at 2 000 g. After measurement of the volume of the packed cells (4 - 5 ml.) they were washed twice with 10 ml. of cold 0.9% NaCl and suspended in 10 ml. of 5% (w/v) trichloroacetic acid at 1°. After extraction of the insoluble residue with cold trichloroacetic acid (2 x 5 ml.) the combined extracts were extracted with ether (3 x 50 ml.) and the aqueous phase was evaporated at 25 - 30°/15 mm. The residue was dissolved in water (0.1 ml./ml. of packed cells used) and 0.05 ml. portions of the solution were subjected to electrophoresis in 0.05 M-citrate (adjusted to pH 4.8 with tris) on Whatman 3MM paper under CCl_4 at 30 v/cm. for 2.5 hr. Areas corresponding to thio-IMP (which was added as an internal standard to replicate samples, and migrated towards the anode directly ahead of AMP) were eluted with 10 ml. of water and extinctions were measured against blanks from corresponding areas of paper after adjustment to pH 5.5 with NaOH. Concentrations of thio-IMP were calculated from the millimolar extinction coefficient of 24.1 at 321.5 mμ (see Section I).

(b) Analysis by anion-exchange chromatography. In one experiment with sensitive cells the final injection was of [8 - ^{14}C]6-mercaptopurine (5 μmoles; 1.6 μC). The acid-soluble fraction, after extraction with ether, was applied to a column (14 cm. x 2 cm.^{2}) of DEAE-

cellulose (bicarbonate form). Water (200 ml.) was passed through the column, followed by linear gradients of NH_4HCO_3 (600 ml., from 0 - 50 mM and 1 litre, from 50 - 200 mM) and then by 250 ml. of 0.4 M-NH_4HCO_3. Extinctions of each 7 ml. fraction were measured at 260 mμ and 320 mμ and the radioactivity of a 0.2 ml. sample from each fraction with $E_{260\ m\mu}$ or $E_{320\ m\mu}$ greater than 0.05 was measured by liquid scintillation counting with a scintillator suitable for aqueous solutions (Herberg, 1960). [8-^{14}C]Thio-IMP was eluted between ADP and ATP by 140 - 165 mM-NH_4HCO_3 and after removal of volatile material at 25°/15 mm. was further purified by electrophoresis in the tris-citrate system described above. The spectroscopic, chromatographic, and electrophoretic properties of the product were identical with those of synthetic thio-IMP.

3. <u>Substrates and inhibitors</u>.

<u>Purines</u>. A sample of guanine from British Drug Houses Ltd. contained about 0.1 mol. prop. of adenine. A 0.5% solution of this material in boiling 3.6 N-H_2SO_4 was filtered and the guanine sulphate which crystallized on cooling was converted into the silver salt of guanine with silver sulphate. The guanine was freed of silver by extraction into 1.5 N-HCl and precipitated by adjustment of the solution to pH 7 with NaOH. After being washed with water and with ethanol the guanine was dried; it was found to be free of contaminants that absorbed light at 254 mμ on chromatography in propan-2-ol -

1.4 N-NH_4OH (85:16.5, v/v; R_F 0.45) or in freshly mixed butan-1-ol - propionic acid - water (2.1:1:1.4, by vol.; R_F 0.48).

[8-^{14}C]Guanine, [8-^{14}C]adenine, and [8-^{14}C]6-mercaptopurine from California Corp. for Biochemical Research, contained no contaminants that could be detected by spectroscopy or autoradiography after chromatography in the systems described above.

[8-^{14}C]Hypoxanthine was prepared from [8-^{14}C]adenine by deamination. Sodium nitrite (30 mg.) in about 1 ml. of water was added slowly to a solution of [8-^{14}C]adenine (3 mg.; 50 µC) in 0.5 ml. of 2 N-acetic acid. After 3 hr. at 40° the solution was diluted with 10 ml. of water, brought to pH 8 with 2 N-NaOH and passed through a column (7 cm. x 1 cm.^{2}) of Amberlite CG-120 (H^+ form). The column was washed with 20 ml. of water and a mixture of hypoxanthine and adenine was then eluted with 15 ml. of 17 N-NH_4OH. The residue obtained on evaporation of the eluate was dissolved in water (5 ml.) and the solution was again evaporated. This procedure was repeated once more to remove ammonia. After electrophoresis as a band 20 cm. wide on Whatman 3MM paper in 0.05 M-formate (NH_4^+, pH 3.5) the hypoxanthine was obtained free of adenine by elution with 40 ml. of water. On autoradiography and measurement of the adenine and hypoxanthine separated in this way it was found that 85 - 90% of the adenine had been deaminated.

Non-radioactive adenine and hypoxanthine (California Corp. for Biochemical Research) and 6-mercaptopurine (Sigma Chemical Co.) contained no detectable impurities when examined by spectrophotometry or by chromatography in the systems described above.

5-Phosphoribosyl pyrophosphate. Analysis of the magnesium salt with orotic acid phosphoribosyltransferase (Kornberg, Lieberman & Simms, 1955b) confirmed the analysis (76%, corrected for hydration) reported by the manufacturers (Pabst Laboratories).

4. Preparation of extracts for measurement of purine phosphoribosyltransferase activities.

(a) Extracts for assays with radioactive purines. Extracts were prepared from Ehrlich ascites-tumour cells as described before (see the Materials and Methods part of section I) except that 10 mM-tris (Cl^-, pH 7.8) was used instead of phosphate buffer. The supernatant obtained on centrifuging at 20 000 g was further clarified by centrifuging at 100 000 g for 1 hr. at 2° and dialysed against 50 vol. of 10 mM-tris (Cl^-, pH 7.8) for 24 hr. at 2°. The supernatant obtained on centrifuging the dialysis residue at 20 000 g for 15 min. ('dialysed first extract'; Table II.1.) was used in assays of conversion of radioactive purines into their nucleotides with PRPP.

(b) Extracts for spectrophotometric assays. The supernatant obtained on centrifuging at 100 000 g as described above ('first extract'; Table II.1.) was mixed with 1 ml. of 5% (w/v) streptomycin sulphate/40 mg. of protein (calculated from $E_{260\ m\mu}$ and $E_{280\ m\mu}$; Warburg & Christian, 1942). After 5 min. at 1° the suspension was centrifuged at 10 000 g for 10 min. at 2°. The supernatant ('streptomycin supernatant'; Table II.1.) was kept at 55 - 60° for 3 min., cooled to 2° and centrifuged

Table II.1. Properties of fractions obtained in the purification of 6-mercaptopurine phosphoribosyltransferase from Ehrlich ascites-tumour cells

Details are given in the text. Protein content was calculated from $E_{260\ m\mu}$ and $E_{280\ m\mu}$ (Warburg & Christian, 1942).

Fraction	Volume (ml.)	$\frac{E_{280\ m\mu}}{E_{260\ m\mu}}$	Protein (mg.)	Net formation of thio-IMP (μm-moles/min.)
First extract	48	0.64	549	180
Dialysed first extract	52	0.66	530	180
Streptomycin supernatant	60	0.85	343	170
First heated supernatant	58	0.88	160	145
$(NH_4)_2SO_4$ fraction	7.0	1.40	60	106
Dialysed enzyme	8.5	1.60	52	100
Second heated supernatant	6.5	1.61	35	80

as before ('first heated supernatant'; Table II.1.). Protein which precipitated between 0.3 and 0.7 saturation of $(NH_4)_2SO_4$ was dissolved in 3 mM-tris (Cl^-, pH 7.8). The solution ('$(NH_4)_2SO_4$ fraction'; Table II.1.) was dialysed for 16 hr. against 300 vol. of 3 mM-tris (Cl^-, pH 7.8). The dialysis residue ('dialysed enzyme'; Table II.1.) was heated rapidly to 70°, kept at this temperature for 2.5 min., cooled to 2°, and centrifuged at 60 000 g for 30 min. The supernatant ('second heated supernatant'; Table II.1.) was used for spectrophotometric assays of guanine and 6-mercaptopurine phosphoribosyltransferases. After 3 weeks at -15° this fraction had 80% of its initial activity with 6-mercaptopurine.

5. <u>Measurement of purine phosphoribosyltransferase activities.</u>

(a) <u>Assays with radioactive purines.</u> Solutions containing 0.25 μmole of PRPP, 10 μmoles of $MgCl_2$ and 40 μmoles of tris, adjusted to pH 7.8 with HCl, were mixed with the phosphoribosyltransferase preparation (about 0.45, 1.8, 3.6, and 3.0 mg. of protein for assays with adenine, guanine, hypoxanthine, and 6-mercaptopurine respectively). After 5 min. at 25° a solution of [8-^{14}C]adenine, [8-^{14}C]guanine, [8-^{14}C]hypoxanthine or [8-^{14}C]6-mercaptopurine (specific activities 2.85, 0.28, 1.25, and 0.47 μC/μmole respectively) in 0.01 - 0.15 ml. was added to give the concentration of purine

needed for the kinetic studies, in a final volume of 1.4 ml. Guanine was added as a solution in 0.01 N-HCl and with this purine the reaction mixtures contained 100 μmoles of tris buffer. When non-radioactive hypoxanthine or 6-mercaptopurine were used as inhibitors they were added to the enzyme and buffered PRPP 10 sec. before the radioactive purines. Reactions were stopped after the required period at 25° by dilution with 50 ml. of water at 1°. The reaction tubes were rinsed twice with 5 ml. portions of cold water and the combined solutions were passed through 4 cm. discs of DEAE-cellulose paper in a Millipore filtration apparatus (Millipore Filter Corp., Bedford, Mass., U.S.A.). Filtration was completed within 30 sec. of the initial dilution. Zero-time samples were obtained by adding the radioactive purines after the cold water. The filtrates were passed through the filters twice more and the discs of paper were then washed with 50 ml. of 4 mM-NH_4HCO_3 and with water (10 x 150 ml.). In assays with [8-^{14}C]6-mercaptopurine the discs of DEAE-cellulose were washed with 8 mM-NH_4HCO_3; 6-mercaptopurine is a stronger acid than hypoxanthine and is not completely removed by 4 mM-NH_4HCO_3. The paper discs were dried without heating for about 2 hr. and the radioactive nucleotide on the paper was measured by liquid scintillation counting (cf. Wang & Jones, 1959; Sherman, 1963). Discs were immersed in 10 ml. of 0.3% 2,5-diphenyloxazole - 0.02% p-bis-2,5-diphenyloxazolylbenzene in toluene and counted with a photomultiplier (EM 9514S) attached to an N530F scaler (Ecko Electronics Ltd.). The counting efficiency of this system was 55 $\pm$ 2%.

Enzymic activities are expressed as μm-moles of nucleotide formed/min. /mg. of protein.

(b) Spectrophotometric assays. Enzyme was added to blank and test cells containing $MgCl_2$ (20 μmoles), guanine (0 - 0.43 μmole), 6-mercaptopurine (0 - 0.44 μmole) and tris (100 μmoles, adjusted to pH 7.8 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 0.1 ml. of 5 mM-PRPP to the test cell (final volume of cell contents, 2.5 ml.). Changes of extinction were recorded at 255 mμ and 320 mμ at 25°. Initial rates of increase were maintained for at least 30 min. but the rates were usually measured for 10 min. The rate of conversion of guanine into GMP was calculated by the use of 3.6×10^3 as the net change of molecular extinction coefficients at 255 mμ (Pabst Laboratories, 1961a). The rate of formation of thio-IMP from 6-mercaptopurine was calculated by the use of 5.4×10^3 as the net change of extinction coefficients at 320 mμ (from measured extinction coefficients of 18.0×10^3 for the purine and 23.4×10^3 for its nucleotide at pH 7.8). When mixtures of guanine and 6-mercaptopurine were examined the interference due to extinction changes at 320 mμ arising from the reaction 6-mercaptopurine $\longrightarrow$ thio-IMP and at 225 mμ arising from the reaction guanine $\longrightarrow$ GMP were negligible ($\Delta m\epsilon_{320\ m\mu}$, 0.02 and $\Delta m\epsilon_{225\ m\mu}$, 0.01 respectively).

(c) Calculation of kinetic parameters. Michaelis constants (K_m), inhibitor constants (K_i), and extrapolated maximum velocities in the absence (V), and presence (V_p) of inhibitor, together with their coefficients of variation, were calculated as described before (see section I, p. 46).

6. Analysis of the products formed in the presence of the purine phosphoribosyltransferase preparations.

(a) Identification of the products formed from radioactive purines. Solutions containing 40 µmoles of tris, 8 µmoles of $MgCl_2$, 0.5 µmole of PRPP and 17.4 µm-moles of [8-^{14}C]adenine, 64.5 µm-moles of [8-^{14}C] guanine or 106 µm-moles of [8-^{14}C]hypoxanthine, adjusted to pH 7.8 with HCl, were kept at 25° for 4 min. after addition of 'dialysed first extract' (cf. Table II.1.; 1.0, 3.6, and 7.2 mg. of protein respectively in the final volume of 1.2 ml.). The specific activities of the purines were the same as in the kinetic experiments and the final protein concentrations were twice those used in the corresponding kinetic experiments. The test solutions and controls without PRPP were treated with 1 ml. of 8% (w/v) trichloroacetic acid (2°). After centrifuging, the residues were washed with cold 5% trichloroacetic acid (2 x 1 ml.) and the combined extracts were extracted with ether (3 x 20 ml.) and evaporated to dryness. The residues were dissolved in a small volume of water and two equal portions (approx. 40% of the total fraction) from each reaction mixture were chromatographed on Whatman 3MM paper

in the butanol - propionic acid - water system described above and in butan-1-ol - acetic acid - water (20:3:7, by vol.). Guanine, guanosine, GMP, hypoxanthine, inosine, IMP, adenine, adenosine, and AMP were chromatographed as reference compounds. The purines were detected with a low pressure mercury lamp and radioactive compounds by autoradiography for 8 days. The radioactivity in the products was measured by liquid scintillation counting as described above (see the Results and Discussion section). Discs of DEAE-cellulose used in the assay of hypoxanthine phosphoribosyltransferase were extracted with acetone and with ether and radioactive material was eluted with M-NH_4HCO. After removal of NH_4HCO_3 under reduced pressure the residue was chromatographed in isobutyric acid - 0.19 N-NH_4OH (661:339, v/v). This system separates IMP (R_F 0.21) and AMP (R_F 0.48).

To test for the validity of the assay with [8-^{14}C]6-mercaptopurine, 95 μm-moles (56 μmC) of this purine was treated with extracts of sensitive and of resistant cells (6.0 and 6.6 mg. of protein respectively) in the conditions described above. On chromatography of the products in the butanol - propionic acid - water system, the only radioactive compounds detected were thio-IMP (R_F 0.14) and unchanged 6-mercaptopurine.

(b) Tests for cleavage of AMP, IMP, GMP, and thio-IMP. In separate experiments the reaction mixtures containing PRPP and [8-^{14}C] adenine or [8-^{14}C]hypoxanthine were treated with trichloroacetic acid after 4 and 8 min. respectively. The acid-soluble fractions, after

removal of trichloroacetic acid as before, were subjected to electrophoresis on Whatman 3MM paper (17 cm. wide) in 0.05 M-formate (NH_4^+, pH 3.5) at 17 v/cm. for 3 hr. at 2° and the [8-^{14}C]-AMP and [8-^{14}C]-IMP were located by comparison with reference compounds and eluted with 15 ml. of water. After removal of ammonium formate by evaporation the [8-^{14}C]-AMP (5.2 μm-moles; 21.7 μmC) and [8-^{14}C]-IMP (4.3 μm-moles; 0.99 μmC) were treated with 'dialysed first extract' (cf. Table II.1.) of ascites-tumour cells in solutions containing 4 μmoles of $MgCl_2$, 0.5 μmole of PRPP, 25 μmoles of tris (Cl^-, pH 7.8) and non-radioactive adenine (16 μm-moles) or non-radioactive hypoxanthine (98 μm-moles). The reaction mixture with AMP contained 0.26 mg. of protein in a final volume of 1.25 ml. and was kept at 25° for 4 min. before addition of 1 ml. of 10% trichloroacetic acid. The reaction mixture with IMP contained 2.6 mg. of protein in a final volume of 1.7 ml. and was kept at 25° for 6 min. before addition of 1 ml. of 10% trichloracetic acid. After electrophoresis of the acid-soluble fractions as described above the distribution of radioactivity in regions corresponding to AMP, adenosine, adenine, IMP, and (inosine + hypoxanthine) was measured by scintillation counting. To test for the presence of hypoxanthine and inosine in the region containing AMP and of AMP in the region containing hypoxanthine the corresponding pieces of paper were freed of scintillator by elution with 5 ml. of acetone and 5 ml. of ether; purine derivatives were then eluted with 10 ml. of water. The residue obtained on evaporation of the aqueous

eluates were chromatographed in the butanol - acetic acid - water system described above and the distribution of radioactivity was examined as before.

When a solution containing 4 μmoles of $MgCl_2$, 100 μmoles of tris (Cl^-, pH 7.8) and 70 μm-moles of GMP or 62 μm-moles of thio-IMP was mixed with 'dialysed first extract' (Table II.1.; 0.52 mg. of protein) in a final volume of 3 ml. at 25° the rates of change of $E_{255\ m\mu}$ or $E_{320\ m\mu}$ respectively were less than 0.001/min. in a cell of 1 cm. light path. Similar tests for cleavage of AMP, IMP, GMP, and thio-IMP were carried out with an extract from cells that were resistant to 6-mercaptopurine.

7. Electrophoresis of purine phosphoribosyltransferases in starch gel.

A portion of 'dialysed first extract' (Table II.1.) was subjected to electrophoresis in starch gel as described by Atkinson, Jackson & Morton (1964); the initial pH of the system was 9.5 and the concentration of tris was 76 mM. Purine phosphoribosyltransferases were detected by placing DEAE-cellulose paper soaked in PRPP, $MgCl_2$, tris chloride and the appropriate radioactive purine, in contact with the cut surface of the gel for 1 hr. at 25° and then removing radioactive compounds other than nucleotides by washing the papers. [8-^{14}C]Adenine (17.7 μm-moles; 50.5 μmC) and [8-^{14}C]hypoxanthine (212 μm-moles; 53 μmC) were applied to the paper as solutions containing 0.4 μmole of PRPP, 8 μmoles of $MgCl_2$ and 20 μmoles of tris

(Cl^-, pH 7.8) in a final volume of 2.9 ml. For [8-^{14}C] guanine (129 μm-moles; 36 μmC) the solution contained an extra 80 μmoles of tris chloride. The papers were washed with water (200 ml.), 4 mM-NH_4HCO_3 (200 ml.), and again with water (4 x 200 ml.), and were dried. The distribution of radioactivity in 5 mm. x 15 mm. segments of the papers was measured by scintillation counting and a qualitative confirmation of the results was obtained by autoradiography of duplicate strips.

B. RESULTS AND DISCUSSION

1. Purine phosphoribosyltransferases from sensitive cells

(a) Assay of purine phosphoribosyltransferases

The spectrophotometric assay of guanine phosphoribosyltransferase and 6-mercaptopurine phosphoribosyltransferase (Carter, 1959), was satisfactory for studies of the conversion of these two purines, alone or in admixture, into their nucleotides in the presence of a partially purified fraction ('second heated supernatant'; Table II.1. see p. 88) from Ehrlich ascites-tumour cells. Both purines were converted quantitatively into their nucleotides and the K_m of guanine and K_i of 6-mercaptopurine (acting as a competitive inhibitor of the conversion of guanine into GMP) did not differ significantly from those measured in assays with radioactive guanine (Table II.2.). In the conditions of the assay there was no change in $E_{255\ m\mu}$ when guanine or GMP was mixed with the enzyme in the absence of PRPP nor was there

detectable change in $E_{320\ m\mu}$ with 6-mercaptopurine or thio-IMP without PRPP; these results indicate the absence of interference by guanase or by enzymes that catalyse the cleavage of the glycosidic linkage in GMP or thio-IMP. In the experiments with radioactive purines, chromatographic and electrophoretic examination of the products formed from [8-^{14}C]hypoxanthine and PRPP in the presence of 'dialysed first extract' from the ascites-tumour cells showed quantitative conversion of the purine into nucleotide. The only radioactive product on the DEAE-cellulose discs in experiments with hypoxanthine was IMP. With [8-^{14}C]guanine the only radioactive product detected was GMP in the conditions of the kinetic experiments, but with higher concentrations of protein (3.6 mg. in 1.2 ml. of reaction mixtures; for details see the Materials and Methods section) about a tenth as much guanosine as GMP was formed. No guanosine was detected in the absence of PRPP. With twice the concentration of protein used for kinetic experiments 5% as much adenosine as AMP was formed from [8-^{14}C]adenine in the presence of PRPP. Half this quantity of adenosine was formed in the absence of added PRPP.

When 4.3 µM-[8-^{14}C]-IMP was treated, in conditions similar to those used in kinetic studies, with 'dialysed first extract', PRPP and non-radioactive hypoxanthine (for details see the Materials and Methods section) 4% of the nucleotide was converted into hypoxanthine and no inosine was detected. When 5.2 µm-[8-^{14}C]-AMP was allowed to

react with the 'dialysed first extract' in the presence of PRPP and non-radioactive adenine the upper limits for conversion of the nucleotide into IMP, adenosine and adenine were 0.8%, 0.4% and 0.2% respectively. From these results it is unlikely that any serious interference with the kinetic measurements resulted from hydrolysis or phosphorolysis of bonds in the nucleotides formed during the assays.

