KINETIC AND METABOLIC STUDIES IN HPRT DEFICIENCY

A THESIS PRESENTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
AT THE UNIVERSITY OF CAPE TOWM

December, 1983

Lafras Marais Steyn MBChB (Stell), BSc (Med)(Hons) (Cape Town)

Preface

The patient (T.K.), was first diagnosed as having a partial hypoxanthine-guanine phosphoribosyltransferase deficiency in 1978 when he was 20 years old. At presentation, he complained of a colicky loin-pain which radiated into his groin, and that he had had dark urine for a month. He was shown to have haematuria and urate crystalluria, and had a serum urate of 0.8mmol/l (reference range 0.12-0.5mmol/l). The diagnosis was confirmed by demonstrating a haemolysate hypoxanthine-guanine phosphoribosyltransferase activity of 550μU/mg Hb (reference range 1680-2480μU/mg Hb). Studies to determine whether the low hypoxanthine-guanine phosphoribosyltransferase activity was caused by an altered K_m of the enzyme for one of its substrates, showed that there was substrate inhibition of the enzyme activity by hypoxanthine.
This thesis examines the patient and the variant HPRT at three levels.

Firstly, a detailed and comprehensive study of the the kinetic properties of the variant enzyme was made. The novel feature of the kinetics is the presence of substrate inhibition by the purine bases, with a true K_i value for hypoxanthine of 80+- 20μM, and a normal value for the true K_m. The pattern of substrate inhibition is characteristic of that associated with the formation of a dead-end complex and double inhibition experiments indicate that the form of this complex is enzyme-hypoxanthine-PPi. These unusual kinetic properties provided an opportunity to study the order of substrate binding in a way not possible for the normal enzyme and showed an ordered sequential reaction mechanism. Some limited protein-structural studies were performed and showed an altered electrophoretic mobility for the variant enzyme in non-denaturing gels.

Secondly, the purine metabolic pathways in cultured cells, derived from T.K., from a patient with the Lesch-Nyhan syndrome, and from normal individuals, were studied. The cells were labelled with precursors of the **de novo** or of the salvage pathways, usually in the presence of a reference label, and sometimes in the presence of inhibitors of the various steps in the purine metabolic pathways. Hypoxanthine salvage was about 10% of that of control cultures. The growth of cells in a variety of selective media was also studied.

Thirdly, as physician in charge of T.K., I could monitor the progress of his hyperuricaemia and observe the effects of therapy throughout the duration of this project.

Acknowledgements

I am indebted to Prof. E. H. Harley, my supervisor, for his help, encouragement, and constructive criticism during the course of this project. I am grateful to Dr. P. Adams for the use of his computer-programs, and to Dr. L. R. Purves for the use of his computing facilities and for his help in compiling this manuscript with the DOC Pocessor on the Univac computer. I am grateful to Mr. R. Schadle for his technical assistance with the tissue culture experiments . I would like to thank Dr. R. Rubinstein and my wife, Kate, for reading through the manuscript and for correcting my many errors, and Mrs. E. Brown for typing the references.

I would like to express my appreciation to Prof. M. C. Berman and the other members of the Department of Chemical Pathology for a most interesting and pleasant work-enviroment and the members of the Department of Medical Microbiology for their patience and indulgence while I was preparing this manuscript.

Furthermore I would like to thank the Trustees of the Guy Elliot Research Fellowship who appointed me as a Research Fellow in 1979.

Abbreviations

ADA,	adenosine deaminase;
AMP,	adenosine-5'-monophosphate;
ATP,	adenosine-5'-triphosphate;
APRT,	adenine phosphoribosyltransferase;
ART,	amidophosphoribosyltransferase;
Ci,	curie (2.2×10^{12} disintegrations/minute);
cm,	centimetre;
DE81,	diethylaminoethyl cellulose;
DNA,	deoxyribonucleic acid;
dpm,	disintegrations/minute;
DTT,	dithiothreitol;
EBV,	Epstein-Barr Virus;
EDTA,	ethylenediamine tetraacetic acid;
g,	acceleration due to gravity;
GMP,	guanosine-5'-monophosphate;
HPLC,	high pressure liquid chromatography;
HPRT,	hypoxanthine-guanine phosphoribosyltransferase;
IMP,	inosine-5'-monophosphate;
K_i,	dissociation constant of the enzyme and inhibitor
K_m,	Michaelis-Menten constant;
mg,	milligram;
ml,	millilitre;
mRNA	messenger RNA;
NADH,	nicotinamide adenine dinucleotide (reduced)
PCA,	perchloric acid;
PEG,	polyethylene glycol;
PEI,	polyethylene-imine;
PNP,	purine nucleoside phosphorylase;
PP-ribose-P,	5-phosphoribose-1-pyrophosphate;
RNA,	ribonucleic acid;
TCA,	trichloroacetic acid
T.K.,	initials of the patient from whom the variant HPRT was obtained;
TLC,	thin layer chromatography;
tris,	tris (hydroxymethyl) amino methane;
μ (prefix)	micro (10^{-6});
V_{max},	maximum velocity;
XMP,	xanthosine-5'-monophosphate.