In the absence of added PRPP, [8-^{14}C]adenine, [8-^{14}]guanine, [8-^{14}C]hypoxanthine or [8-^{14}C]6-mercaptopurine were not converted into materials that were bound to DEAE-cellulose; with PRPP the formation of bound products (shown by the chromatographic analysis to be 5'-phosphoribosyl derivatives) was linear for a sufficient period (Fig. II.1.) to permit calculation of initial rates in the presence or absence of 6-mercaptopurine or of [^{12}C]hypoxanthine. With adenine the mean rate was measured from values at 0, 0.5, 1 and 2 min; with guanine, hypoxanthine and 6-mercaptopurine the mean rate was measured from values at 0, 1, 2 and 4 min. On prolonged incubation the rate of conversion of guanine into GMP and of hypoxanthine in IMP in the presence of 6-mercaptopurine increased. In these conditions much of the 6-mercaptopurine had been converted into thio-IMP, as discussed by Carter (1959). With each of the radioactive purines the activity of the corresponding purine phosphoribyosyltransferase was proportional to the concentration of protein in the reaction mixture (Fig. II.2.).

(b) <u>The effect of pH</u>

The effect of varying the pH over the range 6.8 - 8.7 on

Fig. II.1. Synthesis of purine nucleotides by an extract of sensitive Ehrlich ascites-tumour cells in the presence of PRPP and 12.4 μM-adenine (○), 45 μM-guanine (△), 132 μM-hypoxanthine (●) or 128 μM-6-mercaptopurine (□). For details of protein concentrations and conditions of the assays with radioactive purines see the Materials and Methods section.

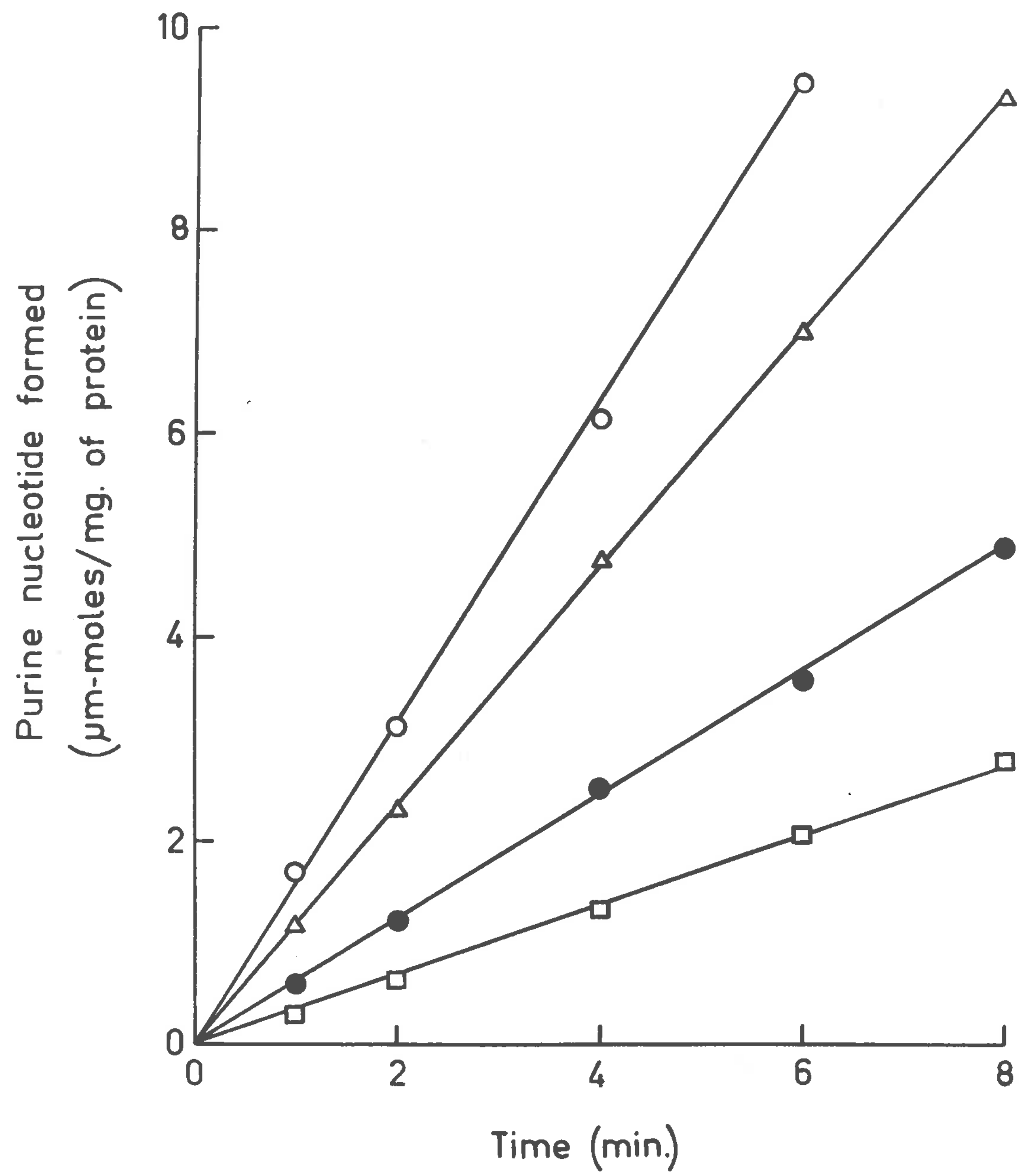
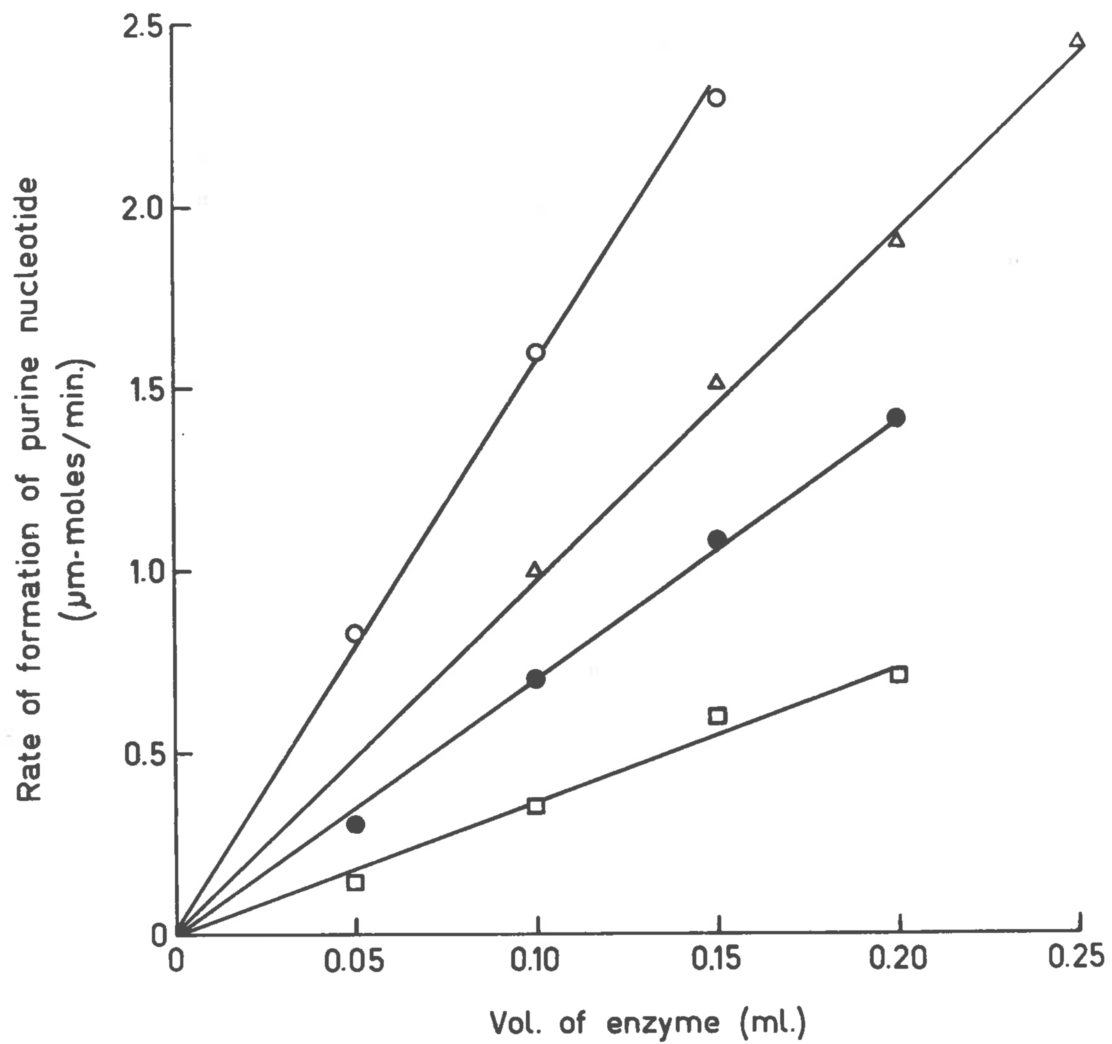

Purine nucleotide formed
(µm-moles/mg. of protein)
Time (min.)
10
8
6
4
2
0
0
2
4
6
8

Fig. II.2. Variation of the rate of synthesis of purine nucleotides with the concentration of protein in the presence of an extract from Ehrlich ascites-tumour cells. The 'dialysed first extract' used in this experiment contained 5 mg. of protein/ml. and assays were carried out as described in the Materials and Methods section with 12.4 μM-adenine (○), 45 μM-guanine (△), 132 μM-hypoxanthine (●), or 128 μM-6-mercaptopurine (□)



Rate of formation of purine nucleotide
(µm-moles/min.)
2.5
2.0
1.5
1.0
0.5
0
0
0.05
0.10
0.15
0.20
0.25
Vol. of enzyme (ml.)

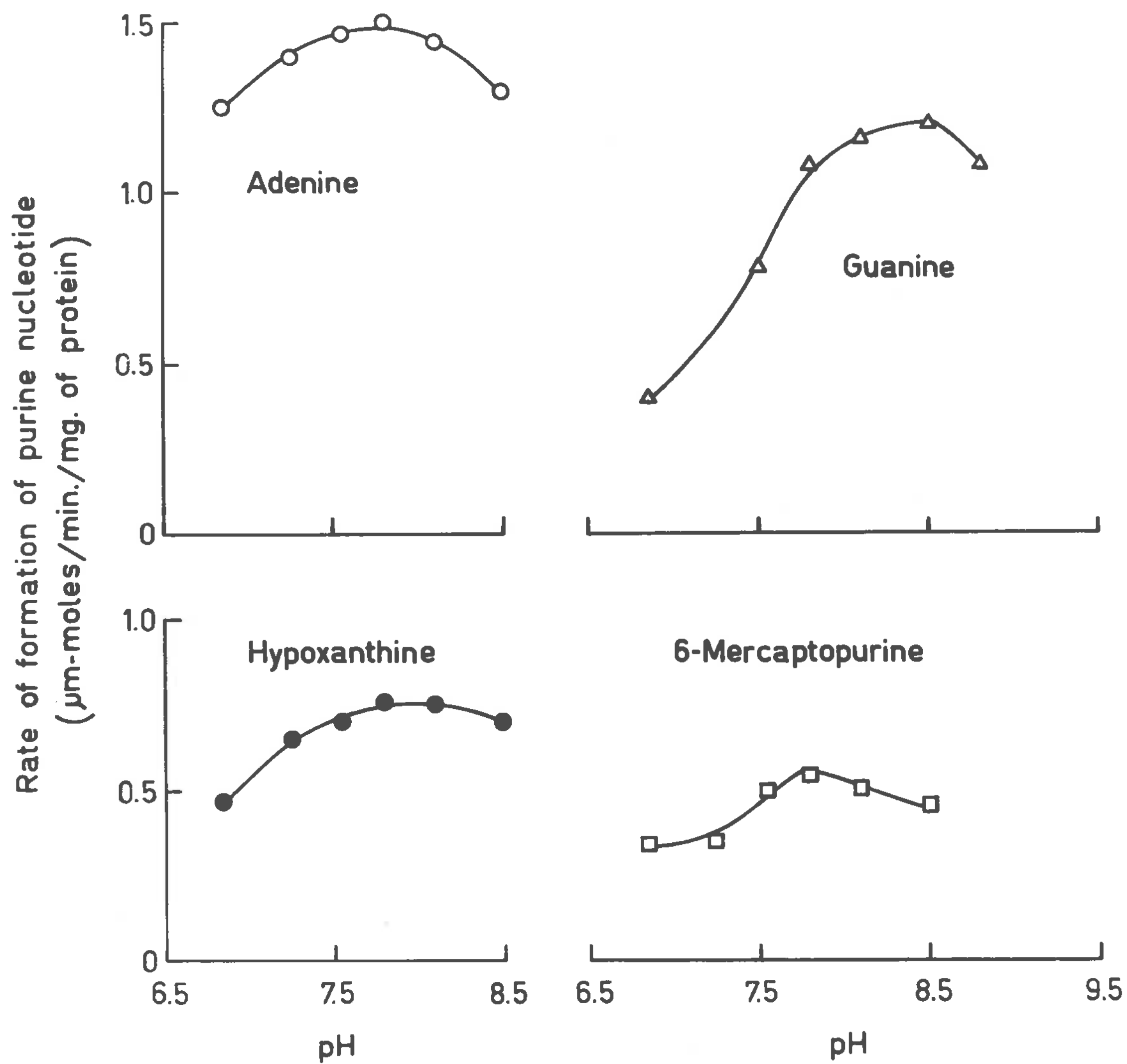
the initial velocities of adenine, guanine, hypoxanthine and 6-mercaptopurine phosphoribosyltransferases is shown in Fig. II.3. The standard assay with radioactive purines was used (see the Materials and Methods section) and the required pH was obtained by adjustment with HCl. It is clear that variation of the pH within this range has relatively little effect on the initial rate of conversion of adenine, hypoxanthine and 6-mercaptopurine into AMP, IMP and thio-IMP respectively; the optimum pH in each case is near 7.8. The conversion of guanine into GMP has a more sharply defined optimum near pH 8.5. These results are in good agreement with those found by other workers. Thus Brockman, Debavadi, Stutts and Hutchison (1961b) reported that adenine, hypoxanthine and 6-mercaptopurine had a broad pH optimum in the range 7.5 - 8.5 and that the optimum for guanine was in the range 8.5 - 9.0 with enzyme from _S. faecalis_. Flaks _et al_. (1957) reported a pH optimum of 7.6 - 8.4 for adenine phosphoribosyltransferase from ox liver, and Kornberg _et al_. (1955a) found an optimum near pH 7.6 with a preparation of this enzyme from yeast.

(c) _Evidence for the separate identity of adenine, guanine and hypoxanthine phosphoribosyltransferases in extracts of ascites-tumour cells_

(i) _Mixed substrate experiments_

If adenine, guanine and hypoxanthine were converted into their nucleotides by the same enzyme, it is probable that the same active centre

Fig. II.3. The effect of pH on the reactions catalysed by purine phosphoribosyltransferases.
The required pH values were obtained by adjustment of the tris buffer system with HCl, and did not vary by more than 0.05 during any particular assay. Assays were carried out as described in the Materials and Methods section with 12.4 µM-adenine (○), 46 µM-guanine (△) 132 µM-hypoxanthine (●), or 128 µM-6-mercaptopurine (□).



Rate of formation of purine nucleotide
(µm-moles/min./mg. of protein)
1.5
1.0
0.5
0
Adenine
Guanine
Hypoxanthine
6-Mercaptopurine
6.5
7.5
8.5
9.5
pH
pH

would be involved for each purine base and that there would be competition between the purines. Assays with radioactive purines provide an excellent means for determining whether this is the case.

With 'dialysed first extract' from ascites cells it was found that the rate of conversion of a mixture of 17.4 μm-M-[8-^{14}C] adenine and of 64.5 μm-M-[8-^{14}C]guanine into nucleotides (124 counts/sec./mg. of protein after incubation for 4 min. with PRPP; for details see the Materials and Methods section) was not significantly less than the sum of the rates observed with [8-^{14}C]adenine (114 counts/sec./mg. of protein) and [8-^{14}C]guanine (9.4 counts/sec./mg. of protein) when these purines were tested separately in the assay with PRPP. Similarly, with a different preparation, the rate of conversion of a mixture of 26.5 μm-M-[8-^{14}C]adenine and 142 μm-M-[8-^{14}C]hypoxanthine into nucleotides (108 counts/sec./mg. of protein) was as great as the sum of the rates observed when the purines were tested separately (88 and 22 counts/sec./mg. of protein respectively). With another preparation of dialysed extract the rate of conversion of a mixture of 64.5 μm-M-[8-^{14}C]guanine and 106 μm-M-[8-^{14}C]hypoxanthine into GMP and IMP (15.5 counts/sec./mg. of protein) was slightly greater than the rate with hypoxanthine alone (14.2 counts/sec./mg. of protein) and less than the rate with guanine alone (31.2 counts/sec./mg. of protein).

The results clearly show that adenine phosphoribosyltransferase is distinct from either guanine or hypoxanthine phosphoribosyltransferase activity. If the same enzyme were involved the rate of reaction with adenine mixed with either guanine or hypoxanthine could not have been

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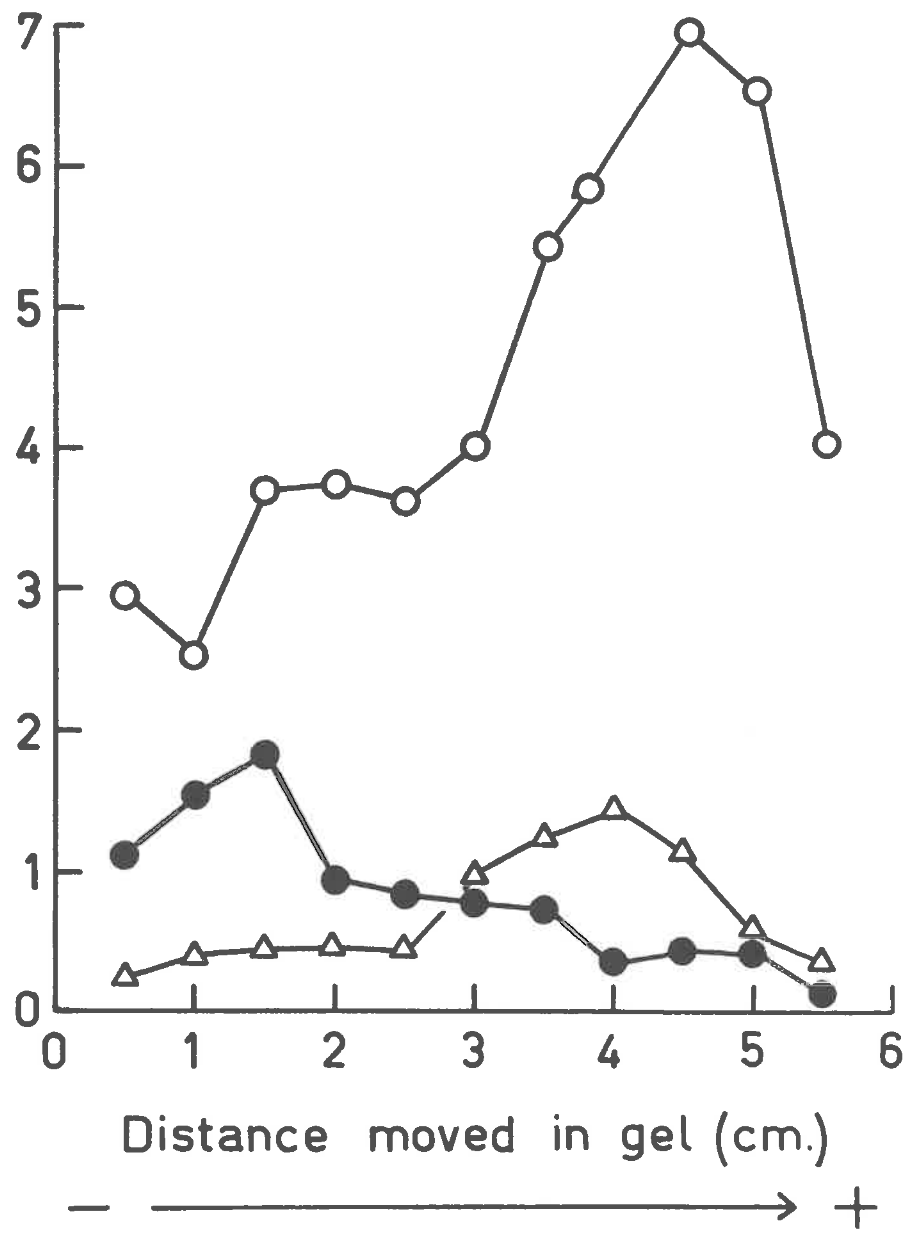
greater than the rate with adenine alone, as there would have been competition between the substrates at the concentrations used (see Dixon & Webb, 1958, p.91). It has been shown previously that different enzymes convert adenine and hypoxanthine into their nucleotides in extracts from yeast (Kornberg _et al._, 1955a) and liver (Flaks _et al._, 1957). A mixture of guanine and hypoxanthine gave a rate intermediate between the rates with either base alone. This does not necessarily mean that a single enzyme catalyses the reaction of guanine and hypoxanthine as the compounds could be mutually inhibitory and still be substrates of separate enzymes.

(ii) _Electrophoresis on starch gel_

The distribution of adenine, guanine and hypoxanthine phosphoribosyltransferase after electrophoresis in starch gel (see the Materials and Methods section) is shown in Fig. II.4. The most rapid conversion of adenine into AMP was catalysed by enzyme with high anionic mobility; some material with lower mobility was also active and may represent isoenzymes of adenine phosphoribosyltransferase. The guanine and hypoxanthine phosphoribosyltransferases had maxima in different regions of the gel, suggesting that at least three phosphoribosyltrans-ferases are involved in the conversion of adenine, guanine and hypoxanthine into their nucleotides. This conclusion is supported by the different values of K_i for 6-mercaptopurine as an inhibitor of these enzymes (see below). High recoveries of the three transferase activities were obtained after electrophoresis in acrylamide gel but the activities

Fig. II.4. Distribution of adenine phosphoribosyltransferase (○), guanine phosphoribosyltransferase (△), and hypoxanthine phosphoribosyltransferase (●) activities of an extract from Ehrlich ascites-tumour cells after electrophoresis in starch gel. For conditions of electrophoresis and details of the assays see the Materials and Methods section.



Formation of purine nuceotides
(10⁻³counts/50min./segment)
0
1
2
3
4
5
6
7
0
1
2
3
4
5
6
Distance moved in gel (cm.)
−
+

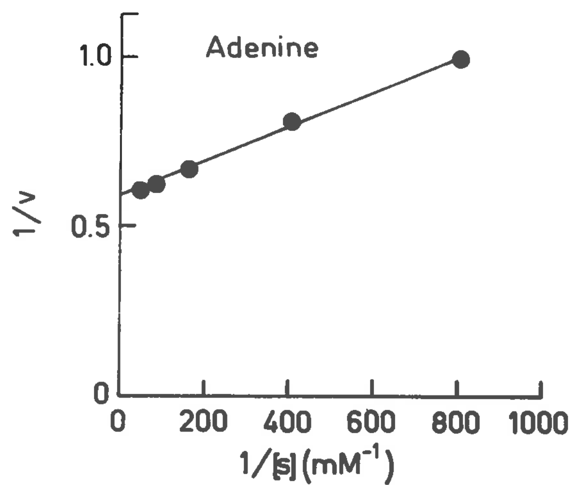
were not separated from each other in this system.

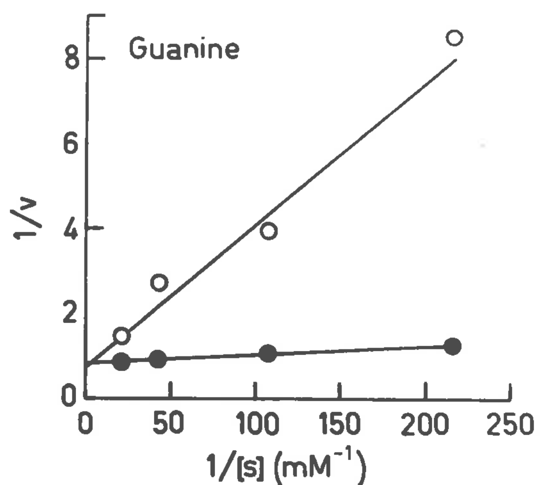
(d) Kinetic studies

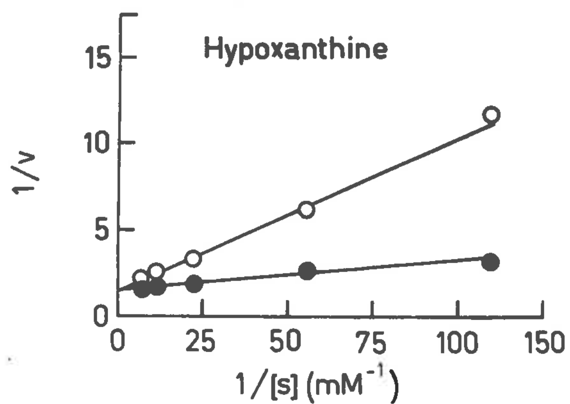
The reciprocal of the rate of conversion of each purine into its 5'-phosphoribosyl derivative was a linear function of the reciprocal of the concentration of that purine. Table II.2. lists Michaelis constants and extrapolated maximum velocities for conversion of adenine, guanine, hypoxanthine and 6-mercaptopurine into AMP, GMP, IMP and thio-IMP (measured by the assay with radioactive purines). It also lists the values found, by the spectrophotometric assay, for conversion of guanine and 6-mercaptopurine into GMP and thio-IMP. The data from which these parameters were calculated is given in Table II.3., and the corresponding reciprocal plots are shown in Fig. II.5.

The assay with radioactive adenine was particularly suitable for measurement of the very low Michaelis constant (0.89 ± 0.04 μM) of this purine. Kornberg *et al*. (1955a) found no variation in activity of adenine phosphoribosyltransferase from yeast with adenine concentrations above 45 μM nor did Flaks *et al*. (1957) find an increased rate with the enzyme from liver at concentrations above 100 μM-adenine. The extrapolated maximum rate of conversion of adenine into AMP (1.69 ± 0.02 μm-moles/min./mg. of protein) obtained with the 'dialysed first extract' (Table II.1.; see p.88) was higher than the extrapolated maximum rates of conversion of guanine into GMP and of hypoxanthine into IMP with the same sample of extract (1.21 ± 0.02 and 0.66 ± 0.01 μm-moles/min./mg. of protein respectively). In the presence of

Fig. II.5. The reaction of adenine, guanine, hypoxanthine, and 6-mercaptopurine phosphoribosyltransferase. Assays with guanine were carried out in the absence (●) and presence (○) of 74 μM-6-mercaptopurine and assays with hypoxanthine in the absence (●) and presence (○) of 38 μM-6-mercaptopurine. With adenine the points overlap and only those in the absence of inhibitor are shown.


Adenine
1/v
1.0
0.5
0
0
200
400
600
800
1000
1/[s] (mM^{-1})


Guanine
1/v
8
6
4
2
0
0
50
100
150
200
250
1/[s] (mM^{-1})


Hypoxanthine
1/v
15
10
5
0
0
25
50
75
100
150
1/[s] (mM^{-1})

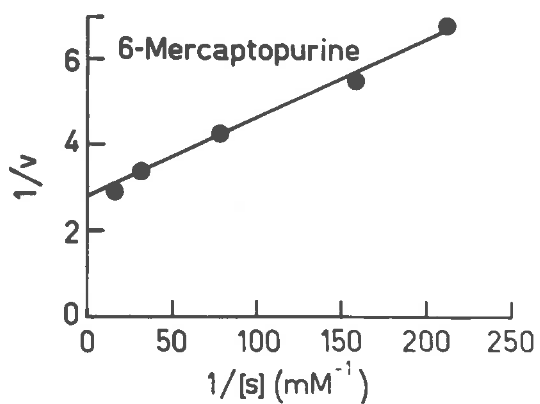

6-Mercaptopurine
1/v
6
4
2
0
0
50
100
150
200
250
1/[s] (mM^{-1})

Table II.2. Inhibition of purine phosphoribosyltransferases from sensitive Ehrlich ascites-tumour cells by 6-mercaptopurine

Values in parentheses are the coefficients of variation of the means.

	Substrate	pH	K_m (µM)	V (µm-moles/min./mg. of protein)	Inhibitor	Inhibitor concentration (µM)	K_p (µM)	V_p (µm-moles/min./mg. of protein)	K_i (µM)
A*	Adenine †	7.8	0.89 (0.04)	1.69 (0.01)	6-mercaptopurine	25	0.89 (0.05)	1.68 (0.01)	-
A*	Hypoxanthine †	7.8	11.0 (0.08)	0.66 (0.02)	6-mercaptopurine	38	60 (0.09)	0.68 (0.04)	8.3 (0.21)
A*	Guanine †	7.8	2.5 (0.11)	1.21 (0.02)	6-mercaptopurine	74	45 (0.16)	1.39 (0.09)	4.4 (0.20)
B*	Guanine †	7.8	3.3 (0.05)	0.80 (0.01)	hypoxanthine	40	42 (0.07)	0.68 (0.04)	3.4 (0.10)
C*	6-Mercaptopurine †	7.8	7.55 (0.17)	0.38 (0.05)	-	-	-	-	-
D*	Guanine ≠	7.8	2.6 (0.10)	7.93 (0.03)	6-mercaptopurine	18	11.7 (0.30)	6.58 (0.16)	5.1 (0.37)
D*	6-Mercaptopurine ≠	7.9	10.9 (0.04)	1.82 (0.04)	-	-	-	-	-

* Different enzyme preparations were used for experiments, A, B, C, and D.

† Assay with radioactive substrate.

≠ Spectrophotometric assay.

Table II.3. Initial velocities used in the calculation of kinetic parameters of purine phosphoribosyltransferases from sensitive ascites-tumour cells (see Table II.2.)

Assays were carried out with radioactive substrates and velocities are given as μm-moles of nucleotide formed/min./mg. of protein.

Adenine concentration (μM)	v*	v_p†	guanine concentration (μM)	v*	v_p†	guanine concentration (μM)	v*	v_p≠	hypoxanthine concentration (μM)	v*	v_p†	6-mercaptopurine concentration (μM)	v*
1.24	0.99	0.98	4.60	0.795	0.123	4.6	0.469	0.063	9.7	0.308	0.085	4.7	0.147
2.48	1.24	1.25	9.20	0.942	0.254	11.5	0.613	0.145	19.1	0.394	0.162	6.3	0.180
6.20	1.47	1.45	23.00	1.075	0.458	23.0	0.700	0.244	47.0	0.536	0.289	12.6	0.236
12.40	1.59	1.57	46.00	1.165	0.708	46.0	0.751	0.354	91.0	0.580	0.386	31.5	0.294
18.60	1.65	-	-	-	-	-	-	-	13.0	0.609	0.463	63.0	0.351

* Initial velocity.

† Initial velocities for adenine, guanine, and hypoxanthine in the presence of 25, 74, and 38 μM-6-mercaptopurine respectively.

≠ Initial velocity in the presence of 40 μm-hypoxanthine.

Table II.3. (continued)

Assays were carried out by the spectrophotometric method and velocities are given as µm-moles of nucleotide formed/min./mg. of protein.

Guanine concentration (µM)	v*	v_p †	6-mercaptopurine concentration (µM)	v*
2.33	3.73	0.98	18.2	1.13
4.66	4.94	1.70	36.0	1.40
9.00	6.28	2.20	70.4	1.59
17.30	6.80	3.40	109.0	1.64
-	-	-	135.0	1.68

* Initial velocity.

† Initial velocity in the presence of 18 µM-6-mercaptopurine.

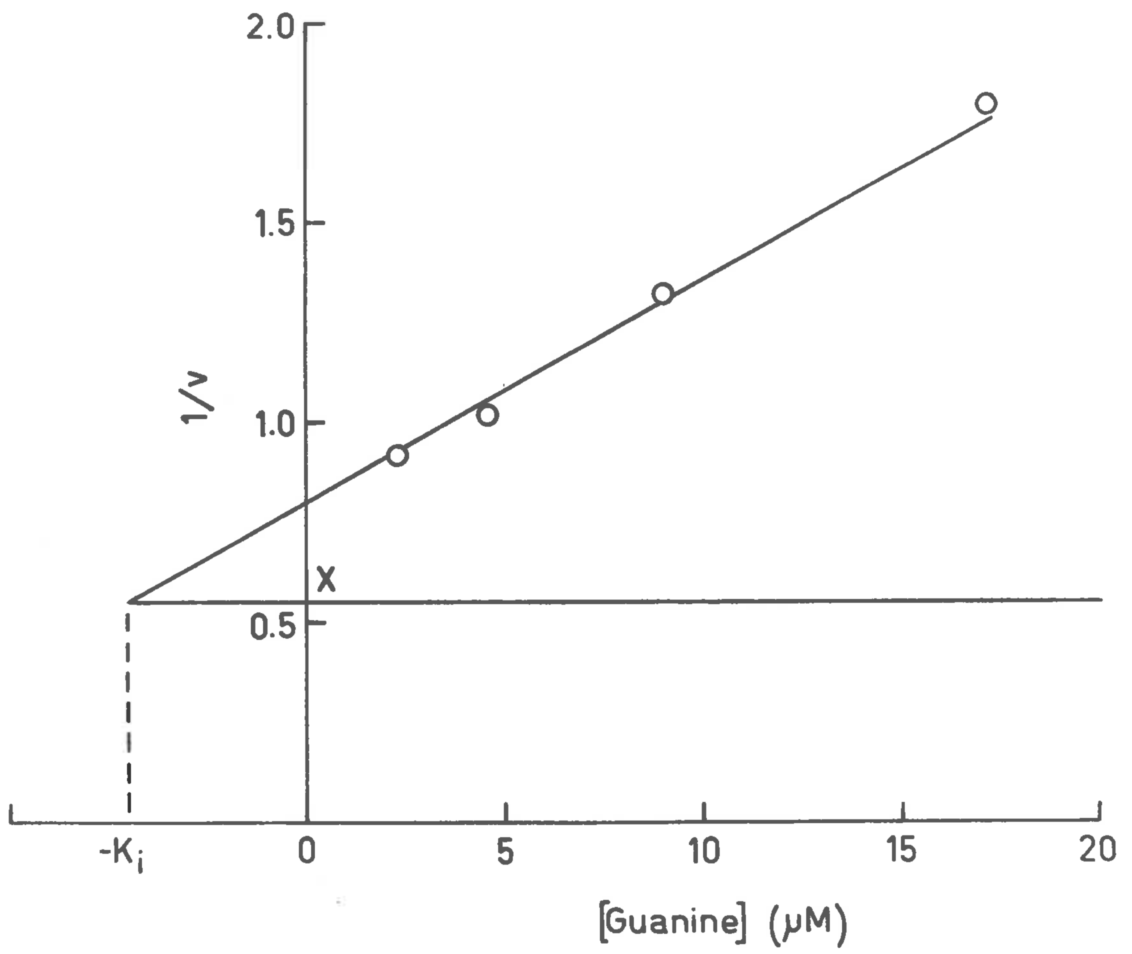
38 μM-mercaptopurine the apparent Michaelis constant for conversion of hypoxanthine into IMP was increased from 11.0 $\pm$ 0.9 μM to 60 $\pm$ 5 μM-hypoxanthine but there was no significant change in the extrapolated maximum velocity (V_p, 0.68 $\pm$ 0.03 μm-moles/min./mg. of protein; Table II.2.). 6-Mercaptopurine is therefore a competitive inhibitor of hypoxanthine phosphoribosyltransferase from Ehrlich ascites-tumour cells (K_i, 8.3 $\pm$ 1.7 μM). Similarly, from the assays with radioactive guanine (Table II.2.), 6-mercaptopurine and hypoxanthine are competitive inhibitors of guanine phosphoribosyltransferase from the tumour cells (K_i, 4.4 $\pm$ 0.9 μM and 3.4 $\pm$ 0.3μM respectively). In the spectrophotometric assay with a partially-purified fraction from the ascites-tumour cells 6- mercaptopurine was again a competitive inhibitor of conversion of guanine into GMP. The value of K_i (5.1 $\pm$ 1.9 μM). did not differ significantly from that found in the assay with radioactive guanine. The Michaelis constant for conversion of 6-mercaptopurine into thio-IMP was 10.9 $\pm$ 0.44 μM with the partially-purified fraction, and the extrapolated maximum rate (1.82 $\pm$ 0.2 μm-moles/min./mg. of protein) was 23% of that with guanine. In this experiment the final pH was 7.9 while the final pH in the other kinetic experiments was 7.8. The value of K_m for 6-mercaptopurine in the assay with radioactive purine was 7.55 $\pm$ 1.3 at pH 7.8, and the extrapolated maximum rate was 0.38 $\pm$ 0.02 μm-moles/min./mg. of protein.

Using the spectrophotometric assay, the rate of formation of thio-IMP at 320 mμ and of GMP at 255 mμ could both be followed in cuvettes containing a mixture of guanine and 6-mercaptopurine. Thus the results

from the experiment determining the inhibition of guanine phosphoribosyltransferase by 6-mercaptopurine could also be used to estimate inhibition of the conversion of 6-mercaptopurine into thio-IMP by guanine. In this case the concentration of the substrate (6-mercaptopurine) was kept constant and the concentration of the inhibitor (guanine) was varied. A plot of 1/initial rate of thio-IMP formation against guanine concentration resulted in a straight line (see Fig.II.6.). The maximum velocity for 6-mercaptopurine was known for this enzyme preparation (1.82 μm-moles of thio-IMP/min./mg. of protein; see Table II.2.) and a horizontal line through this point (X) on the 1/v axis cut the extrapolated graph at a guanine concentration equal to $-K_i$ (see Fig. II.6. and Dixon & Webb, 1958, p. 22). The value of K_i found was 4.4 μM; this is similar to the value of K_i (5.1 μM) found for the inhibition of the conversion of guanine into GMP by 6-mercaptopurine in the same set of experiments.

The Michaelis constants for the conversion of 6-mercaptopurine and hypoxanthine into their 5'-phosphoribosyl derivatives do not differ significantly from each other or from the K_i of 6-mercaptopurine when it acts as a competitive inhibitor of the latter reaction. These parameters do differ significantly ($P < 0.05$) from the K_i values of 6-mercaptopurine and hypoxanthine when these purines act as competitive inhibitors of conversion of guanine into GMP. This provides further evidence that more than one enzyme is involved in the conversion of guanine and hypoxanthine into GMP and IMP respectively.

Fig. II.6. Plot of 1/v against [guanine] used to calculate the K_i for guanine as an inhibitor of 6-mercaptopurine phosphoribosyltransferase. The point marked X on the ordinate represents the reciprocal of the maximum velocity for 6-mercaptopurine determined with the same sample of enzyme (1.82 µm-moles/min./mg. of protein). See the text for details of calculation.



2.0
1.5
1.0
0.5
1/v
X
-K
0
5
10
15
20
[Guanine] (μM)

There is some evidence that Ehrlich ascites-tumour cells convert purines, derived from the tissues of the host, into nucleotides and nucleic acids (Henderson & LePage, 1959; Smellie, Thomas, Goutier & Davidson, 1956; Kimball & LePage, 1963). Smellie *et al.* (1956) showed that rabbit bone marrow and Ehrlich ascites-tumour cells incorporated [^{14}C]formate extensively into purines *in vivo* but very poorly *in vitro*. The *in vitro* incorporation could be increased by the addition of a soluble extract from pigeon liver containing the enzymes for *de novo* purine biosynthesis. It was suggested that, in these cells, nucleic acid synthesis normally proceeds by utilization of purines synthesised elsewhere in the body. This idea was extended by Lajtha & Vane (1958) who showed a rapid incorporation of [^{14}C]formate into the purines of rabbit bone marrow *in vivo* but almost no incorporation after hepatectomy or portal occlusion. These experiments indicated that bone marrow (and probably Ehrlich ascites-tumour cells) depends, at least to some extent, on a supply of preformed purines from the liver (see also section III of this thesis). From the low values of K_i reported here it is evident that 6-mercaptopurine is a potent competitive inhibitor of the conversion of hypoxanthine and guanine into their nucleotides. In cells which use the 'salvage' pathway of nucleotide biosynthesis from free purines (Kornberg, 1957) this inhibition is of potential importance in the control of growth by 6-mercaptopurine.

The results provide a quantitative basis for observations

made by other workers. Thus Carter (1959) showed that guanine, hypoxanthine, and 6-mercaptopurine were mutually competitive with a purine phosphoribosyltransferase preparation from _E. coli_. Similarly inhibition of the conversion of hypoxanthine into nucleotides by 6-mercaptopurine in _S. faecalis_ (Brockman _et al._, 1957a) is probably due to competition between the two purines. 6-Mercaptopurine has been shown to markedly inhibit the incorporation of [8-^{14}C]hypoxanthine and [8-^{14}C]guanine into the acid-soluble nucleotides of exponentially growing cultures of _S. faecalis_ (Debavadi, C.S. & Brockman, R.W., cited by Brockman, 1963); in agreement with the results presented here, 6-mercaptopurine did not inhibit the incorporation of [8-^{14}C]adenine. The formation of hypoxanthine from 6-mercaptopurine has been reported in microorganisms (Balis _et al._, 1958a; Carey & Mandel, 1960), and to a lesser extent in mice (Elion _et al._, 1954) and in tumour cells (Brockman 1960). In cells in which this occurs to a significant extent, competition between the two bases would reduce the effectiveness of 6-mercaptopurine as a growth inhibitor by the mechanism discussed here.