For Kate, Taillefer and Lesca

Table of contents

Table of contents

Table of contents

Table of contents

SECTION 2

Table of contents

Table of contents

Table of contents

Table of contents

Table of contents

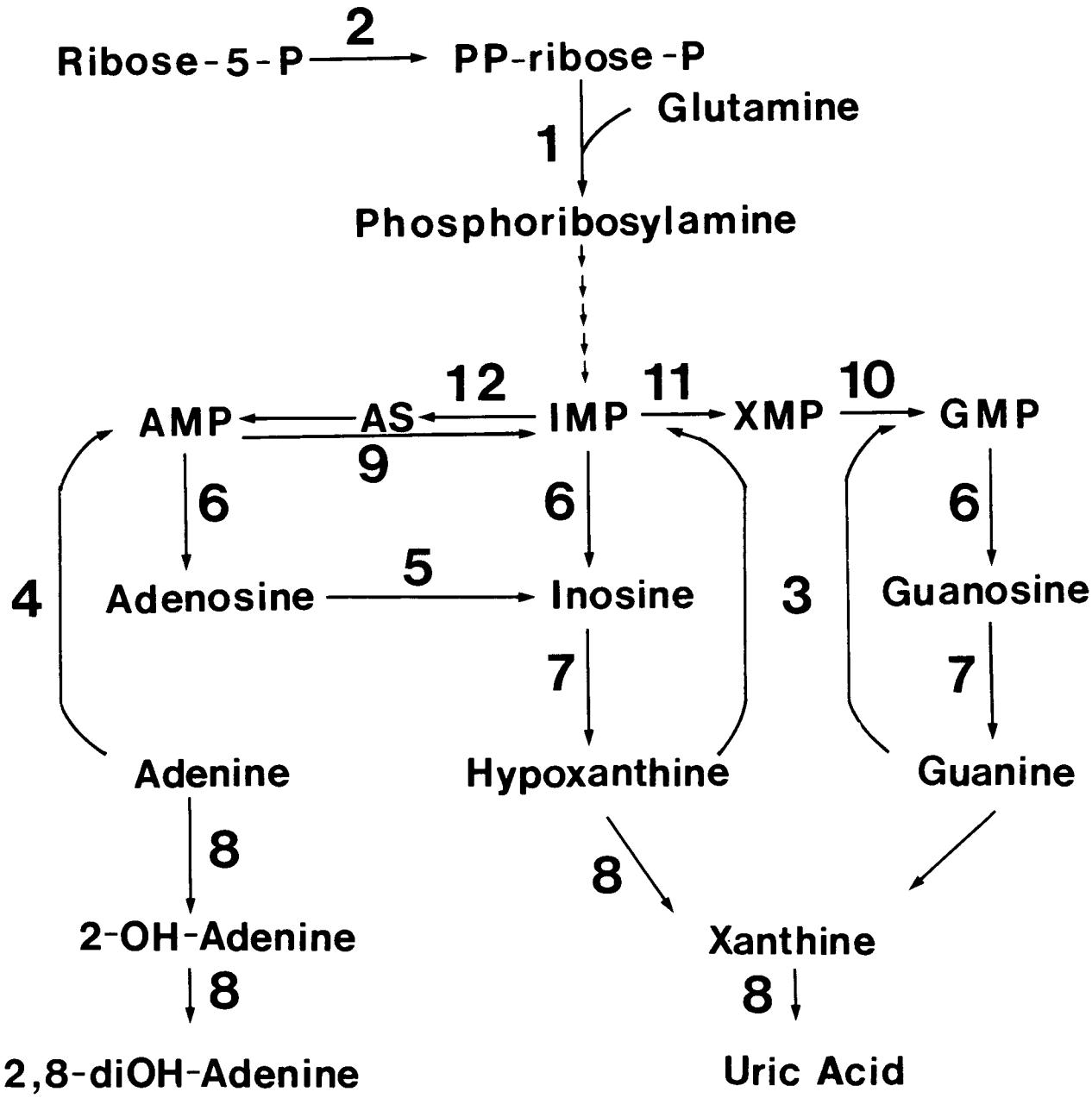


Fig. 1. Outline of purine metabolism.
1) Amidophosphoribosyltransferase; 2) PP-ribose-P synthetase 3) hypoxanthine-guanine phosphoribosyltransferase; 4) adenine phosphoribosyltransferase; 5) adenosine deaminase; 6) 5'-nucleotidase; 7) purine nucleoside phosphorylase; 8) xanthine oxidase; 9) adenosine-5'-monophosphate deaminase; 10) guanosine-5'-monophosphate synthetase; 11) inosine-5'-monophosphate deaminase; 12) adenylosuccinate synthetase.

1. INTRODUCTION

1.1. Purine metabolism

Purine nucleotides are synthesised from two sources (Fig. 1):

1) by the **de novo** synthetic pathway from the small precursor molecules, PP-ribose-P, glutamine, formate, aspartate and bicarbonate. The adenine and guanine nucleotides are formed from IMP, the first nucleotide product of the **de novo** pathway;

2) by the activity of the phosphoribosyltransferases, hypoxanthine-guanine phosphoribosyltransferase (HPRT) and adenine phosphoribosyltransferase (APRT), from pre-formed purine bases and PP-ribose-P.