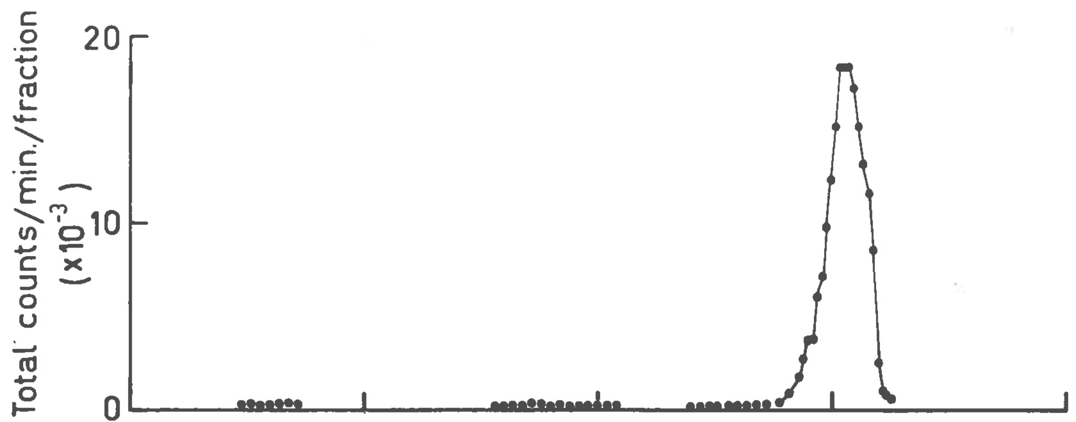
2. Purine phosphoribosyltransferases from ascites-tumour cells resistant to 6-mercaptopurine

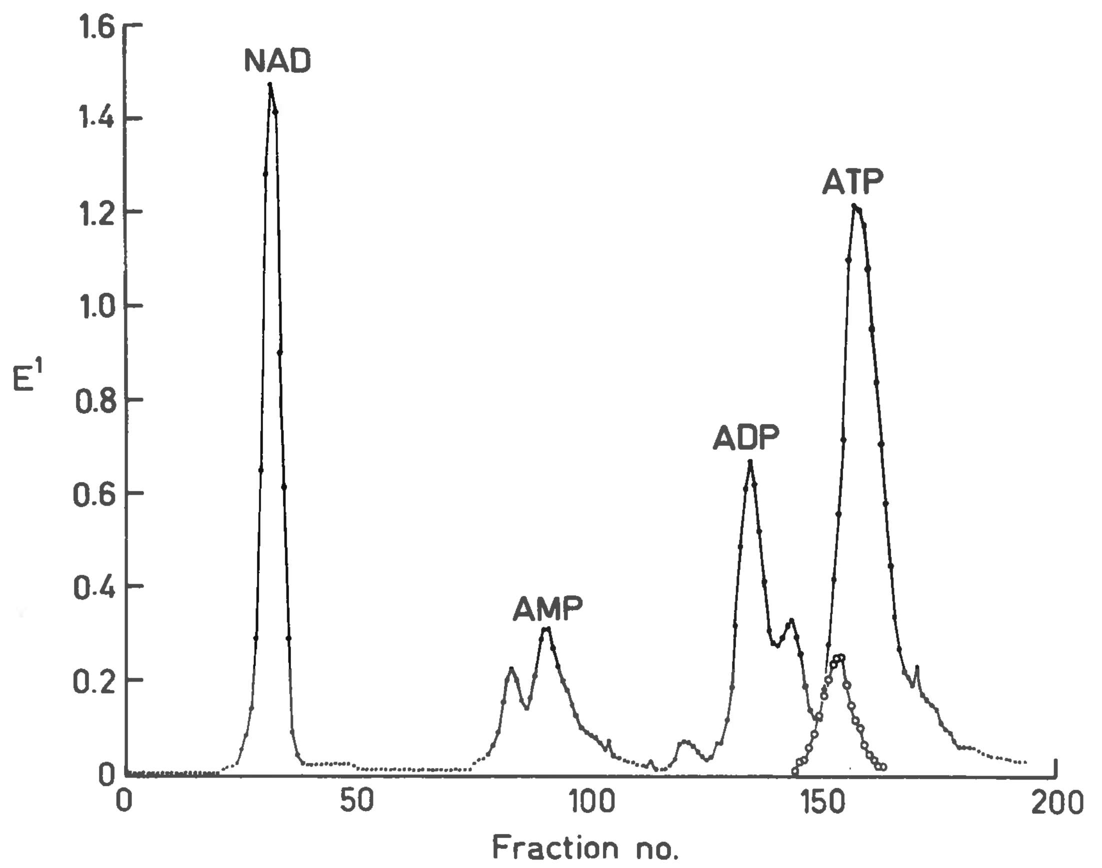
(a) The formation of thio-IMP from 6-mercaptopurine by sensitive and resistant cells in vivo.

In the experiment with [8-^{14}C]6-mercaptopurine (see the Materials and Methods section), 0.27 μmole of thio-IMP/ml. of packed cells (specific activity, 0.124 μC/μmole) was isolated 1 hr. after injection

of the radioactive drug. In similar experiments, Paterson (1959) found 0.25 - 0.3 μmole of thio-IMP/ml. of packed cells (mice were pretreated with 6-mercaptopurine for 4 days before the final injection). In the experiments described here (see the Materials and Methods section), 8% of the added 6-mercaptopurine and 6.6% of the added radioactivity was recovered as thio-IMP. The elution pattern of radioactivity and of extinction at 260 mμ and 320 mμ, after passage of the acid-soluble extract from ascites-tumour cells through a DEAE-cellulose column, is shown in Fig. II.7. (see the Materials and Methods section). The major components other than thio-IMP (NAD, AMP, ADP, and ATP) were identified by their spectra and by electrophoresis at pH 4.8. There was little radioactivity in any component other than thio-IMP (a trace of activity was found in the position expected for free 6-mercaptopurine) indicating that 6-mercaptopurine was not converted into hypoxanthine, with consequent incorporation into adenine and guanine nucleotides, under these conditions. Continued washing of the column with 0.4 M-NH_4HCO_3 after the elution of thio-IMP did not remove any components which contained radioactivity or which absorbed light at 320 mμ. Thus it is unlikely that significant amounts of 6-thioinosine 5'-di- or triphosphates were formed (the formation of these compounds would be a prerequisite to the incorporation of a 6-mercaptopurine nucleotide into nucleic acids by known enzymic reactions). Similarly, Paterson (1959) was unable to detect further phosphorylated derivatives of thio-IMP in acid extracts of Ehrlich ascites-tumour cells exposed to 6-mercaptopurine.

Fig. II.7. Isolation of [8-^{14}C]-thio-IMP from an acid-soluble extract of ascites-tumour cells 1 hr. after treatment with [8-^{14}C]6-mercaptopurine. The extract was prepared and fractionated on a DEAE-cellulose column as described in the Materials and Methods section. Fractions (7 ml.) were collected and the extinction at 260 mμ (●) and 320 mμ (○) was read in a cuvette with a 1 cm. light path. The total radioactivity in each fraction with an extinction at 260 mμ or 320 mμ of greater than 0.05 is given as counts/min. (see the Materials and Methods section).





0-0.05M-NH_4HCO_3 0.05-0.2M-NH_4HCO_3

When analyses for thio-IMP were carried out by electrophoresis (see the Materials and Methods section), 0.23 and 0.16 μmole of thio-IMP/ml. of packed cells was isolated, in two separate experiments, 1 hr. after the final injection of 6-mercaptopurine. Two similar experiments with resistant ascites-tumour cells resulted in the isolation of 0.011 and 0.007 μmole of thio-IMP/ml. of packed cells. It is not known if this small synthesis of thio-IMP was due to a small proportion of sensitive cells in the resistant population. If this were the case the proportion of sensitive cells could have been no more than 5%, and the corresponding contamination of the transferase preparation used in these studies would not have caused serious errors in measurement of kinetic parameters.

(b) Assay of purine phosphoribosyltransferases

As with the extracts from sensitive cells, there was no evidence that extracts from resistant cells contained enzymes that would interfere to a significant extent with the assays of purine phosphoribosyltransferases. When the standard assay systems with radioactive adenine, guanine, hypoxanthine, and 6-mercaptopurine were allowed to react with an extract from resistant cells for 15 min. the extent of conversion of these purines into their nucleotides was 95%, 93%, 96% and 98%, respectively. When 5.2 μM-[8-^{14}C]-IMP was treated with the extract of resistant cells in the presence of PRPP and non-radioactive hypoxanthine in the conditions of the assays (see the Materials and Methods section) 6-7% of the nucleotide was converted

into hypoxanthine and 0.1 - 0.2% of inosine was detected. In a corresponding experiment with 6 μM-[8-^{14}C]-AMP, 1% of the nucleotide was converted into adenine and 0.4% was converted into IMP. In the conditions of the assay, but without PRPP, the change in the extinction at 255 mμ was less than 0.002/10 min. in a 1 cm. cell, with 25 μM-guanine or with 23 μM-GMP; in corresponding experiments with 24 μM-6-mercaptopurine or with 19 μM-thio-IMP there was no detectable change in the extinction at 320 mμ. From these results it is unlikely that resistance to 6-mercaptopurine is due to more rapid destruction of thio-IMP by soluble enzymes, in the cells. Insoluble components of the homogenate were not examined in this respect, but Paterson & Hori (1963) have reported that a 5'-nucleotidase which catalyses the cleavage of thio-IMP, and which is localized in the insoluble fraction of ascites-tumour cell homogenates, has decreased activity in resistant cells.

(c) Kinetic studies

The specific activities of adenine, guanine, hypoxanthine, and 6-mercaptopurine phosphoribosyltransferases (1.08, 0.55, 0.87, and 0.34 μm-moles/min./mg. of protein respectively; see Table II.4.) were 64%, 45%, 130%, and 89% of values found with corresponding extracts from sensitive cells (see Table II.2.). Paterson (1962) reported no significant difference in the last of these activities with extracts from sensitive or from resistant cells.

With the extract from resistant cells, extrapolated maximum specific activities of guanine phosphoribosyltransferase in the presence or in the absence of 34 μM-6-mercaptopurine (0.56 ± 0.03 and 0.55 ± 0.01

µm-moles/min./mg. of protein respectively) did not differ significantly, nor did the corresponding specific activities of hypoxanthine phosphoribosyltransferase (0.78 $\pm$ 0.02 and 0.87 $\pm$ 0.03 µm-moles/min./mg. of protein) in the presence or absence of 32 µM-6-mercaptopurine. However, the apparent values of the Michaelis constants were increased from 1.60 $\pm$ 0.2 µM-guanine to 30.7 $\pm$ 2.1 µM-guanine and from 7.84 $\pm$ 1.2 µM-hypoxanthine to 37.3 $\pm$ 1.9 µM-hypoxanthine in the presence of 34 and 32 µM-6-mercaptopurine respectively. 6-Mercaptopurine was therefore a competitive inhibitor of guanine and hypoxanthine phosphorribosyltransferases from resistant Ehrlich ascites-tumour cells. The inhibitor constants (1.8 $\pm$ 0.3 µM and 8.5 $\pm$ 1.6 µM-6-mercaptopurine respectively) were similar to the corresponding values found with the extract of sensitive cells (see Table II.2.). The Michaelis constants found with guanine and hypoxanthine with the extract of resistant cells (1.6 $\pm$ 0.2 µM and 7.85 $\pm$ µM respectively) were also not significantly different from those found with extracts of sensitive cells.

The extrapolated maximum specific activities of adenine phosphoribosyltransferase in the extract from resistant cells and the Michaelis constants for adenine were not significantly different in the presence of 32 µM-6-mercaptopurine or in the absence of this purine (1.06 $\pm$ 0.03 and 1.08 $\pm$ 0.01 µm-moles/min./mg. of protein; 0.91 $\pm$ 0.1 µM and 0.89 $\pm$ 0.04 µM-adenine; cf. Table II.2.). The corresponding enzyme in extracts of sensitive cells was also not inhibited by 6-mercaptopurine and had the same Michaelis constant (0.89 µM-adenine)

as that reported here. The kinetic parameters found for purine phosphoribosyltransferases from resistant cells are summarised and compared with the parameters found with extracts from sensitive cells in Table II.4. The parameters were calculated from rate data listed in Table II.5.

These kinetic studies provide no evidence of major qualitative or quantitative differences between the purine phosphoribosyltransferases of Ehrlich ascites-tumour cells that are resistant or sensitive to 6-mercaptopurine and indicate that the mechanism of resistance is not related to the properties of these enzymes. Paterson (1962) reported resistance to 6-mercaptopurine in Ehrlich ascites-tumour cells in which intact resistant cells did not form thio-IMP; however, extracts of resistant cells contained 6-mercaptopurine phosphoribosyltransferase activity and it was postulated that resistance was due to a specific permeability barrier preventing the entry of 6-mercaptopurine into the cell. It is likely that a similar mechanism would account for resistance in the ascites-tumour cells used in these experiments. These resistant cells differ from the resistant Ehrlich ascites-tumour cells that Kimball & LePage (1964) have reported to contain no 6-mercaptopurine phosphoribosyltransferase.

Table II.4. Inhibition of purine phosphoribosyltransferases from resistant Ehrlich ascites-tumour cells by 6-mercaptopurine

Values in parentheses are the coefficients of variation of the means, and values in square brackets the corresponding values found with sensitive cells (cf. Table II.2.).

Substrate	K_m (μM)	V (μm-moles/min./ mg. of protein)	6-mercaptopurine concentration (μM)	K_p (μM)	V_p (μm-moles/min./ mg. of protein)	K_i (μM)
Adenine	0.89 (0.046)	1.08 (0.01)	32	0.91 (0.10)	1.06 (0.03)	-
	[0.89]	[1.69]	[25]	[0.89]	[1.68]	-
Guanine	1.60 (0.13)	0.55 (0.02)	34	30.7 (0.07)	0.56 (0.05)	1.82 (0.16)
	[2.5]	[1.21]	[74]	[45]	[1.39]	[4.4]
Hypoxanthine	7.85 (0.15)	0.87 (0.03)	32	37.3 (0.05)	0.78 (0.03)	8.49 (0.19)
	[11.0]	[0.66]	[38]	[60]	[0.68]	[8.3]
6-Mercaptopurine	3.56 (0.11)	0.34 (0.03)	-	-	-	-
	[7.55]	[0.38]	-	-	-	-

Table II.5. <u>Initial velocities used in the calculation of kinetic parameters of purine phosphoribosyltransferases from ascites-tumour cells resistant to 6-mercaptopurine</u> (see Table II.4.)

Assays were carried out with radioactive substrates and velocities are given as μm-moles of nucleotide formed/min./mg. of protein.

Adenine concentration (μM)	v*	v_p †	Guanine concentration (μM)	v*	v_p †	Hypoxanthine concentration (μM)	v*	v_p †	6-Mercaptopurine concentration (μM)	v*
1.22	0.616	0.618	2.49	0.343	0.044	6.79	0.475	0.125	6.29	0.148
2.44	0.802	0.798	6.23	0.439	0.092	16.90	0.647	0.242	12.60	0.205
6.10	0.944	0.943	12.50	0.477	0.163	33.90	0.715	0.366	31.50	0.261
12.20	1.005	0.999	24.90	0.530	0.250	67.90	0.822	0.504	63.00	0.308

* Initial velocity.

† Initial velocities of adenine, guanine, and hypoxanthine in the presence of 32, 34, and 32 μM-6-mercaptopurine respectively.

SECTION III

BIOCHEMICAL STUDIES WITH INTACT EHRLICH ASCITES-TUMOUR CELLS

In this section the effect of 6-mercaptopurine on the incorporation of [U-^{14}C]glycine, [8-^{14}C]adenine, and [8-^{14}C] hypoxanthine into the nucleotides of intact ascites-tumour cells is described. The effect of prolonged treatment with 6-mercaptopurine on the amount of purine nucleotides in ascites-tumour cells has also been studied.

A. MATERIALS AND METHODS

1. Purine bases.

[8-^{14}C]Adenine and [8-^{14}C]hypoxanthine were of the same purity as described in section II. [U-^{14}C]Glycine was obtained from California Corp. for Biochemical Research and contained no contaminants that could be detected by radioautography or by spraying with 1% ninhydrin (w/v) in butan-1-ol after chromatography in 5% Na_2HPO_4 (w/v) saturated with isoamylalcohol (R_F 0.86). Non-labelled adenine, guanine, hypoxanthine, and 6-mercaptopurine were of the same purity as described in section II.

2. Estimation of purine bases.

Adenine, guanine, and hypoxanthine in the acid-soluble nucleotide fraction. Ascitic fluid and cells were collected from individual mice, the peritoneal cavity was washed with 4 ml. of 0.9% NaCl (cf. Paterson, 1960a), and the packed volume of cells from ascitic fluid and washings (about 1-3 ml.) was measured. The cells were suspended in 6 ml. of 5% trichloroacetic acid (w/v; 2°) and centrifuged after stirring for 5 min. The residue was washed with cold 5% trichloroacetic acid (3 x 5 ml.) and the combined extracts were extracted with ether (3 x 50 ml.). After evaporation to dryness at 30°, the extract was chromatographed on Whatman 3MM paper in butan-1-ol - acetic acid - water (20:3:7; 8 hr.) at 22°. The nucleotides,

which remained on the origin, were eluted with 50 ml. of water and the eluate was evaporated to dryness. The residue was transferred to a glass hydrolysis tube (4 cm. x 0.25 cm.^{2}) with a small volume of 0.01 N-HCl and the liquid was removed with a stream of nitrogen. After dissolving the residue in 0.25 ml. of N-HCl, the hydrolysis tube was sealed and completely immersed in a boiling-water bath for 1 hr. Samples (0.04 - 0.20 ml.) were applied as bands to Whatman No. 1 paper and chromatographed in 5% (w/v) Na_2HPO_4 saturated with isoamylalcohol (descending; 12 hr.) at 22°. After location of the purines with ultra-violet light, areas containing adenine (R_F 0.42), and guanine (remained on the origin) were eluted from the paper with 5 ml. of 0.1 N-HCl (papers were left in test-tubes with the acid for 6 hr. at 22°). Hypoxanthine (R_F 0.61) was eluted from the paper in the same way with 5 ml. of phosphate buffer (Na^+, pH 7.0). Adenine, guanine, and hypoxanthine were determined by measuring the extinction at 262 mμ, 249 mμ, and 249.5 mμ respectively against suitable blanks eluted from the paper at an R_F similar to that of the corresponding purine. Concentrations were calculated by the use of an extinction coefficient of 13.1×10^3 for adenine, of 11.1×10^3 for guanine, and of 10.7×10^3 for hypoxanthine.

Nucleic acid adenine and guanine. The residue remaining after extraction with cold trichloroacetic acid (see above) was suspended in 6 ml. of 5% (w/v) trichloroacetic acid and heated at 90°

for 15 min. After centrifuging the residue was washed with 5% trichloroacetic acid (2 x 5 ml.) and the combined extracts were extracted with ether (3 x 40 ml.). After evaporation to dryness, the residue was dissolved in 2 ml. of N-HCl and portions (0.02 - 0.05 ml.) were assayed for adenine and guanine by chromatography as described above. An alternative method of assay was to pass the hot-acid extracts, after removal of trichloroacetic acid with ether, through columns (3 cm. x 0.25 $cm.^2$) of Amberlite CG-120 (H^+ form). The columns retained adenine and guanine, and the pyrimidine nucleotides were washed through with N-HCl (2 x 10 ml.). Adenine and guanine were eluted with 6 N-HCl (2 x 10 ml.) and, after dilution of portions to a final HCl concentration of 0.1 N, the relative amounts of each purine were determined by differential spectrophotometry (Loring, 1960).

3. Determination of radioactivity.

The radioactivity of adenine, guanine, and hypoxanthine of the acid-soluble nucleotide fractions and of adenine and guanine of the nucleic acid fractions was measured directly by liquid scintillation counting of discs cut from the paper chromatograms (see Section II, p. 90) after separation of the purine bases as described above. Blank discs of paper of the same size were cut from corresponding areas of the chromatograms and counted to provide a control value for each radioactivity determination.

B. RESULTS AND DISCUSSION

1. The validity of the assay procedures

The method of preliminary chromatography in butanol-acetic acid - water has been found useful for separation of nucleotides from nucleosides and free bases (cf. Section I). This procedure was essential in these experiments so that free purine bases could be distinguished from purines derived from cellular nucleotides. To check the assay procedure, a mixture of 5 μmoles of AMP and IMP and 1 μmole of GMP were dissolved in 5 ml. of 5% trichloroacetic acid and extracted with ether (3 x 20 ml.). The nucleotides were subjected to chromatography in butanol - acetic acid - water, hydrolysis in N-HCl, and chromatography in isoamylalcohol - phosphate buffer as described in the Materials and Methods section. After elution from the paper and estimation by spectrophotometry, 98%, 96%, and 102% of the added AMP, GMP, and IMP were recovered as adenine, guanine, and hypoxanthine respectively. Quantitative recoveries of adenine and guanine after hydrolysis in N-HCl for 1 hr. at 100° have been obtained by Vischer & Chargaff (1948) and Loring, Fairley & Seagran (1952) from AMP and GMP, and by Smith & Markham (1950) from yeast RNA. It has been reported that as much as 7 - 8% of both adenine and guanine may be destroyed by N-HCl at 100° (Abrams, 1951a). Similarly when a mixture of 20 μmoles each of adenine, guanine, cytidine 5'-phosphate, and

uridine 5'-phosphate in 20 ml. of N-HCl were passed through an Amberlite CG-120 column (see the Materials and Methods section) quantitative recoveries of adenine and guanine were obtained by differential spectrophotometry. The nucleic acid adenine and guanine from about 2.5 ml. of packed ascites-tumour cells was obtained in 20 ml. of 6 N-HCl after elution from the Amberlite columns. Dilution of 0.05 ml. to 3 ml. with water resulted in a final HCl concentration of 0.1 N and also gave a convenient concentration for accurate extinction readings.

No attempt was made to separate RNA from DNA, and only figures for total nucleic acid adenine and guanine were obtained. Adequate separations of relatively pure adenine and guanine could be obtained without prior removal of phospholipid material. LePage (1953) found similar recoveries of nucleic acid purines either with or without extraction with alcohol - ether mixtures to remove the phospholipids.

2. <u>The affect of 6-mercaptopurine on the incorporation of</u> [U-^{14}C] <u>glycine into the purine nucleotides of Ehrlich ascites-tumour cells</u>.