1.2. Regulation of purine metabolism

Purine synthesis **de novo** is regulated by feedback inhibition on the sequential reactions catalysed by PP-ribose-P synthetase and by amidophosphoribosyltransferase (ART), (Wyngaarden and Kelley 1983). The activities of both these enzymes are modified by the concentrations of the ribonucleotide end-products of the **de novo** pathway (Fox and Kelley 1972, Holmes **et al** 1973). There are two molecular forms of ART (Holmes **et al** 1973), a high molecular weight (270 000) inactive form and a low molecular weight (133 000) active form. The interconversion of the inactive form to the active form is stimulated by the presence of PP-ribose-P, while

AMP and GMP have the opposite effect (Holmes **et al** 1973). The ribonucleotides inhibit the activity of PP-ribose-P synthetase (Fox and Kelley 1972), and may therefore lower the concentration of PP-ribose-P, with a further reduction in the amount of active ART. The regulatory role of PP-ribose-P has been further established by observations that have shown that high intracellular levels of PP-ribose-P are associated with an increased rate of synthesis **de novo**, while low levels are associated with a reduced rate of synthesis **de novo** (Kelley and Wyngaarden 1983). The nucleotides, GMP and AMP, regulate their rate of synthesis from IMP by feedback inhibition on the IMP dehydrogenase and adenylosuccinate synthetase activities, respectively (Mager and Magasanik 1960, Wyngaarden and Greenland 1963). The formation of GMP requires ATP hydrolysis at the GMP synthetase step, while the formation of adenylosuccinate requires GTP hydrolysis, therefore high intracellular levels of either of these nucleotide triphosphates will favour the formation of the other. However, GTP appears to have the major role in determining the relative rate of interconversion of IMP to either GMP or AMP (Kelley and Wyngaarden 1983). High levels of GTP inhibit the formation of XMP from IMP, and stimulate adenylosuccinate synthetase activity, while low levels favour the formation of GMP from IMP. Further control may be found at the level of the phosphoribosyltransferases, which are inhibited by their respective nucleotide monophosphates (Henderson 1968).

1.3. Purine synthesis as a cyclic process

Purine synthesis has been described as being a cyclic process with PP-ribose-P (or ribose) acting as a carrier on which the purine ring is assembled (Mapes and Krebs 1978). The PP-ribose-P can be

released from IMP by the pyrophosphorylase action of HPRT. HPRT may therefore play a role in channelling IMP to PP-ribose-P synthesis and hypoxanthine to purine excretion, especially in uricotelic animals.

1.4. Deficiencies in purine metabolising enzymes

In recent years deficiencies in a number of purine metabolising enzymes have been recognised. The clinical and biochemical consequences of these enzyme deficiencies have increased the understanding of the role of these enzymes in purine metabolism, and has led to a greater insight into purine metabolism in general.

1.4.1. Adenosine deaminase deficiency

A deficiency in the activity of adenosine deaminase (ADA) has been shown to result in an altered function in T-lymphocytes (Giblett **et al** 1972), with a resultant combined immune deficiency. A number of theories have been proposed to explain the mechanism of the altered lymphocyte function. Adenosine is normally converted to inosine by ADA in human cells (Krenitsky **et al** 1968). In the absence of ADA activity, there is an accumulation of adenine and deoxyadenine nucleotides (Kredick and Hershfield 1983) which inhibit DNA synthesis in the cells. The inhibition of

S-adenosylmethionine synthesis is another cause of T-lymphocyte dysfunction in ADA deficiency (Kredirch and Hershfield 1983). ADA degrades a number of adenine analogues, including arabinosyl adenine, formycin and cordycepin (Agarwel **et al** 1975). These anti-metabolites have a chemotherapeutic potential which has been enhanced by the co-administration of deoxycoformycin, an inhibitor of ADA activity, in experimental animals (Johns and Adamson 1976).

1.4.2. Purine nucleoside phosphorylase deficiency

Patients with a deficiency in purine nucleoside phosphorylase (PNP), have been described (Giblett **et al** 1975). They also have a deficiency in their T-lymphocyte function, and in contrast to the accumulation of adenine and deoxyadenine nucleotides in ADA deficiency, there is an accumulation of guanine and deoxyguanine nucleotides (Kredirch and Hershfield 1983).

1.4.3. AMP deaminase deficiency

A few cases of muscle dystrophy are due to a deficiency in AMP deaminase activity (Fishbein **et al** 1978); an enzyme with a role in the purine nucleotide cycle (Lowenstein and Tornheim 1971).

1.4.4. Phosphoribosyltransferase deficiencies

The metabolic consequences of a deficiency in HPRT activity are related to the extent of the intracellular enzyme deficiency (Page **et al** 1981). Purine overproduction with hyperuricaemia, gout, and uric acid renal stones occurs in patients with less than 50% of the normal activity. Whereas patients with the most marked reduction in HPRT activity develop the Lesch-Nyhan Syndrome (Lesch and Nyhan 1964), with its characteristic neurological features, including episodes of compulsive self-mutilation. Since the gene for HPRT is on the X-chromosome, these diseases are sex-linked, and only affect males. A deficiency in APRT activity has also been described (Debray **et al** 1976, Simmonds **et al** 1976). These patients present with renal stones consisting of 2,8 dihydroxyadenine, which must be distinguished from uric acid stones for the correct diagnosis.

1.4.5. Xanthine oxidase deficiency

Patients with a deficiency in xanthine oxidase activity are prone to renal stone formation (Dent and Philpot 1954). The stones consist of xanthine, which is a major excretory form of purines in this deficiency.

1.4.6. PP-ribose-P synthetase overactivity

A number of families with changes in the activity of PP-ribose-P synthetase have been described (Sperling **et al** 1972, Becker **et al** 1974, Zoref **et al** 1975). These patients have an increased rate of purine production **de novo**, and an overactivity of PP-ribose-P synthetase, resulting in an elavation of PP-ribose-P levels in their cells (Zoref **et al** 1975). This is unusual since genetic alterations normally result in reduced activity of the affected enzyme.

1.5. Gout

Gout is the commonest clinical disorder associated with the metabolism of purines. It is a crystal induced arthritis, resulting from the deposition of monosodium urate in the synovial fluids of various joints (Wyngaarden and Kelley 1983). Urate is the end product of purine degradation in man and the primates, and there is a strong association between hyperuricaemia and gout. However, not all patients are hyperuricaemic during an attack of gout, and not all hyperuricaemic patients develop gout. The underlying changes in purine metabolism are unclear in the majority of patients with primary gout. There are two general mechanisms by which hyperuricaemia can arise: 1) a generalised overproduction of purines, and therefore of urate; 2) an underexcretion of urate by the kidneys. These two mechanisms form the bases for the classification of hyperuricaemia given in Table 1.

Table 1

Mechanistic classification of hyperuricaemia

1) Increased production of uric acid

1.1 Mechanism unknown, but likely to be a consequence of one of the following:

1.1.1. Increase in PP-ribose-P

1.1.2. Increase in glutamine

1.1.3. Defect in regulation of amidophosphoribosyl-transferase

1.2. Increase in nucleic acid turnover:
e.g., tumours, infection, haemolytic anaemia, psoriasis

1.3. Specific heritable enzyme defects
e.g., HPRT deficiency, PP-ribose-P synthetase overactivity, glucose-6-phosphatase deficiency.

2. Decreased excretion of uric acid:

2.1. Specific renal tubular defect, either:

2.1.1. Enhanced reabsorption, or

2.1.2. Decreased secretion

2.2 Decrease in renal functional mass
e.g., acute or chronic renal failure

2.3. Drugs or metabolites altering tubular secretion of urate
e.g., thiazides, ethanol, salicylates (in low dosage), lactate, B-hydroxy-butyrate

Table reproduced by courtesy of Prof. E. H. Harley

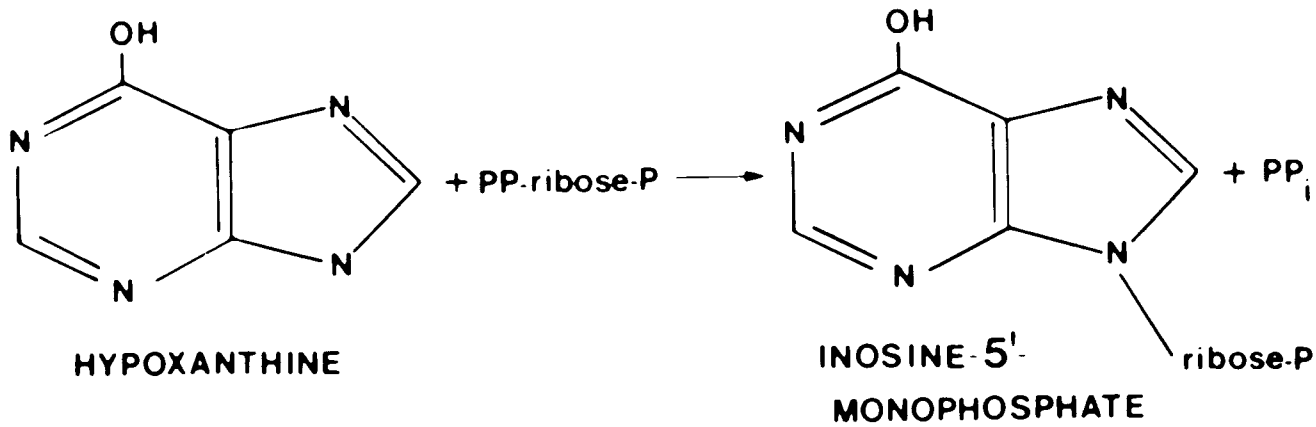


Fig. 2a. Reaction catalysed by HPRT with hypoxanthine as substrate.