(a) <u>In vivo</u>.

For each experiment four female Swiss Albino mice were inoculated with Ehrlich ascites-tumour cells (see sections I and II). Each mouse was given an intraperitoneal injection of 6-mercaptopurine (30 mg./Kg. of body weight) on the 8th and 9th day after inoculation. On the 10th day animals were given a further injection of 6-mercaptopurine

immediately followed by 10 μC of [U-^{14}C]glycine (0.8 μmole). Control animals were injected with equivalent volumes of 0.9% NaCl and were also treated with 10 μC of [U-^{14}C]glycine. Control and test animals were killed after 2 hr. and the total adenine and guanine nucleotides in the acid-soluble and nucleic acid fractions from the pooled ascites-tumour cells was determined by the chromatographic technique described in the Materials and Methods section. The total acid-soluble hypoxanthine derived from the nucleotide fraction was also determined by this method. The results from two such experiments are tabulated in Table III.1. Fig. III.1. shows a radioautograph of acid-soluble nucleotide material from Experiment I (see Table III.1.) after hydrolysis and chromatography in isoamylalcohol - phosphate (see the Materials and Methods section). The specific activity of isolated guanine did not change on elution and rechromatography in isopropanol - conc. HCl - water (170:44:36; R_F 0.26). This check was necessary as guanine did not move from the origin in the isoamylalcohol - phosphate solvent.

The specific activities (Table III.1.) of adenine and guanine in both acid-soluble and nucleic acid fractions were less in the presence of 6-mercaptopurine. More experiments of this type would be needed to determine whether 6-mercaptopurine had a greater effect on glycine incorporation into adenine or into guanine.

(b) *In vitro*.

An attempt was made to study the incorporation of [U-^{14}C] glycine into ascites-tumour cells *in vitro* so that any effects of

Fig. III.1. Radioautograph showing the incorporation of [U-^{14}C]glycine into the acid-soluble purine nucleotides of Ehrlich ascites-tumour cells. A cold trichloroacetic acid extract was made of ascites-tumour cells 2 hr. after injection of [U-^{14}C]glycine; material in the extract was hydrolysed in N-HCl (see the Materials and Methods section) and chromatographed in 5% (w/v) Na_2HPO_4 saturated with isoamylalcohol. The left-hand strip shows the ultra-violet absorption print of marker guanine, adenine, and hypoxanthine in order of increasing R_F values. The radioautograph shows, from left to right, material isolated from untreated and from 6-mercaptopurine treated ascites-tumour cells after exposure to radioactive glycine.

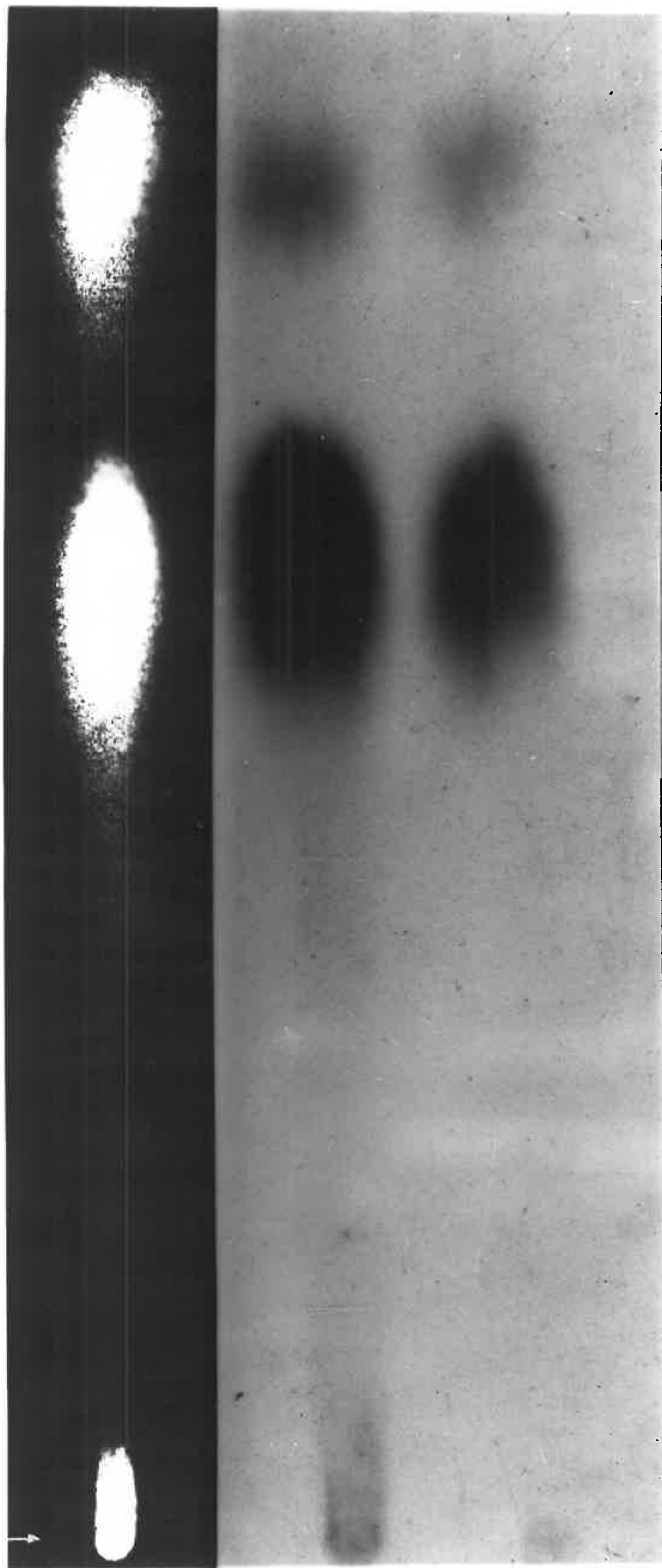


Table III.1. The effect of 6-mercaptopurine on incorporation of [U-^{14}C] glycine into the acid-soluble nucleotides and nucleic acid purines of Ehrlich ascites-tumour cells in vivo.

Expt. 1

		Acid-soluble purines of nucleotide fraction		Nucleic acid purines	
		µmoles/ml. of packed cells	cts./min./* µmole	µmoles/ml. of packed cells	cts./min./* µmole
Control	adenine	1.950	4,960 (9,700)	4.7	210 (410)
	guanine	0.020	13,980 (280)	4.8	306 (6)
	hypoxanthine	0.008	14,100 (112)	-	-
6-Mercaptopurine	adenine	1.470	3,430 (5,070; 52%)	4.7	115 (169; 41%)
	guanine	0.014	8,040 (112; 40%)	5.2	153 (2; 33%)
	hypoxanthine	0.007	13,380 (95; 85%)	-	-

Expt. 2

		µmoles/ml. of packed cells	cts./min./* µmole	µmoles/ml. of packed cells	cts./min./* µmole
Control	adenine	1.560	7,020 (11,100)	4.3	318 (496)
	guanine	0.013	12,900 (168)	6.2	370 (5)
	hypoxanthine	0.021	13,800 (290)	-	-
6-Mercaptopurine	adenine	1.62	2,900 (4850; 44%)	4.4	170 (275; 55%)
	guanine	0.012	4,800 (58; 34%)	5.9	196 (2; 40%)
	hypoxanthine	0.018	15,000 (270; 93%)	-	-

* Values in parentheses are counts/min./ml. of cells and percentages of control value.

the host would be removed. Ascitic fluid and cells were collected from mice 7 days after inoculation and the suspension was centrifuged at 2 000 g for 5 min. to collect the ascites-tumour cells (about 3 ml.). The cells were resuspended in an equal volume of Robinson's (1949) medium and half a volume of the original ascitic fluid was added. The suspension was divided into two equal portions, was made 0.25% with respect to glucose, and 10 μC of [U-^{14}C]glycine (0.8 μmole) was added to each portion. The flasks were flushed with either oxygen or nitrogen and incubated at 37° for 2 hr. with gentle shaking. Incorporation of radioactivity into acid-soluble and nucleic acid nucleotides was measured as described above. The results of a typical experiment are shown in Table III.2., and indicate very little incorporation of [U-^{14}C] glycine into adenine or guanine nucleotides under these conditions. Incorporation of radioactivity into acid-soluble adenine, guanine, and hypoxanthine nucleotides was less than one-tenth of that *in vivo*. Little difference in incorporation was obtained by inclusion of 0.5mM-glutamine and 0.5 mM-sodium bicarbonate in the incubation flasks. This decrease in the incorporation of non-cyclic precursors into purine nucleotides *in vitro* may simply be the result of collecting the cells and incubating them in a non-physiological environment or it may mean that the host itself contributes to the observed incorporation of [U-^{14}C] glycine into purine nucleotides *in vivo*. Evidence has been obtained by other workers that this is probably the case (see section II, p.108). This adds to the suggestion, made in section II, that some of the

Table III.2. <u>The incorporation of</u> [U-^{14}C]<u>glycine into acid-soluble and nucleic acid nucleotides of Ehrlich ascites-tumour cells</u> in vitro.

	acid-soluble purine nucleotide fraction				nucleic acid purines			
	µmoles/ml. packed cells		counts/min. /µmole*		µmoles/ml. packed cells		counts/min. /µmole	
	O_2	N_2	O_2	N_2	O_2	N_2	O_2	N_2
adenine	2.00	2.410	228 (456)	368 (880)	5.23	5.10	only traces of activity were detectable	
guanine	0.032	0.030	740 (23)	860 (26)	5.70	5.76		
hypoxanthine	0.098	0.140	1044 (102)	1120 (157)	-	-		

*Values in parentheses are counts/min./ml. of packed cells.

metabolic effect of 6-mercaptopurine may be accounted for by inhibition of the conversion of preformed purines into nucleotides.

Even when experiments were done _in vivo_ there was only a low level of incorporation of radioactivity from the added [U-^{14}C]glycine into purine nucleotides (less than 0.5% of the added radioactivity). In addition about 0.5% of the radioactivity was associated with material insoluble in hot 5% trichloroacetic acid (this fraction was assumed to be mainly protein). Ehrlich ascites-tumour cells are known to contain relatively high concentrations of glycine (Christensen & Riggs, 1952) and the low incorporation may in part be due to dilution of the added [U-^{14}C]glycine by the intracellular 'pool' of this amino acid.

3. _The effect of 6-mercaptopurine on the incorporation of [8-^{14}C] adenine and [8-^{14}C]hypoxanthine into the purine nucleotides of Ehrlich ascites-tumour cells_

Studies on the effect of 6-mercaptopurine on the incorporation of preformed purines have been carried out by numerous investigators, and this aspect has not been examined in detail in this thesis. The experiments were carried out in an attempt to establish the relative capacities of ascites-tumour cells to utilize preformed purines and to synthesise purines from non-cyclic precursors.

For experiments on the incorporation of [8-^{14}C]adenine, mice were injected intraperitoneally with 6-mercaptopurine (30 mg./Kg. of body weight) on the 7th and 8th day after inoculation with ascites-tumour

cells; control mice received an equal volume of 0.9% NaCl. On the 10th day test mice received a further dose of 6-mercaptopurine, and 15 min. later, were injected with 2.5 μmoles of [8-^{14}C]adenine (5 μC). Control mice were treated with saline and with [8-^{14}C]adenine in the same way. The mice were killed after 1 hr. and acid-soluble adenine, guanine, and hypoxanthine nucleotides, and nucleic acid adenine and guanine were estimated as described in the Materials and Methods section (see Table III.3.). In vitro incorporation was measured by incubating the collected cells with the required purines under the conditions described before for [U-^{14}C]glycine (the gas phase was nitrogen). The results for the incorporation of [8-^{14}C]adenine in vitro are also given in Table III.3. There was extensive incorporation of [8-^{14}C]adenine into acid-soluble nucleotides both in vivo and in vitro, and this incorporation was not inhibited by 6-mercaptopurine. In one hour, 50% and 60% of the added radioactivity was incorporated into nucleotide (material remaining on the origin after chromatography in butanol - acetic acid - water; see the Materials and Methods section) in vivo and 42% and 48% in vitro in the absence and presence of 6-mercaptopurine respectively. Under similar conditions less than 0.5% and 0.05% of added [U-^{14}C]glycine was incorporated into nucleotides in vivo and in vitro respectively (see Table III.1. and Table III.2.).

These results suggest that utilization of preformed purines by the ascites-tumour cells used in these experiments is a more efficient process than biosynthesis of the purine ring from non-cyclic

Table III.3. The effect of 6-mercaptopurine on [8-^{14}C]adenine incorporation into the acid-soluble nucleotides and the nucleic acid purines of Ehrlich ascites-tumour cells

IN VIVO

		Acid-soluble purine nucleotides		Nucleic acid purines	
		µmoles/ml. of packed cells	counts/min.* /µmole	µmoles/ml. of packed cells	counts/min.* /µmole
Control	adenine	1.730	540,000 (935,000)	5.81	1680 (2900)
	guanine	0.020	4,350 (87)	6.90	38 (0.8)
	hypoxanthine	0.049	44,400 (2,170)	-	-
6-Mercaptopurine	adenine	1.400	864,000 (1,210,000)	5.71	2440 (3400)
	guanine	0.016	4,200 (67)	6.70	62 (1.1)
	hypoxanthine	0.039	77,900 (3,040)	-	-

IN VITRO

		Acid-soluble purine nucleotides		Nucleic acid purines	
		µmoles/ml. of packed cells	counts/min.* /µmole	µmoles/ml. of packed cells	counts/min.* /µmole
Control	adenine	1.210	380,000 (460,000)	4.23	980 (1190)
	guanine	0.011	3,900 (43)	5.60	30 (0.3)
	hypoxanthine	0.066	40,000 (2,640)	-	-
6-Mercaptopurine	adenine	1.480	549,000 (810,000)	4.78	1980 (2900)
	guanine	0.018	4,200 (76)	5.78	26 (0.5)
	hypoxanthine	0.040	41,000 (1,640)	-	-

* Values in parentheses are counts/min./ml. of cells.

precursors; evidence suggesting that preformed purines may be available to the ascites-tumour cells was reviewed in the previous section. The ability to use preformed purines would be advantageous to rapidly dividing cells as the enzymes involved do not seem to be under control by 'feedback' mechanisms as may be the case with the synthesis of purine nucleotides from non-cyclic precursors.

Acid-soluble extracts from the ascitic fluid of untreated mice contained only traces of material corresponding to adenine and guanine on chromatography in isoamylalcohol-phosphate (for details of the solvent see the Materials and Methods section); however free purines would not be expected to accumulate because of their rapid uptake into the purine 'pool' of the cell.

A small amount (about 0.2%) of the incorporated radioactivity from [8-^{14}C]adenine was associated with hypoxanthine of the hydrolysed extracts, indicating only a limited conversion of AMP into IMP, either directly or indirectly at the nucleoside level, by deamination. In addition, soluble extracts of ascites-tumour cells, prepared as described in section I, did not contain detectable adenylate deaminase activity (assayed by the method of Nikiforuk & Colowick, 1955). These results differ from those of Paterson (1962) who found that the specific activities of AMP and IMP were similar after treatment of Ehrlich ascites-tumour cells with [8-^{14}C]adenine _in vitro_. A soluble extract from these cells was shown to contain an active adenylate deaminase.

One similar _in vivo_ experiment was carried out with [8-^{14}C]

hypoxanthine in which mice were pretreated with 6-mercaptopurine as described for [8-^{14}C]adenine incorporation. Total activity was determined in the acid-soluble nucleotide fraction, but this was not further fractionated into adenine, guanine, and hypoxanthine. The control nucleotide fraction contained 319,000 counts/min. (75% of the added radioactivity), 2 hr. after injection with [8-^{14}C]hypoxanthine (2.5 μmoles, 0.19 μC) and the nucleotide fraction from the ascites-tumour cells treated with 6-mercaptopurine contained 800 counts/min. The decrease in incorporation is almost certainly due to inhibition of hypoxanthine phosphoribosyltransferase by 6-mercaptopurine and, together with the lack of an inhibition of [8-^{14}C]adenine incorporation into nucleotides, provides confirmation _in vivo_ of the results found in section II with soluble extracts from ascites-tumour cells.

4. _The effect of 6-mercaptopurine on the levels of acid-soluble and nucleic acid adenine and guanine nucleotides of Ehrlich ascites-tumour cells_

Ten female Swiss Albino mice were given intraperitoneal injections of 6-mercaptopurine (30 mg./Kg. of body weight) on the 6th, 7th, 8th, 9th and 10th day after inoculation with ascites-tumour cells. Ten control mice were injected with an equivalent volume of 0.9% NaCl. One hour after the last injection the mice were killed by decapitation and total acid-soluble adenine and guanine nucleotides of the ascites-tumour cells from each mouse were separately determined as described

in the Materials and Methods section (see Table III.4.). Hypoxanthine was not detected in measurable amounts in the hydrolysates of the nucleotide fractions, indicating only low concentrations of IMP in these cells, either in the presence or absence of 6-mercaptopurine. Nucleic acid adenine and guanine from the same samples of cells were determined by differential spectrophotometry (see the Materials and Methods section); the results are listed in Table III.4. Samples of ascitic fluid and cells from each mouse were taken for dry weight determinations (cellular material was collected by centrifuging and dried at 100° for 16 hr.) and the results are given as μmoles of adenine or guanine/ml. of packed cells and as μmoles/g. dry weight of cells. Significance tests indicated a significant difference between the packed cell volume of 6-mercaptopurine treated and untreated mice ($P < 0.01$). The decrease in packed-cell volume after 6-mercaptopurine treatment is substantially less than when treatment was started only one day after inoculation with ascites-tumour cells (see section II, p. 83). 6-Mercaptopurine treatment resulted in a significant decrease in the amount of adenine and guanine derived from the acid-soluble nucleotide fraction ($P < 0.05$). This decrease was apparent when the results were expressed per unit volume of packed cells or per unit dry weight of cells. There was no decrease in the adenine and guanine of the nucleic acid fraction when the results were expressed in the same way. These results show that the inhibition of purine biosynthesis or interconversion by 6-mercaptopurine results in lower concentrations of

Table III.4. The effect of 6-mercaptopurine on the levels of acid-soluble and nucleic acid adenine and guanine nucleotides of Ehrlich ascites-tumour cells.

Mice were treated with 6-mercaptopurine for five days and the total adenine and guanine of the acid-soluble nucleotide and nucleic acid fraction was determined after hydrolysis as described in the Materials and Methods section. The table has been divided into two parts. This page gives the total amounts of acid-soluble purine nucleotides, and the following page the total amounts of nucleic acid purines. The mean value for each set of determinations is also listed, together with values for the standard deviation from the mean.

Expt. no.	Packed cell vol. (ml.)	Dry weight of cells/ ml. (g.)	Adenine µmoles/ml. of packed cells	Adenine µmoles/g. dry weight	Guanine µmoles/ml. of packed cells	Guanine µmoles/g. dry weight	adenine / guanine
ACID-SOLUBLE PURINE NUCLEOTIDES (CONTROL)							
1	2.4	0.24	1.01	4.20	0.0137	0.0570	73.9
2	2.5	0.23	0.72	3.14	0.0137	0.0595	52.6
3	2.5	0.24	1.45	6.02	0.0173	0.0720	83.9
4	2.5	0.22	1.82	8.26	0.0104	0.0472	175.0
5	2.5	0.23	1.46	6.35	0.0152	0.0661	102.5
6	2.0	0.32	2.40	7.28	0.0146	0.0456	144.5
7	2.6	0.26	1.94	7.49	0.0164	0.0630	118.0
8	2.5	0.24	1.54	6.41	0.0122	0.0509	126.0
9	2.7	0.22	1.32	6.00	0.0138	0.0628	95.6
10	2.6	0.24	1.84	7.67	0.0128	0.0534	144.0
Mean	2.5 ± 0.2	0.24 ± 0.08	1.55 ± 0.55	6.30 ± 1.5	0.0140 ± 0.002	0.0577 ± 0.008	111.6 ± 37
ACID-SOLUBLE PURINE NUCLEOTIDES (6-MERCAPTOPURINE TREATED)							
1	2.1	0.23	0.94	4.00	0.0089	0.0387	105.9
2	1.1	0.21	0.77	3.66	0.0160	0.0761	48.1
3	1.4	0.22	0.82	3.72	0.0100	0.0455	82.0
4	2.0	0.26	0.68	2.62	0.0062	0.0238	110.0
5	1.8	0.26	0.89	3.42	0.0130	0.0500	68.5
6	1.4	0.25	0.69	2.76	0.0054	0.0216	128.0
7	1.2	0.23	0.86	3.74	0.0068	0.0295	126.4
8	1.8	0.22	0.98	4.45	0.0124	0.0564	79.0
9	1.6	0.23	0.66	2.86	0.0088	0.0382	74.9
10	1.5	0.24	0.72	3.00	0.0105	0.0437	68.8
Mean	1.6 ± 0.3	0.23 ± 0.02	0.80 ± 0.11	3.40 ± 0.57	0.0098 ± 0.003	0.0424 ± 0.016	89.2 ± 27

Table III.4. (continued)

Expt. no.	Adenine		Guanine		
	μmoles/ml. of packed cells	μmoles/g. dry weight	μmoles/ml. of packed cells	μmoles/g. dry weight	adenine/guanine
NUCLEIC ACID PURINES (CONTROL)					
1	4.60	19.2	5.23	21.8	0.73
2	4.84	21.0	5.70	24.7	0.85
3	5.15	21.4	6.78	28.2	0.76
4	4.55	20.6	5.32	24.2	0.85
5	4.92	21.4	6.00	26.0	0.82
6	5.20	16.3	6.65	20.8	0.78
7	4.68	18.0	6.22	23.9	0.75
8	5.00	20.8	5.95	24.8	0.84
9	4.31	19.6	6.00	27.2	0.72
10	4.47	18.6	5.40	22.5	0.83
Mean	4.77 ± 0.28	19.7 ± 1.5	5.93 ± 0.45	24.4 ± 2.2	0.79 ± 0.04
NUCLEIC ACID PURINES (6-MERCAPTOPURINE TREATED)					
1	4.34	18.8	4.95	21.5	0.88
2	4.65	22.1	5.80	27.6	0.80
3	3.93	17.8	6.25	28.4	0.63
4	4.27	16.4	4.72	18.2	0.90
5	4.03	15.5	5.61	21.6	0.72
6	4.23	16.9	6.38	25.4	0.62
7	4.13	18.0	4.96	21.6	0.83
8	4.76	21.7	6.35	28.8	0.75
9	4.25	18.5	5.60	24.3	0.76
10	4.15	17.3	4.88	20.3	0.85
Mean	4.27 ± 0.24	18.3 ± 2.0	5.55 ± 0.61	23.8 ± 3.5	0.77 ± 0.08

adenine and guanine nucleotides within the cell and suggests that these inhibitions may be causally related to the decreased rate of growth of ascites-tumour cells observed in the presence of 6-mercaptopurine.