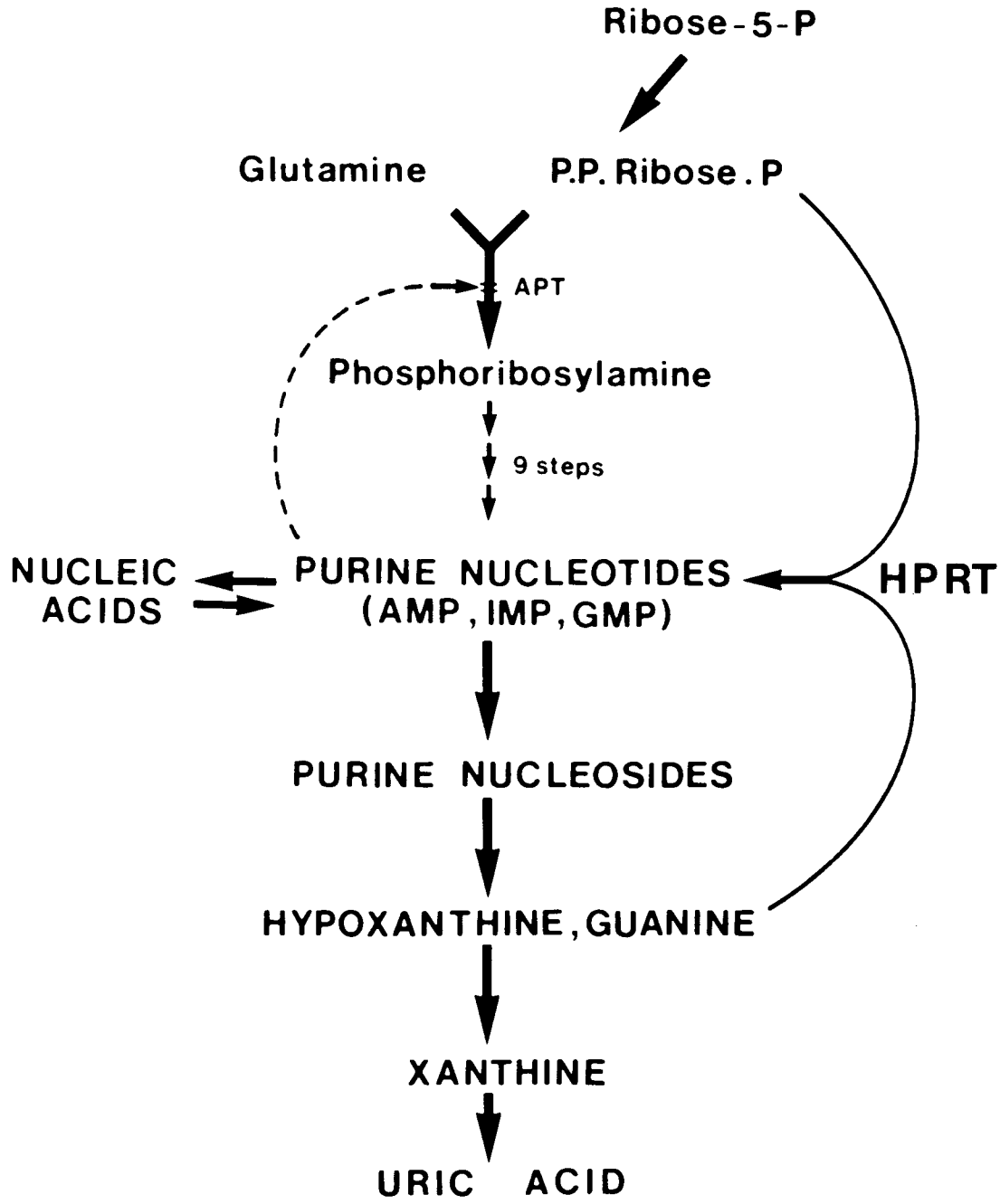


Fig. 2b. Reaction catalysed by HPRT in purine metabolism in general.

2. HUMAN HYPOXANTHINE-GUANINE PHOSPHORIBOSYLTRANSFERASE

Hypoxanthine-guanine phosphoribosyltransferase (HPRT, inosinate-guanylate: pyrophosphate phosphoribosyltransferase, E.C. 2.4.2.8), catalyses the transfer of the 5-phosphoribosyl moiety of PP-ribose-P to the purine bases hypoxanthine and guanine, to form the nucleotides, IMP and GMP, respectively (Fig. 2). Although the function of the enzyme is assumed to be the salvage of the preformed purine bases, hypoxanthine and guanine, other roles for the enzyme are possible: 1) the interconversion of IMP and GMP (Salerno and Giacomello 1979); 2) the synthesis of PP-ribose-P from IMP by pyrophosphorolysis, with hypoxanthine directed towards excretion (Mapes and Krebs 1978).

2.1. Localisation of HPRT

HPRT is a cytoplasmic enzyme, with the highest activities in the basal ganglia of the brain (Rosenbloom **et al** 1967), fibroblasts, lymphocytes and gonadal tissue.

2.2. Kinetics and reaction mechanism of HPRT

The kinetics of erythrocyte HPRT has been investigated extensively (Henderson **et al** 1968, Krenitsky and Papaioannou 1969, Craft **et al** 1970, Giacomello and Salerno 1978). While the HPRT activity shows a hyperbolic relationship to the purine base

concentration, the relationship to PP-ribose-P concentration is dependant on the magnesium concentration; a hyperbolic curve having been obtained at high magnesium concentrations and a sigmoidal curve having been obtained at low magnesium concentrations (Krenitsky and Papaioannou 1969). A sigmoidal relationship of enzyme activity to PP-ribose-P concentration, regardless of the magnesium concentration, was reported when the enzyme was assayed below pH 7.5 (Craft **et al** 1970). The dimagnesium salt of PP-ribose-P appears to be the true substrate for the enzyme (Salerno and Giacomello 1981). A sequential reaction mechanism for HPRT was proposed by Henderson **et al** (1968), on the basis of ^{14}C-guanine and GMP exchange studies and on product inhibition studies. However, the initial velocity plots appeared to be parallel, and this indicated a ping-pong mechanism. They suggested that the ratio of the K_i to the K_m for PP-ribose-P was small (<0.1), and that caused the initial velocity plots to have similar slopes. However, Krenitsky and Papaioannou determined that the ratio was greater than 0.1, and showed that there was an initial burst of IMP synthesis when the enzyme was incubated with hypoxanthine after it had been preincubated with PP-ribose-P and an excess of magnesium (Krenitsky and Papaioannou 1970). This indicated the existence of an enzyme-ribosylphosphate intermediate. They proposed two mechanisms for the HPRT reaction: 1) at low magnesium concentrations the reaction is ordered sequential; 2) at high magnesium concentrations, the reaction is ping-pong, with PP_i being released before the binding of hypoxanthine. Giacomello and Salerno (1978), showed that the magnesium complexes of IMP and PP_i bind to the enzyme in a rapid equilibrium random fashion, while hypoxanthine and PP-ribose-P,

dissociate from the enzyme in an ordered sequence. Studies with the enzyme isolated from yeast cells, have shown that the reaction for the binding of purine base and PP-ribose-P is ordered, and that the enzyme can exist in an enzyme-phosphoribosyl form during the phosphoribosyltransferase reaction, but not during the pyrophosphorolysis reaction (Ali and Sloan 1982). The purine bases can therefore bind to both an enzyme-PP-ribose-P form, and an enzyme-phosphoribosyl form.

2.3. Kinetic parameters of human HPRT

The human erythrocyte HPRT has a K_m value for hypoxanthine of 9.9μM, and for PP-ribose-P of 240μM (Henderson **et al** 1968, Krenitsky and Papaioannou 1969). The K_m value for guanine is 4μM (Henderson **et al** 1968, Benke **et al** 1973).

2.4. Structure of human HPRT

The complete aminoacid sequence of human erythrocyte HPRT has been determined (Wilson **et al** 1982). The enzyme has 217 residues and a molecular weight of 24 470. The NH_2-terminal alanine is acetylated . The native enzyme may exist as a tetramer of identical subunits (Holden and Kelley 1978), or more accurately, a dimer of dimers (Holden and Kelley 1978, Johnson et al 1979). However, proposals that the subunit composition of the native enzyme is a trimer (Hughes **et al** 1975, Olsen and Milman 1977, Muensch and Yoshida 1977), or a dimer (Arnold and Kelley 1971), have been made.

2.5. Electrophoresis of human HPRT

The human erythrocyte HPRT exists in multiple electrophoretic forms (Arnold and Kelley 1971, Bakay and Nyhan 1972). These multiple forms appear to be due to post-transcriptional modifications of the enzyme (Kelley and Wyngaarden 1983). The basis of the post-transcriptional change has to be determined, but it may be due to phosphorylation of the enzyme or deamination of asparagine in the enzyme (Wilson **et al** 1982).

2.6. Mutant forms of human HPRT

The first report linking a deficiency in HPRT activity to the Lesch-Nyhan Syndrome was made in 1967 (Seegmiller **et al** 1967), while later that year, a partial deficiency in the enzyme activity was demonstrated in a number of patients with gout (Kelley **et al** 1967). Since then a number of reports of patients with HPRT activities ranging from almost undetectable to about 50% of the normal, have appeared. The structure of a HPRT mutant ($HPRT_{London}$), has been reported (Wilson **et al** 1983). The enzyme was purified from the erythrocytes of a patient with a severe form of gout. There were reduced amounts of immunologically detectable enzyme in his erythrocytes (Wilson **et al** 1981) and in his transformed lymphoblasts (Wilson **et al** 1982). This variant enzyme has previously been shown to have an abnormal K_m for hypoxanthine,

a normal isoelectric point, and smaller subunit molecular weight on electrophoresis (Wilson **et al** 1981, Wilson **et al** 1982). The mutant enzyme has an aminoacid substitution (Leu for Ser) at position 109. This substitution can be explained by a single nucleotide change in the codon for serine (UCA to UUA). Wilson **et al** (1981, 1982) were able to provide evidence for the existence of five structural variants of HPRT on the basis of changes in the size, electrophoretic, and isoelectric properties of variant enzyme subunits. Earlier studies demonstrated that there were reduced levels of cross reactive material (CRM) in the majority of haemolysates prepared from patients deficient in HPRT activity (Upchurch **et al** 1975, Ghangas and Milman 1975). In the cases were there were normal amounts of CRM, the enzyme was shown to have an increased lability in the intact cells (Balis **et al** 1974), or to have an altered K_m for one or more of its substrates (Gutensohn and Jahn 1978, Henderson **et al** 1976). Several HPRT variants have been shown to have an altered electrophoretic mobility (Bakay and Nyhan 1972, Sweetman **et al** 1978, Steyn and Harley 1981, Wilson **et al** 1982), and an altered isoelectric focusing (Gutensohn and Jahn 1978, Wilson **et al** 1982). The variant HPRT activities generally migrate faster than normal enzyme activities during electrophoresis. A number of HPRT variants have an increased K_m value for PP-ribose-P, or for the purine base substrates (McDonald and Kelley 1971, Benke **et al** 1973, Sweetman **et al** 1978). While the variant described by Gutensohn and Jahn (1978) has an increase in its K_m values for the purine base substrates, it has a reduced K_m value for PP-ribose-P and a reduced susceptibility to the effects of active site inhibitors. Some variants are more susceptible to inhibition by NaF than the