It should be remembered, in all of these experiments, that only the total adenine and guanine nucleotides of the cell was measured. This was made up of various nucleotides and coenzymes and an effect of 6-mercaptopurine on the relative amounts of individual components would not have been detected.

The actual amount of acid-soluble adenine nucleotide isolated from untreated Ehrlich ascites-tumour cells (0.2 µmole /35 mg. dry weight of cells) is of the same order (0.5 µmole /35 mg. dry weight of cells) as that reported by LePage (1953); the latter value includes base, nucleoside and nucleotide fractions. Bennett et al. (1960) reported a ratio of acid-soluble purines to total nucleic acid purines of 1:9 which is similar to the ratio of 1:7 found here. The ratio of total nucleic acid adenine to nucleic acid guanine found by LePage (1953) was approximately 0.5, compared with a value of 0.79 found in these studies. This may indicate a greater amount of RNA than DNA in ascites-tumour cells as it has been shown for many tissues that the adenine/guanine ratio is less than one for RNA and greater than one for DNA (see Kit, 1960).

SECTION IV

FURTHER CHEMICAL AND BIOCHEMICAL EXPERIMENTS WITH DERIVATIVES OF 6-MERCAPTOPURINE

This section describes the synthesis and some of the properties of 6-thioinosine 5'-triphosphate and nicotinamide-6-mercaptopurine dinucleotide.

A. MATERIALS AND METHODS

1. Preparation of 6-thioinosine 5'-triphosphate.

The following synthesis of 6-thioinosine 5'-triphosphate is essentially the same as described by Roy, Kvam, Dahl & Parks (1961). A solution of triethylammonium thio-IMP (146 μmoles) was passed through a column (5 cm. x 1.5 cm.^{2}) of Amberlite IRC-20 (pyridinium salt) and washed with water (about 100 ml.) until the extinction of the effluent was less than 0.2 when measured against water in a cell with a 1 cm. light path. Pyridine (1 ml.) was added and the solution was freeze-dried. The resultant residue was rinsed into the reaction flask with a few ml. of water, evaporated to dryness at 30°, resuspended in a small volume of dry pyridine and again evaporated to dryness. After addition of 15 ml. of dry pyridine and 1.5 ml. of tri-n-butylamine, the tube was stoppered and shaken at 25° until all the thio-IMP had dissolved (about 2 hr.). Then 280 mg. of 85% phosphoric acid and 3.09 g. of N, N'-dicyclohexylcarbodiimide were added; the tube was well stoppered and placed in a plastic bag containing silica gel. After 48 hr. at 25° the solution was filtered through a sintered glass plate (No. 3 porosity) and the crystals of dicyclohexylurea were washed with water (2 x 10 ml.); the filtrate obtained was turbid and contained numerous oily droplets. Pyridine was removed by shaking with ether (3 x 50 ml.) and after extraction of the ether with water (2 x

10 ml.) the washings and the original aqueous solution were combined and evaporated at 30°. The residue was freeze-dried with further portions of water (2 x 10 ml.) and the final residue was dissolved in 250 ml. of water. The solution was passed through a column (12 cm. x 1.5 $cm.^2$) of ECTEOLA (formate form) at a rate of about 4 ml./min. and the ion-exchanger was washed with water until the extinction of the effluent was less than 0.05 when measured against water in a cell with a 0.5 cm. light path. Elution with a linear gradient, obtained by mixing 600 ml. of water and 600 ml. of 0.5 M-triethylammonium formate (pH 4.5), removed thio-IMP (0.21 - 0.26 M buffer), 6-thioinosine 5'-diphosphate (0.36 - 0.42 M buffer) and two unidentified compounds (0.29 - 0.35 and 0.42 - 0.5 M buffer) both of which had an absorption maximum near 290 mμ (these compounds had slightly greater anionic mobility than thio-IMP and 6-thioinosine 5'-diphosphate respectively on electrophoresis at pH 4.1 and were converted into compounds absorbing at 320 mμ on treatment with 0.5 mM-mercaptoethanol; it is likely that these reaction products are the disulphides of thio-IMP and 6-thioinosine 5'-diphosphate (see Section I). Elution with 300 ml. of 1 M-triethylammonium formate (pH 4.5) removed 6-thioinosine 5'-triphosphate, which was collected and concentrated by freeze-drying. The residue was dissolved in a small volume of water and chromatographed on Whatman 3MM paper in isobutyric acid - 0.19 N-NH_4OH (661:339, v/v). The major component absorbing light at 254 mμ was 6-thioinosine 5'-triphosphate (R_F 0.17) and this was eluted with 30 ml. of water and freeze-dried. The residue was freeze-

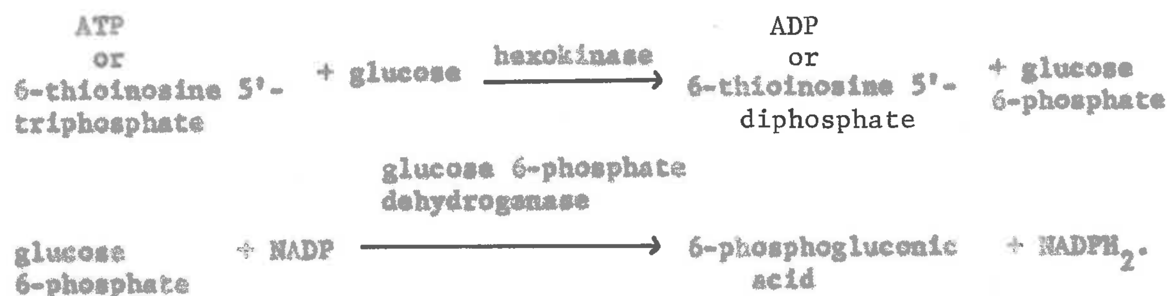
dried with further portions of water (3 x 30 ml.) and finally dissolved in 5 ml. of water (yield 20 μmoles).

2. Nicotinamide-6-mercaptopurine dinucleotide.

A solution of 30 mM-pyridinium thio-IMP (5 ml.) in pyridine was mixed with 7.9 ml. of 19 mM-NMN. The solution was concentrated to 0.8 ml. at 30° and rinsed into the reaction flask (final volume about 3.5 ml.). Dry pyridine (14 ml.) and 1.5 g. of N,N'-dicyclohexylcarbodiimide were added and the flask was attached to an end-over-end shaker at 25°. Further 1g. amounts of N,N'-dicyclohexylcarbodiimide were added to the flask at 24, 48, and 72 hr. After 96 hr. the reaction mixture was poured into 50 ml. of water and kept for 1 hr. at 20°. The mixture was extracted with chloroform (3 x 50 ml.) and briefly centrifuged to remove traces of insoluble material. After filtration and evaporation to dryness at 30° the residue was redissolved in 10 ml. of water and the solution was passed through a column (17 cm. x 2 cm.^{2}) of Dowex-1 (formate form). The washings contained unreacted NMN and thio-IMP (other compounds absorbing light at 260 mμ and at 320 mμ were also eluted, but were not characterised). Elution with a linear gradient obtained by mixing 1 l. of 0.5 M-formic acid and 1 l. of 4 M-formic acid, removed nicotinamide-6-mercaptopurine dinucleotide (1.0 - 1.35 M acid). After concentration by freeze-drying and electrophoresis in 30 mM-triethylamine - 30 mM-formic acid - 30 mM-acetic acid (pH 4.15), nicotinamide-6-mercaptopurine dinucleotide (migration 0.4 cm./v hr./cm.) was eluted with 20 ml. of water.

3. **Coupled assay of ATP and 6-thioinosine 5'-triphosphate with hexokinase and glucose 6-phosphate dehydrogenase**

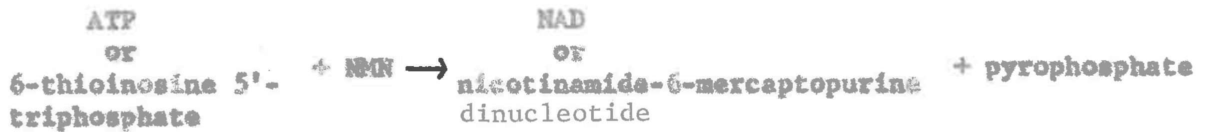
6-Thioinosine 5'-triphosphate was found to be a substrate of hexokinase, and one equivalent of $NADPH_2$ was formed in assays coupling the following reactions (cf. Kornberg, 1950) :



The commercial preparation of glucose 6-phosphate dehydrogenase used contained sufficient hexokinase activity to catalyse the first of these reactions. Assays contained 250 μmoles of glycylglycine buffer, pH 7.6, 100 μmoles of $MgCl_2$, 5 μmoles of glucose, 0.62 μmole of NADP, and 6-thioinosine 5'-triphosphate or ATP (0.02 - 0.2 μmole) in a final volume of 2.5 ml. Glucose 6-phosphate dehydrogenase (0.05 ml. of a solution containing 5 mg. of protein in 0.5 ml. of 0.25 M-glycylglycine buffer, pH 7.6) was added to the test and blank cells; the blank had no 6-thioinosine 5'-triphosphate. The extinction at 340 mμ due to $NADPH_2$ was recorded after equilibrium had been reached. Concentrations of the triphosphate were calculated using an extinction coefficient of 6.22×10^3 for reduced NADP (Horecker & Kornberg, 1948).

4. Assay of ATP:NMN adenylyltransferase in a coupled system with yeast alcohol dehydrogenase.

Assays were carried out by coupling the following reactions:



Assay systems contained 1.25 ml. of a solution containing 6 mM-$MgCl_2$, 0.6 M-ethanol and 0.2 M-tris (Cl^-, pH 8.0), 5 μmoles of NMN and the required concentration of ATP or 6-thioinosine 5'-triphosphate in a final volume of 2.5 ml. The extinction at 340 mμ was followed for 2 min. at 25° after the addition of yeast alcohol dehydrogenase (30 γ of protein), and the reaction was started with 0.02 ml. (about 0.25 mg. of protein) of adenylyltransferase (this enzyme was prepared by Dr. J.F. Jackson and contained approximately 0.4 units/mg. of protein; see Atkinson, Jackson & Morton, 1964). The extinction change at 340 mμ due to the reduction of NAD was recorded and concentrations calculated using an extinction coefficient of 6.22×10^3 for $NADH_2$ (Horecker & Kornberg, 1948). Initial velocities were expressed as μm-moles of NAD reduced/min. in this assay system.

5. Assay of NAD and nicotinamide-6-mercaptopurine dinucleotide

Reaction mixtures contained 200 μmoles of tris (Cl^-, pH 9.5), 600 μmoles of ethanol, 10 μmoles of $MgCl_2$, and either NAD or nicotinamide-6-mercaptopurine dinucleotide in a final volume of 5.5 ml. (assays were carried out in cuvettes with a 2 cm. light path). The reaction was started by the addition of 0.05 ml. of yeast alcohol dehydrogenase (see above) and the extinction was recorded at 340 mμ to obtain initial rates of reduction and the total extinction change after the reaction had gone to completion.

6. Preparation and assay of NAD-ase from ox spleen and Neurospora crassa.

Ox spleen NAD-ase was prepared as described by Zatman, Kaplan & Colowick (1953); the final preparation split 4.2 μmoles of NAD/min./mg. of protein. Assays contained 30 μmoles of phosphate (K^+, pH 7.2), 4 μmoles of NAD and enzyme in a final volume of 0.6 ml. Incubations were carried out at 37° and 0.1 ml. portions were removed at suitable time intervals, cooled rapidly to 1°, and assayed for NAD content as described before. NAD-ase from N. crassa grown on a zinc-deficient medium was purified by Dr. J.F. Jackson according to the method of Kaplan, Colowick & Mason (1951). When assayed as described above the preparation split 666 μmoles of NAD/min./mg. of protein.

7. Potato nucleotide pyrophosphatase

This enzyme was prepared as described by Kornberg & Pricer (1950). The final preparation split 82 μmoles of NAD/min./mg. of protein. Assays contained 2 μmoles of NAD, 100 μmoles of phosphate

(K^+, pH 7.0) and enzyme in a final volume of 1.0 ml. Incubations were carried out at 37° and portions (0.1 ml.) were removed at intervals and assayed for NAD content with alcohol dehydrogenase (see above). A unit of enzymic activity was defined as the amount causing the splitting of 1 µmole of NAD/min. under these conditions.

8. Other reagents.

Yeast alcohol dehydrogenase. A crystalline suspension was purchased from C. F. Boehringer und Soehne, Mannheim, Germany. Dilutions were made in 5 mM-phosphate (Na^+, pH 7.4).

ATP. This compound was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Nicotinamide mononucleotide. The nucleotide was prepared as described by Atkinson, Jackson & Morton (1961).

Glucose 6-phosphate dehydrogenase. The Type II enzyme from Sigma Chemical Co. was used.

Inorganic reagents. These were analytical reagent grade (British Drug Houses, Ltd.).

B. RESULTS AND DISCUSSION

1. Inhibition of adenylyltransferase by 6-thioinosine 5'-triphosphate

The 6-thioinosine 5'-triphosphate used in these studies was purified by paper chromatography (see the Materials and Methods section). This product was homogenous on chromatography and on electrophoresis in 30 mM-triethylamine - 30 mM-formic acid - 30 mM-acetic acid (pH 4.15); the R_F and anionic mobility were similar to those of inosine 5'-triphosphate. However, after storage for 3 days at -15° a second component was apparent on electrophoresis at pH 4.15 with a mobility which would be expected for 6-thioinosine 5'-diphosphate (by comparison of optical density units at 320 mμ, about 5% of the triphosphate may have been hydrolysed). The contaminant was eluted with water and did not inhibit adenylyltransferase; the compound corresponding to 6-thioinosine 5'-triphosphate was eluted from the same electrophoretogram and resulted in 50% inhibition of the transferase (see below). Concentrations of triphosphate solutions were calculated by assaying with hexokinase and glucose 6-phosphate dehydrogenase (see the Materials and Methods section).

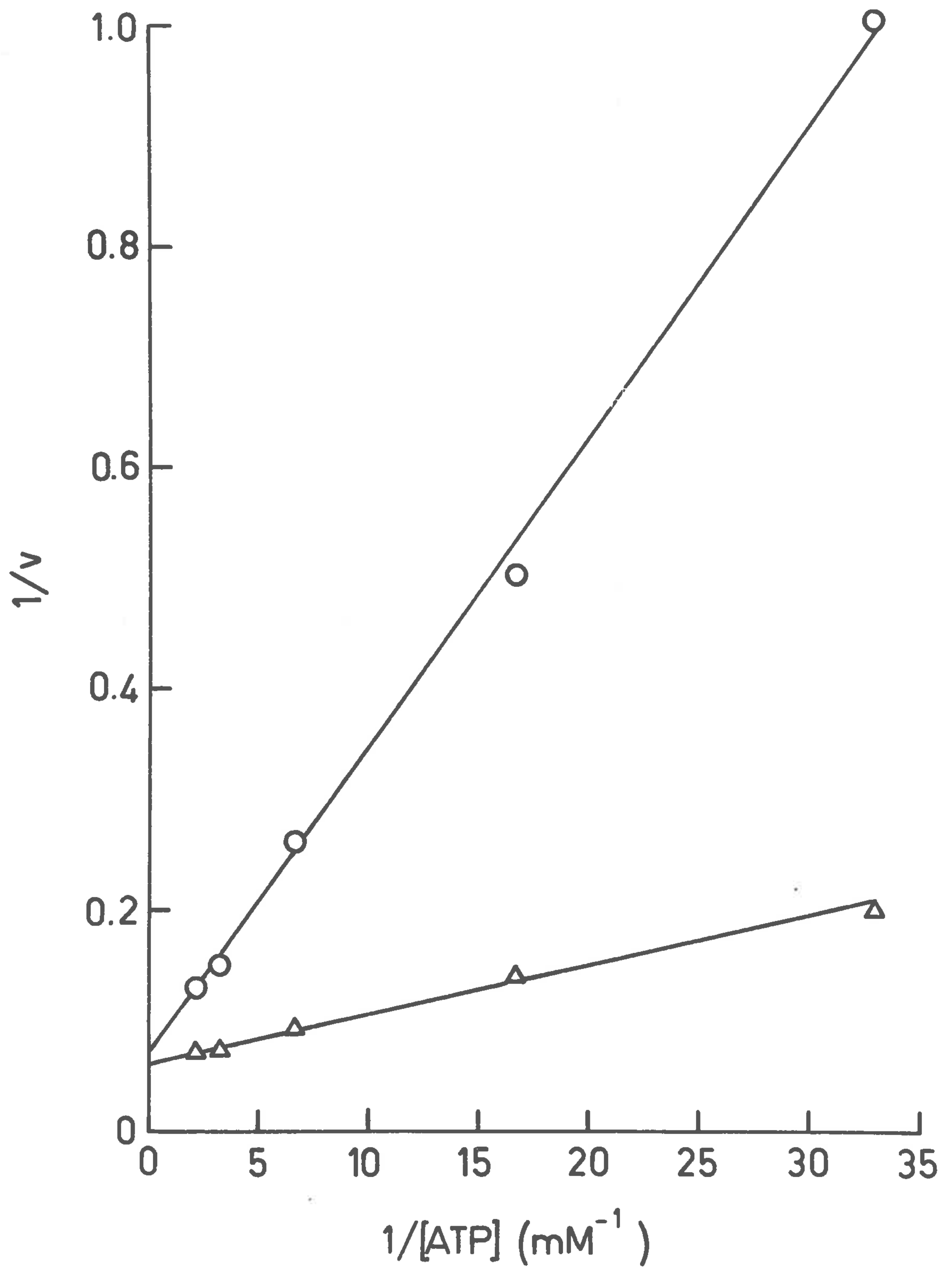
The spectrum of the triphosphate in 0.1 M-acetate (Na^+, pH 5.5) showed a maximum at 320 mμ and a small inflexion near 250 mμ. This inflexion was probably due to inosine 5'-triphosphate in the preparation and calculation indicated an upper limit of 0.10 molar proportion of this contaminant. The resultant concentration of inosine 5'-triphosphate

in inhibition studies (see below) could have been no more than 10 μM. As the minimum ATP concentration used was 30 μM and K_i for inosine 5'-triphosphate (2.1 mM) as a competitive inhibitor of ATP is about five times the K_m for ATP (0.4 mM), (Jackson, 1962), it is likely that the inhibition observed (see below) is almost entirely due to 6-thioinosine 5'-triphosphate.

ATP:NMN adenylyltransferase was assayed in the presence of an excess of yeast alcohol dehydrogenase and alcohol; NAD was reduced as it was formed (see the Materials and Methods section). With this assay system the K_m for ATP was found to be 74 ± 4 μM (see Fig. IV.1. and Table IV.1.). In the presence of 100 μM-6-thioinosine 5'-triphosphate, K_p was 444 ± 40 μM, and V_p did not differ significantly from V in the absence of inhibitor (see Fig. IV.1. and Table IV.1.). 6-Thioinosine 5'-triphosphate was therefore a competitive inhibitor of adenyl transfer to NMN by NMN adenylyltransferase, and the K_i was calculated to be 20 ± 0.36 μM. Using a non-coupled assay system, in which the NAD formed in the presence of the transferase was measured after stopping the reaction with acid, Jackson (1962) found a K_m of 0.4 mM for ATP; in the presence of an impure preparation containing 6-thioinosine 5'-triphosphate a K_i of 0.35 mM was obtained (based on total extinction at 320 mμ). The differences in these parameters compared with those found here may have been due to the different assay systems used; in addition Jackson (1962) assayed at 37°, and the assays here were carried out at 25°. In previous work with the coupled assay

Figure IV.1. Inhibition of ATP:NMN adenylyltransferase from liver nuclei by 6-thioinosine 5'-triphosphate.

Reactions were carried out with a coupled assay system (see the Materials and Methods section) in the presence (○) and absence (△) of 0.1 mM-6-thioinosine 5'-triphosphate.