control enzyme activity is (Benke **et al** 1973, Sweetman **et al** 1978). Variants with an altered susceptiblity to product inhibition have been described (Sperling **et al** 1974, Gutensohn and Jahn 1978). A variant with increased heat stability has been found (Kelley **et al** 1967), while a number of HPRT variants have shown an increased lability on heating (McDonald and Kelley 1971, Balis **et al** 1974, Gutensohn and Jahn 1978).

2.7. Molecular biology of the HPRT gene

The gene coding for HPRT has been cloned from a mouse neuroblastoma cell line (Brennand **et al** 1982), and from a human DNA-library (Jolly **et al** 1982). The HPRT gene is large (>32kb), and contains several intervening sequences. The availability of the cloned gene makes it possible to probe the structure of genes coding for HPRT variants, and will make the characterisation of the genetic heterogeneity of the HPRT locus, possible.

2.8. Biochemical changes at a cellular level, associated with HPRT-deficiency

Changes in a number of other enzyme activities have been described in HPRT deficient cells, these include an elevation in the activities of APRT (Kelley **et al** 1967, Kelley **et al** 1969), IMP dehydrogenase (Pehlke **et al** 1972), some of the enzymes of the pyrimidine biosynthetic pathways (Beardmore **et al** 1973), and PP-ribose-P synthetase (Reem 1975). Not all cells, deficient in HPRT

activity have elevated levels of these enzyme activities, nor has the mechanism of the elevation in these enzyme activities been determined with certainty (Kelley and Wyngaarden 1983). The erythrocytes and fibroblasts from patients with a deficiency in HPRT activity contain increased levels of PP-ribose-P (Fox and Kelley 1971, Greene **et al** 1970). This is due to a lack of utilisation of PP-ribose-P by HPRT. The elevation of PP-ribose-P in their cells may explain, in part, why these patients overproduce purines. While the levels of GTP are normal in erythrocytes from patients deficient in HPRT, the levels of the adenine nucleotides are reduced (Lommen **et al** 1971). The pyrimidine nuleotides levels are elevated levels in such cells (Nuki **et al** 1973).

3. ASSAY OF HPRT ACTIVITY

A number of HPRT assays have been described, but only a few will be reviewed here.

3.1. Separation of substrate and product

The majority of the HPRT assays use radioactive substrates and depend on the detection of labelled products for the determination of the enzyme activity. Several different methods have been used to separate the product and the unreacted substrate. These include various chromatography techniques such as ascending paper chromatography (Henderson **et al** 1968), anion exchange columns with DEAE-cellulose (Olsen and Milman 1978), TLC (Hatanaka **et al** 1975), and HPLC (Ali and Sloan 1982, and Rylance **et al** 1982). Anion exchange paper (Hughes **et al** 1975), or PEI-cellulose plates (Wohlhueter 1975), may be used to bind the product. Electrophoresis has also been used (Craft **et al** 1970, Emmerson **et al** 1972). Most of these methods are effective in separating the products from the substrates, but are tedious and time consuming to perform, since repeated, small aliquots of the sample and cold carriers, have to be applied to the separation system. Furthermore, the recovery of the products may vary, or the radioactivity may be quenched, and therefore errors are possible in the estimation of the amount of product formed.

3.2. Requirements of the HPRT assay

An assay may be required for the detection of the enzyme activity in post-column fractions during the purification of the enzyme or for the accurate measurement of the enzyme activity for its kinetic characterisation. Not all assay methods are suitable under these circumstances. In the first example an easy, rapid assay which can be adapted to a batch approach is needed, while in the second an accurate, sensitive, kinetic assay is required. The following factors were taken into consideration, when deciding to use a particular HPRT assay for the characterisation of the variant enzyme:

1) Intitial rates had to be measured. Rate equations are valid for initial reaction rates, where there is a minimal change in substrate concentration and no accumulation of the product.

2) The reaction had to be linear with time and enzyme concentration.

3) The assay needed accuracy and precision.

3.3. Types of HPRT assays

Assays for HPRT can be divided into two arbitory groups:

1) end point assays, in which the amount of product formed, or the amount of substrate utilised after a fixed period of time, is determined; 2) kinetic assays in which the rate of appearance of product per unit time, is determined.