1.0
0.8
0.6
0.4
0.2
0
1/v
0
5
10
15
20
25
30
35
1/[ATP] (mM^{-1})

Table IV.1. Kinetic parameters for ATP:NMN adenylyltransferase in the presence and absence of 6-thioinosine 5'-triphosphate

v (μm-moles NAD reduced/min. /0.02 ml. enzyme)	v_p* (μm-moles NAD reduced /min./0.02 ml. enzyme)	ATP concentration (μM)
5.0	0.80	30
7.0	2.00	60
10.2	3.80	150
13.7	6.67	300
14.3	7.70	450

* Measured in the presence of 100 μM-6-thioinosine 5'-triphosphate

K_m (ATP)	74 μM
K_p (6-thioinosine 5'-triphosphate + ATP)	444 μM
K_i (6-thioinosine 5'-triphosphate)	20 μM

a Michaelis constant of 71 μM-ATP had been found by Dr. M.R. Atkinson.

It has been shown (Jedeikin & Weinhouse, 1955; Glock & McLean, 1957) that tumour cells have a low content of NAD as compared with normal tissues. In addition the adenylyltransferase activity/ nucleus of spontaneous mammary gland carcinoma was only about 20% of that of normal lactating mammary gland (Branster & Morton, 1956). Activity of this enzyme was also low in Ehrlich ascites-tumour cells (Branster, M.J. & Morton, R.K. cited by Morton, 1958). On the basis of these findings, Morton (1958) proposed that selective inhibition of tumour growth might be obtained by specific inhibition of NMN adenylyltransferase. It is clear that 6-thioinosine 5'-triphosphate is a powerful competitive inhibitor of the transferase (see above). However, the formation of 6-thioinosine 5'-triphosphate from 6-mercaptopurine could not be detected in ascites-tumour cells (see section II), and it is unlikely that this inhibition would have any physiological significance in these cells.

2. <u>The reaction of 6-thioinosine 5'-triphosphate with adenylyltransferase</u>

6-Thioinosine 5'-triphosphate was found to react with adenylyltransferase from pig liver nuclei:

6-thioinosine 5'-triphosphate + NMN ⟶ nicotinamide-6-mercaptopurine dinucleotide + pyrophosphate

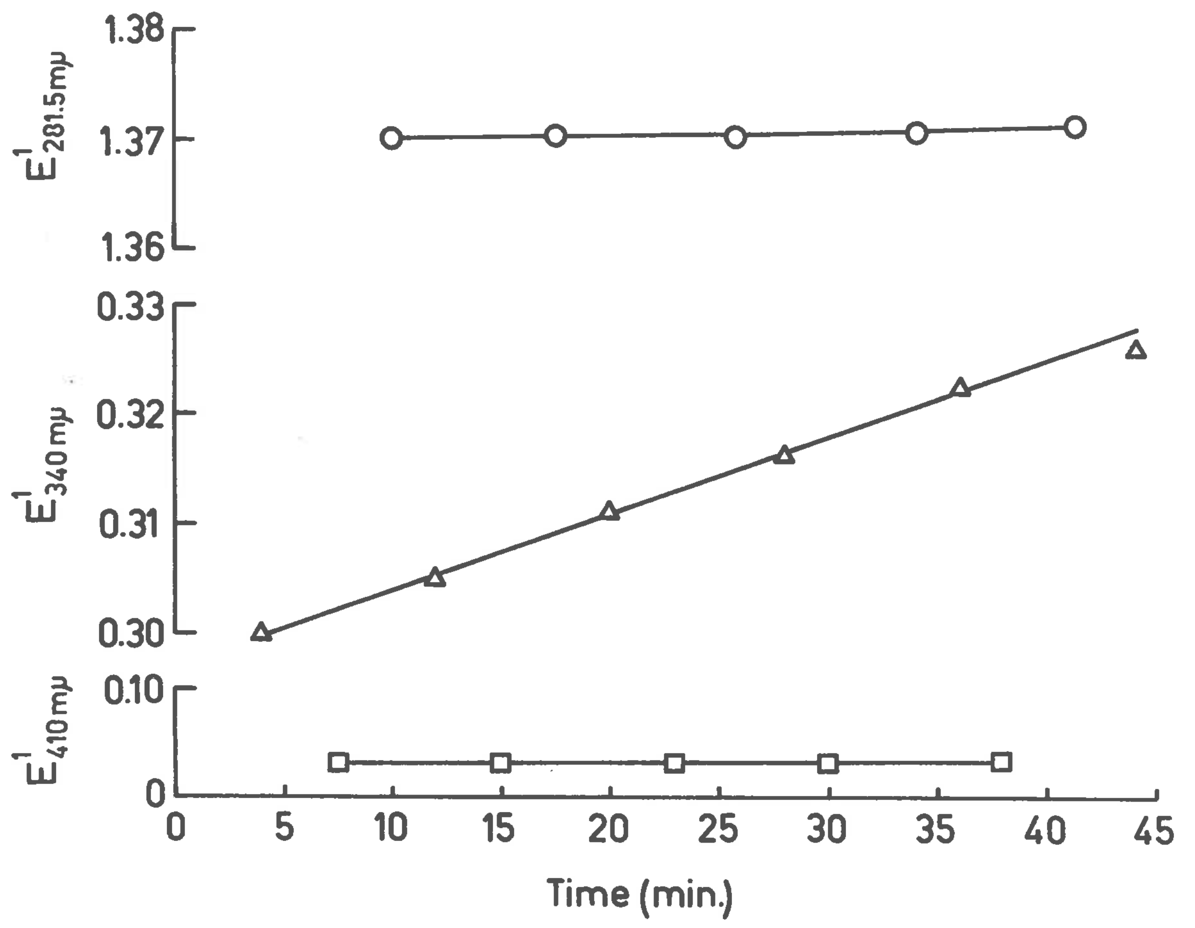
This reaction, which proceeded at only a slow rate, could be followed spectrophotometrically at 340 mμ by adding ethanol and yeast alcohol dehydrogenase to reduce nicotinamide-6-mercaptopurine dinucleotide as it

was formed (see the Materials and Methods section). Such an assay is shown in Fig. IV.2.; the reaction was also followed at 410 mμ to detect changes in turbidity and at 281.5 mμ which is the isosbestic wavelength for the reduction of nicotinamide-6-mercaptopurine dinucleotide (see below). While the extinction at 340 mμ increased continuously there was no increase in turbidity during the reaction and no change in extinction at the isosbestic wavelength. To isolate the products of the reaction, transferase was mixed with 0.2 μmole of NMN, 10 μmoles of tris (Cl^-, pH 8.0), 0.5 μmole of $MgCl_2$, and 0.25 μmole of 6-thioinosine 5'-triphosphate in a final volume of 0.28 ml. The test solution and a blank without added NMN were incubated at 37° for 3 hr. and 0.1 ml. samples were subjected to electrophoresis at pH 4.15 (see above). In the presence of both NMN and 6-thioinosine 5'-triphosphate a compound was formed having the same mobility at this pH as synthetic nicotinamide-6-mercaptopurine dinucleotide (see the Materials and Methods section). Both 6-thioinosine 5'-triphosphate and the new compound gave a positive test for reducing sulphur compounds (Toennies & Kolb, 1951). The reaction with the 6-mercaptopurine analogue was slower than that with ATP and faster than the reaction with ITP. Thus at pH 8.0 the relative rates were : with 0.4 mM-ATP, 100 units, with 0.4 mM-6-thioinosine 5'-triphosphate, 7 units, and with 0.4 mM-ITP, less than 3 units.

In an attempt to show the reverse reaction, 1 μmole of sodium pyrophosphate and 0.09 μmole of nicotinamide-6-mercaptopurine dinucleotide were incubated with 20 μmoles of tris (Cl^-, pH 7.5),

Fig. IV.2. The conversion of 6-thioinosine 5'-triphosphate and NMN into nicotinamide-6-mercaptopurine dinucleotide in the presence of adenylyltransferase from liver nuclei.

See the text for details of the assay system. The assay system without transferase was allowed to react for 4 min. Adenylyltransferase was then added as indicated to start the reaction. The reduction of nicotinamide-6-mercaptopurine dinucleotide by alcohol dehydrogenase was followed at 340 mμ. The reaction was also followed at 410 mμ to detect changes in turbidity and at 281.5 mμ (the isosbestic wavelength for the reduction of nicotinamide-6-mercaptopurine dinucleotide).



$E^1_{281.5 m\mu}$
1.38
1.37
1.36
$E^1_{340 m\mu}$
0.33
0.32
0.31
0.30
$E^1_{410 m\mu}$
0.10
0
0
5
10
15
20
25
30
35
40
45
Time (min.)

0.5 µmole of $MgCl_2$, and adenylyltransferase in a final volume of 0.5 ml. Electrophoresis at pH 4.15 after incubation for 3 hr. at 37° did not separate material with a mobility corresponding to 6-thioinosine 5'-triphosphate. No reaction could be detected spectrophotometrically when it was attempted to measure 6-thioinosine 5'-triphosphate as it was formed by coupling with hexokinase and glucose 6-phosphate dehydrogenase (see the Materials and Methods section).

3. Properties of nicotinamide-6-mercaptopurine dinucleotide

(a) Reaction with yeast alcohol dehydrogenase

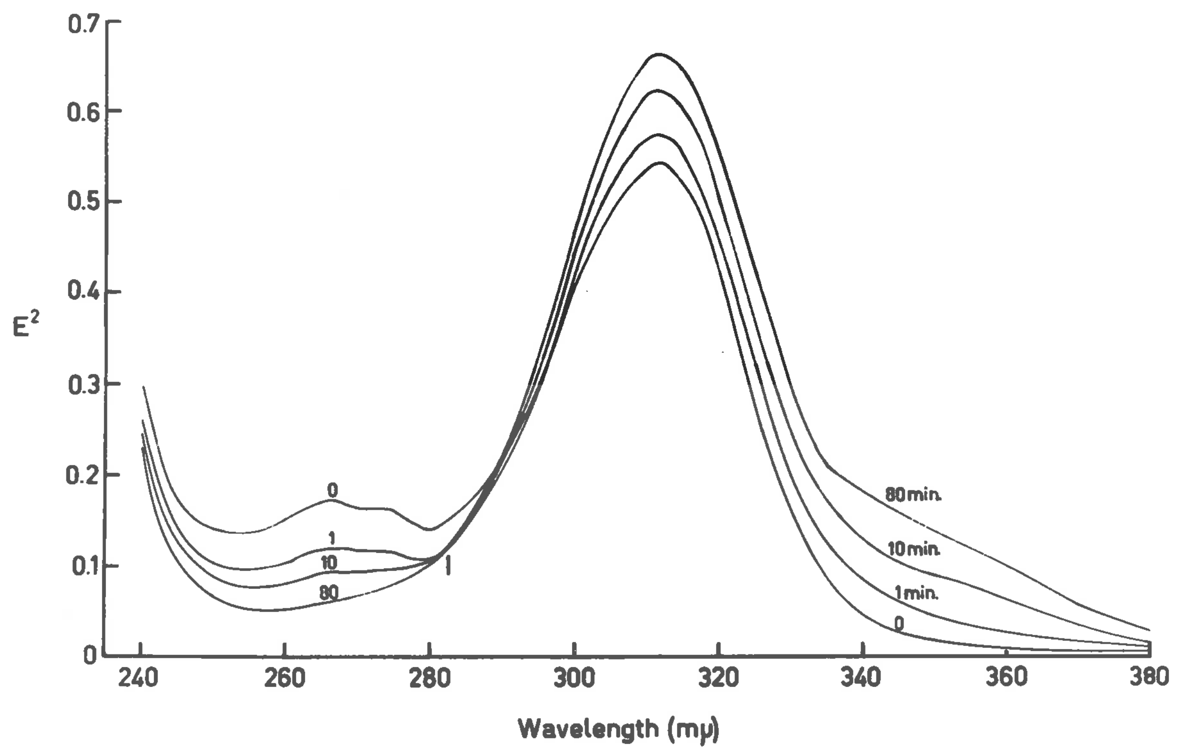
Synthetic nicotinamide-6-mercaptopurine dinucleotide was reduced enzymically with yeast alcohol dehydrogenase at pH 9.5 (see the Materials and Methods section), and was routinely assayed by this method. The initial rate of reduction in assays containing 9 µM-nicotinamide-6-mercaptopurine dinucleotide was one-fifth of the initial rate with the same concentration of NAD; concentrations of nucleotide were, however, well below those needed to obtain maximal velocities. Although insufficient material was available for accurate kinetic studies, preliminary experiments indicated a K_m of approximately 10 µM for the dinucleotide analogue compared with a value of 200 µM found for NAD under these conditions. However, the extrapolated maximal velocity with NAD was about forty times that found with nicotinamide-6-mercaptopurine dinucleotide. Anderson, Anderson, Lee & Stein (1963) studied the reduction of various thionicotinamide analogues of NAD and NADP by several dehydrogenases, and showed that K_m values for the

analogues were usually of the same order or less than the corresponding K_m value for NAD. Conversely the extrapolated maximum velocities for NAD were usually much greater than the values found for the corresponding analogues.

The spectrum of the oxidized form of nicotinamide-6-mercaptopurine dinucleotide at pH 9.5 is shown in Fig. IV.3. (at zero time). The reduction of 0.036 μmole of nicotinamide-6-mercaptopurine dinucleotide by alcohol dehydrogenase at pH 9.5 (for the conditions of assay see the Materials and Methods section) at various times after the addition of dehydrogenase is also shown in Fig. IV.3. The time given for each spectral curve represents the time the recorded spectrum was begun; the scanning time for each spectrum was 2 min. and hence the curves can only be used for qualitative comparison. However, after allowance for the volume of added dehydrogenase, total extinction changes could be obtained from the curve at zero time and the curve obtained at equilibrium (80 min. after the addition of enzyme). Phosphate analyses carried out on the same sample of dinucleotide allowed calculation of the molecular extinction coefficients both before and after the addition of alcohol dehydrogenase; the values obtained are listed in Table IV.2. The maximum extinction of the reduced form was at 311 mμ (ϵ, 27.8×10^3); the increase in extinction at 340 mμ ($\Delta\epsilon$, 5.9×10^3) on reduction of the oxidized form was the same as that reported for a commercial sample of NAD (Pabst Laboratories, 1961a). The spectrum shows clearly the 6-mercaptopurine portion of the molecule ($\epsilon_{max.}$ 311 mμ at pH 9.5) and the NMN portion ($\epsilon_{max.}$ 266 mμ).

Fig. IV.3. The reduction of nicotinamide-6-mercaptopurine dinucleotide with ethanol and yeast alcohol dehydrogenase.

For details of the assay system see the Materials and Methods section. The curve at zero-time was obtained before the addition of alcohol dehydrogenase, and the final curve (80 min.) after the reaction had come to equilibrium. The isosbestic wavelength (I) for the reduction of nicotinamide-6-mercaptopurine dinucleotide under these conditions was 281.5 mμ.



0.7
0.6
0.5
0.4
E²
0.3
0.2
0.1
0
240
260
280
300
320
340
360
380
Wavelength (mμ)
0
1
10
80
1
80 min.
10 min.
1 min.
0

It is of interest that the decrease in molecular extinction coefficient at 266.5 mμ ($\Delta\epsilon$, 4.5×10^3) on reduction with alcohol dehydrogenase is similar to the molecular extinction coefficient of NMN (4.6×10^3 at pH 7.0) at 265.5 mμ (Atkinson et al., 1961). Pfleiderer, Woenckhaus & Scholz (1963) have also described the synthesis of nicotinamide-6-mercaptopurine dinucleotide and have confirmed the observation (Atkinson et al., 1962) that this compound is reduced by ethanol and alcohol dehydrogenase from yeast.

(b) Reaction with nucleotide pyrophosphatase from potato

After incubation of 1 μmole of nicotinamide-6-mercaptopurine dinucleotide with 0.03 units of pyrophosphatase preparation (see the Materials and Methods section) for 30 min., 0.23 μmole of the dinucleotide remained, indicating considerable formation of AMP and thio-IMP under these conditions. After a further 30 min. incubation no nicotinamide-6-mercaptopurine dinucleotide could be detected in the assay mixture, and chromatography in isobutyric acid - 0.19 N-NH_4OH (661:339, v/v) revealed two components that absorbed light at 254 mμ. One had the same mobility as NMN (R_F 0.52), and reacted with methyl ethyl ketone and ammonia to give a fluorescent product. The second component had the same mobility as nicotinamide-6-mercaptopurine dinucleotide and thio-IMP (R_F 0.35), but did not react with methyl ethyl ketone and ammonia. Methyl ethyl ketone and ammonia treatment results in the formation of bluish-white fluorescent substances with quaternary pyridine compounds with a side-chain CO-NH-R in the β position

Table IV.2. Extinction coefficients for the oxidised and reduced forms of nicotinamide-6-mercaptopurine dinucleotide

In 0.1 M-acetate (Na^+, pH 5.5) (oxidised)		In 0.3 M-ethanol, 1.8 mM-$MgCl_2$, 3.6 mM-glycine (Na^+, pH 9.5)		Addition of dehydrogenase (at equilibrium)	
Wavelength (mμ)	ε ($\times 10^{-3}$)	Wavelength (mμ)	ε ($\times 10^{-3}$)	Wavelength (mμ)	ε ($\times 10^{-3}$)
321 (max.)	23.4	311 (max.)	22.4	311 (max.)	27.8
283 (min.)	6.6	280 (min.)	5.8	257.5 (min.)	2.4
272.5 (max.)	8.3	271.5 (inflex)	6.9	266.5	2.6
270.5 (max.)	8.2	266.5 (max.)	7.2	340	8.0
266.5 (max.)	8.6	254	5.6	340 (change)	5.9
253 (min.)	6.5	340	2.1		
340	5.5				

(Kodieck & Reddi, 1951). This tentatively identifies the two components as NMN and thio-IMP respectively.

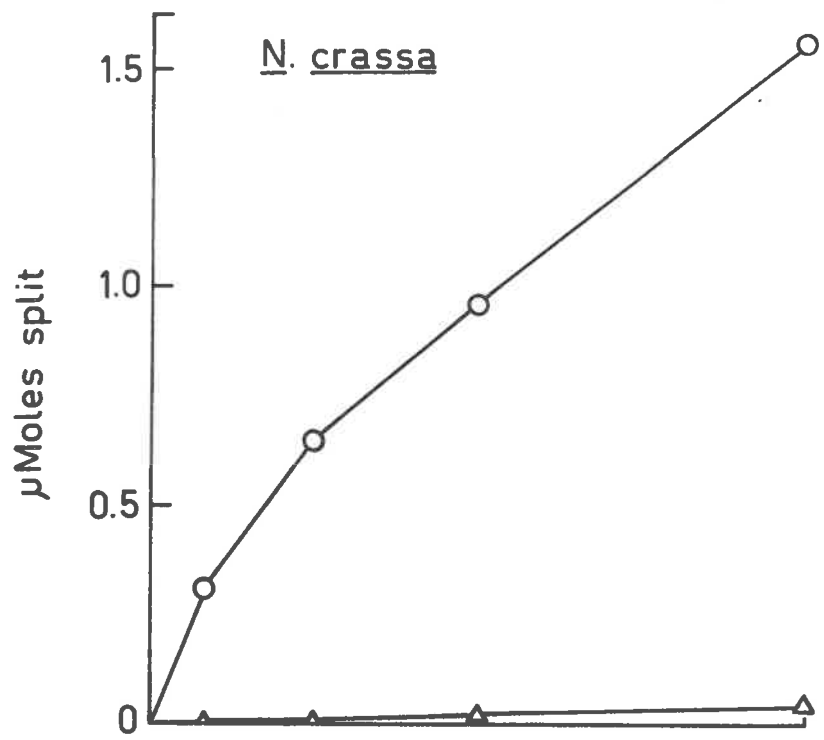
(c) Reaction with NAD-ase

Assays were carried out with 4 μmoles of NAD or 3 μmoles of nicotinamide-6-mercaptopurine dinucleotide as described in the Materials and Methods section (see Fig. IV.4.). The analogue reacted at approximately the same rate as NAD with NAD-ase from ox spleen but reacted at only a very slow rate with NAD-ase from N. crassa. In one assay 0.5 μmole of nicotinamide-6-mercaptopurine dinucleotide was incubated with NAD-ase from ox spleen and was allowed to react until no dinucleotide could be detected in the reaction mixture. The solution was passed through a column (6 cm. x 1.2 cm.^{2}) of ECTEOLA (formate form) and washed with water and with 0.1 M-triethylamine - 0.1 M-formic acid - 0.1 M-acetic acid. Fractions (5 ml.) were collected, and the extinction was read at 260 mμ and 320 mμ in a cuvette with a 1 cm. light path (the elution pattern obtained is shown in Fig. IV.5.). The first compound eluted from the column had the same mobility as nicotinamide (R_F 0.68) on chromatography in butan-1-ol - acetic acid - water (20:3:1, by vol.), gave a positive test with cyanogen bromide and paraminobenzoic acid (Kodicek & Reddi, 1951), and had an absorbtion maximum at 261.5 mμ at pH 4.0 (see Jellinek & Wayne, 1951); these tests indicate that this component was free nicotinamide. The second component absorbed light at 321 mμ and had R_F 0.18 in the butan-ol-acetic acid - water solvent described above (nicotinamide-6-

Fig. IV.4. A comparison of NAD and nicotinamide-6-mercaptopurine dinucleotide as substrates of NAD-ase from *N. crassa* and from ox spleen.

○ NAD

△ nicotinamide-6-mercaptopurine dinucleotide


N. crassa
µMoles split
1.5
1.0
0.5
0

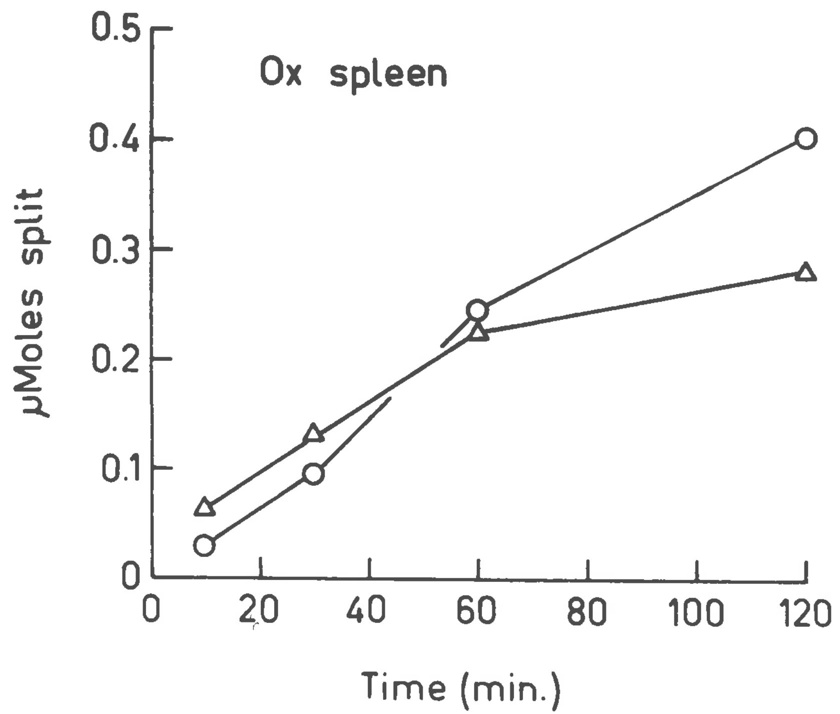
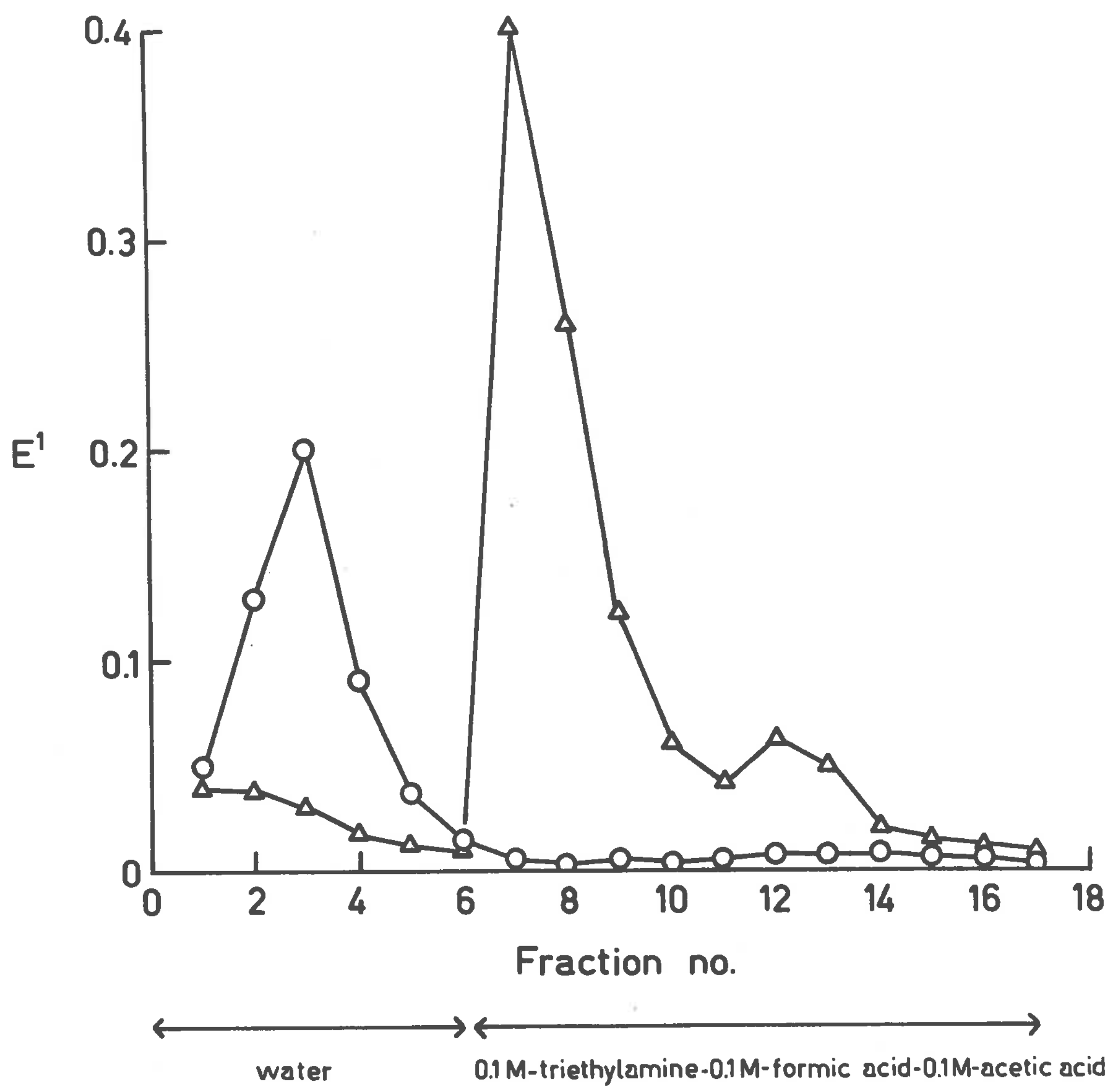

Ox spleen
µMoles split
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
Time (min.)

Fig. IV.5. Separation of the products of the action of ox spleen NAD-ase on nicotinamide-6-mercaptopurine dinucleotide.

An assay system containing NAD-ase and nicotinamide-6-mercaptopurine dinucleotide was allowed to react until all the dinucleotide had been split (see the text), and the reaction mixture was then passed through a column of ECTEOLA-formate. Elution was carried out as described in the figure. Fractions (5 ml.) were collected and their extinction read at 260 mμ (○) and at 320 mμ (△).


0.4
0.3
E[1]
0.2
0.1
0
0
2
4
6
8
10
12
14
16
18
Fraction no.
water
0.1 M-triethylamine-0.1 M-formic acid-0.1 M-acetic acid

mercaptopurine dinucleotide does not move from the origin in this solvent). This component gave a positive test for reducing sulphur compounds (Toennies & Kolb, 1951) and did not react with methyl ethyl ketone and ammonia. It is likely that the two components are nicotinamide and 6-mercaptopurine 5'-diphosphate ribose respectively, formed by the action of ox spleen NAD-ase. No attempt was made to identify the third component eluted from the column after 6-mercaptopurine 5'-diphosphate ribose (see Fig. IV.5.).

C. CONCLUSIONS

Apart from reactions involving oxidation of the sulphur atom, the chemical and physical properties of the derivatives of 6-mercaptopurine described here closely resemble those of the corresponding derivatives of hypoxanthine; their interactions with enzymes are also very similar. Thus 6-thioinosine 5'-triphosphate and ITP are substrates of hexokinase and of ATP:NMN adenylyltransferase. Both nicotinamide-hypoxanthine dinucleotide and nicotinamide-6-mercaptopurine dinucleotide are reduced by ethanol and alcohol dehydrogenase from yeast.

The conversion of nicotinamide-6-mercaptopurine dinucleotide into nicotinamide and 6-thioinosine 5'-diphosphate ribose by spleen NAD-ase and into NMN and thio-IMP by nucleotide pyrophosphatase from potato are further evidence that the presence of the 6-thio group on

the purine ring does not prevent enzymic modification of other parts of the molecule.

The greater reactivity of the 6-thio than the 6-oxo or 6-amino group on purine derivatives can lead to further reactions; the oxidation to disulphides (cf. section I) is an example of this. Hampton (1963) has described an irreversible inhibition of a bacterial IMP dehydrogenase by thio-IMP; this reaction may involve a covalent linkage of the protein to the purine and these thioinosine nucleotides offer the possibility of labelling the active centres of a wide range of enzymes. The failure to observe pyrophosphorolysis of nicotinamide-6-mercaptopurine dinucleotide with the adenylyltransferase from liver nuclei may be due to an inhibition of this kind.

The synthesis of nicotinamide-6-mercaptopurine dinucleotide reported here shows that condensation of thio-IMP in reactions involving carbodiimides offers no technical difficulties. It follows that the great range of syntheses of polynucleotides, phosphodiesters, cyclic phosphates, and other derivatives of nucleotides that Khorana and his co-workers have carried out with carbodiimdies (Khorana, 1961) could be applied to derivatives of thioinosine. Such compounds may be useful antimetabolites, as discussed in the following section.

SECTION V

GENERAL CONCLUSIONS

The results of other workers and those presented in this thesis indicate that 6-mercaptopurine can inhibit the following regions of purine metabolism.

(1) Free 6-mercaptopurine inhibits the conversion of guanine into GMP and of hypoxanthine into IMP by guanine and hypoxanthine phosphoribosyltransferases.

(2) 6-Mercaptopurine is converted into its 5'-phosphoribosyl derivative (6-thioinosine 5'-phosphate) which inhibits the conversion of IMP into AMP and of IMP into XMP.

(3) 6-Mercaptopurine inhibits the synthesis of purine nucleotides from non-cyclic precursors (probably as 6-thioinosine 5'-phosphate).

Reference to the third site of inhibition has been largely made in the Introduction, but two recent reports are of particular interest. Bennett _et al_. (1963) showed that 6-mercaptopurine inhibited the incorporation of formate and glycine into the purines of Ehrlich ascites-tumour cells. Under the same conditions incorporation of 5-amino-

4-imidazole carboxamide was only slightly inhibited, suggesting a block early in purine biosynthesis. More recently Hakala & Nichol (1964) found that a line of Sarcoma-180 cells in culture, dependent on de novo purine biosynthesis for growth, was inhibited by very low concentrations of 6-mercaptopurine. The inhibition was reversed by 5-amino-4-imidazole carboxamide. Higher concentrations of 6-mercaptopurine were required for inhibition when growth was dependent on preformed purines. Thus inhibition of growth could be obtained when the cells were dependent on either purine synthesis from non-cyclic precursors or on utilization of preformed purines, indicating more than one site of 6-mercaptopurine action.

It would seem, then, that the relative importance of reactions inhibited by 6-mercaptopurine depends on the balance between de novo purine biosynthesis and utilization of free purines, in a particular cell type. In the case of some bacteria and mammalian cells in culture, where conditions can be arranged so that cells have no supply of preformed purines, inhibition of growth may be largely due to inhibition of de novo biosynthesis. However, the situation is more complex in Ehrlich ascites-tumour cells (and probably other tumour cells) where the cells are parasitic and derive their nutrients from the host. The relatively high levels of purine phosphoribosyltransferases in ascites-tumour cells would enable them to rapidly metabolise purines derived from the host, into purine nucleotides. That this may, in fact, be the case was suggested by the results of experiments described in section III of this thesis. The potential ability of the body to

make preformed purines available to tissues is suggested by the observation that bone marrow can not synthesize purines efficiently from non-cyclic precursors, and may depend on a supply of preformed purines (Smellie et al., 1956).

The experiments described here showed that 6-mercaptopurine is a powerful competitive inhibitor of guanine and hypoxanthine phosphoribosyltransferases from Ehrlich ascites-tumour cells, and this inhibition may significantly contribute to the inhibition of growth by 6-mercaptopurine in these cells. Similarly this inhibition would probably largely account for the observed toxic effects of 6-mercaptopurine on bone marrow. Any contribution made to growth by free adenine would be unaffected, as adenine phosphoribosyltransferase is not inhibited by 6-mercaptopurine. Hakala & Nichol (1964) observed that very high concentrations of 6-mercaptopurine were needed to inhibit Sarcoma-180 cells in culture when growth was supported by adenine. This suggests that combination therapy with analogues that inhibit adenine phosphoribosyltransferase and with 6-mercaptopurine would more completely inhibit any contribution made to growth by preformed purines.

It is difficult to comment on the possible importance of inhibition of IMP dehydrogenase, adenylosuccinate synthetase and adenylosuccinate lyase by 6-thioinosine 5'-phosphate. Although the dehydrogenase was most strongly inhibited, levels of IMP were always low in the ascites-tumour cells, and it is likely that all three enzymes

would be significantly inhibited. The relative ineffectiveness of 6-mercaptopurine as an inhibitor of incorporation of 5-amino-4-imidazole carboxamide (Bennett *et al*., 1963; Hakala & Michel, 1964) is therefore difficult to understand. The possible existence of separate 'compartments' within the cell, separating purines synthesised by different routes, and of interconversions of purines at the nucleoside or free base level must be considered.

The results with cell-free extracts may be misleading in that no account is taken of the particulate fraction of the cell. Thus Paterson & Hori (1963) showed that the particulate fraction of Ehrlich ascites-tumour cells contained an active 5'-nucleotidase which cleaved 6-thioinosine 5'-phosphate, presumably to 6-thioinosine. Cell-free extracts rapidly converted 6-thioinosine into 6-mercaptopurine (Paterson, 1960b). 6-Thioinosine 5'-phosphate reached a maximum concentration in ascites-tumour cells about 30 min. after injection of 6-mercaptopurine, and the concentration decreased rapidly after this time (Paterson, 1959); the decrease may be due to the above reactions. Removal of sulphur from 6-mercaptopurine or 6-thioinosine 5'-phosphate does not appear to occur to any extent in ascites-tumour cells as no radioactivity could be detected in adenine or guanine nucleotides after injection of [8-^{14}C]6-mercaptopurine (Paterson, 1959; this thesis p.109). It is possible that 6-thioinosine 5'-phosphate itself has relatively little inhibitory effect on these cells, either because of the existence of alternative pathways for purine nucleotide

interconversions or because of its rapid destruction, or a combination of these effects. In this case 6-thioinosine 5'-phosphate may merely serve to provide a further supply of free 6-mercaptopurine which could continue to inhibit purine phosphoribosyltransferase activity when all the 6-mercaptopurine originally injected had been metabolised.

A method widely proposed to overcome resistance to 6-mercaptopurine due to decreased synthesis of 6-thioinosine 5'-phosphate, is introduction of the nucleotide molecule itself into the cell. The negative charges on the phosphate of nucleotides usually prevents movement across membranes and nucleotides probably do not enter cells as such, but are first cleaved to nucleosides or free bases (Roll et al., 1956; Weinfeld et al., 1957). If the negative charge on the molecule could be decreased (as in 3', 5'-cyclic nucleotides) or the molecule made more soluble in lipid (e.g. in nucleotide mono- or diesters as suggested by Montgomery et al., 1961; Thomas & Montgomery, 1962), such modified nucleotides may be able to enter cells. Simple esters of 6-thioinosine 5'-phosphate have shown little advantage in growth inhibition tests with resistant cells (Kelley, G.G., cited by Montgomery et al., 1963), but Montgomery et al. (1963) reported that bis(thioinosine) 5', 5"-phosphate inhibited cultures of human epidermoid cacinoma cells that were resistant to 6-mercaptopurine. It was proposed that the compound entered the cells and was then cleaved to 6-thioinosine 5'-phosphate, which inhibited growth. Similar considerations led to the synthesis of nicotinamide-6-mercaptopurine dinucleotide (see Section IV;

Atkinson <u>et al.</u>, 1962). Nucleotide pyrophosphatase from potato acted on this compound to give 6-thioinosine 5'-phosphate and nicotinamide mononucleotide; similar pyrophosphatases (phosphodiesterases) are widely distributed in animal tissues.

In the Ehrlich ascites-tumour cells studied here resistance is not due to a loss of the phosphoribosyltransferase converting 6-mercaptopurine into 6-thioinosine 5'-phosphate. This was also shown to be the case with leukaemic cells from patients resistant to 6-mercaptopurine (Davidson & Winter, 1964). In addition it follows from the results in this thesis that free 6-mercaptopurine may be of importance in inhibition of the growth of tumour cells <u>in vivo</u>. Consequently attention should be given to the synthesis of analogues of 6-mercaptopurine that could enter resistant cells and then be broken down to free 6-mercaptopurine. If such analogues were only slowly converted into 6-mercaptopurine, either spontaneously or by enzymic reactions, a constant level of the drug may be maintained for longer periods within the cell. This could be of particular importance if the analogues were not acted on by the oxidative enzymes which metabolise 6-mercaptopurine to 6-thiouric acid. Such analogues may also be superior to 6-mercaptopurine in the treatment of tumours sensitive to this drug and in the selective inhibition of immunological cells that lead to the rejection of grafted tissues and organs.

From information available at present it seems most desirable to extend the use of 'combination chemotherapy' so that both free 6-mercaptopurine and 6-thioinosine 5'-phosphate are delivered inside the target cells, preferably in combination with antimetabolites of the adenine nucleotides.

APPENDIX

This section gives the programme used in the calculation of the kinetic parameters given in this thesis. The programme determines the fit of the Michaelis-Menten hyperbola to the observed reaction rates by iterative application of linear regression formulae, as described by Wilkinson (1961). The programme gives estimates of V and K_m with their associated standard errors and provides for the calculation of combined estimates of experimental variability over series of experiments. The programme is written in Fortran Language (University of Adelaide, AFIT system).

```
      FIT OF HYPERBOLA.  WILKINSON, BIOCHEM.J. (1961) 80, 324, W.I. 35
      DIMENSIONS(50), V(50), DH(50), H(50), SVM(20) SKM(20)
   50 PRSS = 0
      PDF = 0
      READ1
    1 FORMAT (49H
      PUNCH1
   40 READ,M,L,N,K
      DF=N-2
      A=0
      B=0
      G=0
      D=0
      E=0
      DO10I=1,N
      READ,V(I),S(I)
      X=V(I)**2
      Y=X/S(I)
      A=A+V(I)*X
      B=B+X**2
      G=G+V(I)*Y
      D=D+X*Y
   10 E=E+Y**2
      JJ=0
      DE=A*E-G*D
      EKM=(B*G-A*D)/DE
      EVM=(B*E-D**2)/DE
   60 RSS=0
      JJ=JJ+1
      DO20I=1,N
      SAD=S(I)+EKM
      H(I)=EVM*S(I)/SAD
      DH(I)=-H(I)/SAD
   20 RSS=RSS+(V(I)-H(I))**2
      IF(JJ-2)4,4,5
    4 IF(JJ-10)9,9,6
    9 A=0
      B=0
      G=0
      D=0
      E=0
      DO30I=1,N
      A=A+H(I)**2
      B=B+DH(I)**2
      G=G+H(I)*DH(I)
      D=D+V(I)*H(I)
   30 E=E+V(I)*DH(I)
      DE=A*B-G**2
```

```
      BI=(B*D-G*E)
      EVM=BI*EVM/DE
      EKM=EKM+(A*E-G*D)/BI
      SVM(L)=EVM*SQRTF(B/DE)
      WVM=1.0/SVM(L)**2
      SKM(L)=(SQRTF(A*DE))/BI
      WKM=1.0/SKM(L)**2
      RSSP=RSS
      GOTO60
    5 IF(RSS)2,6,2
    2 IF((RSSP-RSS)/RSS-1.E-6)6,6,4
    6 SD=SQRTF(RSS/DF)
      SEKM=SKM(L)*SD
      SEVM=SVM(L)*SD
      CVK=G/SQRTF(A*B)
      RVK=EVM/EKM
      CVVM=SEVM/EVM
      CVKM=SEKM/EKM
      SEVK=RVK*SQRTF(CVVM**2+CVKM**2-2.0*CVK*CVVM*CVKM)
      CVVK=SEVK/RVK
      WVK=SD**2/SEVK**2
      PUNCH99,JJ
      PUNCH90,EKM,SEKM,WKM,CVKM,EVM,SEVM,WVM,CVVM,RVK,SEVK,WVK,CVVK
      PUNCH96
      DO7I=1,N
      R=V(I)-H(I)
    7 PUNCH95, H(I),R
      IDF=DF
      PUNCH84,IDF,RSS,SD,CVK
      PRSS=PRSS+RSS
      PDF=PDF+DF
      IF(L-M)40,70,70
   70 PMS=PRSS/PDF
      PSD=SQRTF(PMS)
      IPDF=PDF
      IF(K)50,50,25
   25 PUNCH86
      PUNCH85,IPDF,PRSS,PMS,PSD
      PUNCH81
      DO8J=1,M
      SEKM=SKM(J)*PSD
      SEVM=SVM(J)*PSD
    8 PUNCH85,J,SEKM,SEVM
   81 FORMAT(5H   SET4x4HSEKM10x4HSEVM)
   84 FORMAT(6H D.F.=14,7H,RSS=E14.8, 8H, S.D. =E14.8,8H R(V,K)=E14.8)
   85 FORMAT(15,4E14.8)
   86 FORMAT(42HD.F.,POOLED R.S.S., POOLED M.S., POOLED S.D.)
   90 FORMAT(3X2HKM2F14.6,2E14.8/3X2HVM2F14.6,2E14.8/5HVM/KM2F14.6,2E14.8)
   95 FORMAT(2F18.6)
   96 FORMAT(/19H FITTED VALUES OF V)
   99 FORMAT(/7X8HESTIMATE8x4HS.D.8X6HWEIGHT11X4HC.V.I8,11H ITERATIONS)
      GOTO50
      END
```

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