3.3.1. End-point assays of HPRT activity

End-point assays are widely used, but are not ideally suited to the kinetic characterisation of an enzyme. The amount of product formed after a fixed period of time is an integrated value, and there is no indication whether the rate of production of the product is constant throughout the assay. A latent period for instance, will not be detected. Inadequate separation of the product and substrate may be difficult to detect. Although these problems can be minimised, they may cause uncertainty in individual assays. In an attempt to minimise these problems, it has been suggested that the ratio of product to substrate be determined (Olsen and Milman 1978). However, this approach assumes that the product and substrate are recovered at the same efficiency, and that the separation of the two is complete. An alternative method is to measure the amount of hypoxanthine used by the enzyme in one hour (Johnson **et al** 1977).
The enzyme and IMP are precipitated with $LaCl_3$, and the change in extinction at 249nm due to the reduction in hypoxanthine concentration is measured. This assay is useful for screening samples for HPRT activity, and it was used to detect HPRT activity in the post-column fractions obtained during the partial purification of the variant enzyme.

3.3.2. Kinetic assays of HPRT activity

Giacomello and Salerno (1977), have described a method for linking the rate of formation of GMP by HPRT activity to the oxidation of NADH, and of measuring the rate of change in extinction at 340nm. This assay is convenient and quick to perform. However, when used to determine the HPRT activity in dialysed haemolysates the relationship between the enzyme activity and the increase in protein concentration was not linear. The guanosine-5'-monophosphate kinase, used in the marker enzyme system, was unstable and expensive. However, the initial kinetic characterisation of the variant HPRT, and the studies on the partialy purified variant HPRT, were performed with this assay. A kinetic assay, described by Wohlhueter (1975), was modified and used in the kinetic characterisation of the variant enzyme. The assay was performed as follows: aliquots of the assay mixture were taken at minute intervals, and the amount of product formed at each point, was measured. The data were fitted to the equation for a straight line and the rate of formation of the product was determined from the slope of the line. Non-separation of the product and the substrate was indicated by the value of the intercept on the ordinate. A latent period, instability of the enzyme activity or depletion of the sustrates would have been detected as non-linearity in the rate of product formation. This assay was also used in the double inhibition studies performed to elucidate the mechanism of substrate inhibition found in the variant enzyme. This assay is referred to as the 'multiple end-point assay'.
The details of the various HPRT assays are described in the relevant sections, under 'Methods'.

SECTION 1

4. KINETIC CHARACTERISATION OF THE VARIANT HPRT

4.1. Materials

The radioactive substrates [8-^{14}C]-Hypoxanthine (used at 20.15 dpm/pmol), and [8-^{14}C]-Guanine (114dpm/pmol), were obtained from Amersham and New England Nuclear, respectively. They were checked for purity by TLC and were found to be 96% and 93% pure, respectively. Hypoxanthine, guanine, PP-ribose-P (sodium salt), and IMP were obtained from Sigma, and tetra-sodium pyrophosphate, from B.D.H. The PP-ribose-P was quantitated by assaying the HPRT activity in the presence of a limiting amount of PP-ribose-P, and an excess of hypoxanthine. The actual PP-ribose-P concentration was determined from the amount of product formed when the reaction had run to completion. When assayed in this manner the PP-ribose-P was found to be 94% pure. All other reagents were of analytical grade.

4.2. Enzyme extracts

The enzyme extracts were prepared from 10ml of fresh, heparinised blood from T.K. and from a normal control. The plasma and buffy coat was removed after centifugation at 1 000g for 10 minutes in an MSE desk top centrifuge. The red cells were washed twice with normal saline, and were lysed by adding 4 volumes of cold, distilled water; the stroma was removed by centrifugation.

The haemolysates were dialysed for 16 hours at 4°C against two changes of 5 litres of 50mM tris-HCl, pH 7.8, containing 1mM dithiothreitol. The dialysed lysate was frozen in aliquots of 500µl, and stored at -20°C. After 4 weeks of storage there was a 10% loss in enzyme activity, compared with a loss of over 50% in samples stored for 2 days at 4°C. The thawed lysates were diluted immediately before use to give 100-200µU of enzyme activity per 20µl aliquot. The protein was determined by a modification of the method of Lowry **et al** (1951).

4.3. Methods

4.3.1. Enzyme assay

The HPRT activity was assayed by a modification of the method of Wohlhueter (1975). All assays were performed at 37°C. Each assay contained the following in a final volume of 100µl: 50mM tris-HCl, pH 7.8, 14mM $MgCl_2$, PP-ribose-P and ^{14}C-hypoxanthine or ^{14}C-guanine at the appropriate concentrations. Assay tubes were brought to 37°C for one minute before the reaction, which was started by adding 20µl of the enzyme preparation. Four aliquots of 20µl were taken at one minute intervals, and applied to PEI-cellulose plates (Merck), which were pre-heated to 70°C on a heating block. The PEI-cellulose plates were pre-ruled into 2.5x2.5cm squares, one for each aliquot. In this way twelve assays could be accommodated on each plate. The plates were washed by placing them in a chromatography tank containing 4 litres of tap water for

5 minutes. The plates were removed before the water was changed. Four of these washes were required for assays using hypoxanthine as the substrate, and six for those with guanine as the substrate. Up to four plates were washed at a time. After the plates had dried, the PEI-cellulose was scraped off the plates, and the PEI-bound nucleotide added to 6ml of Instagel (Packard), the radioactivity was counted in a Beckman LS-250 scintillation counter. DE81 paper (Whatman) was used as an alternative anion exchanger to PEI-cellulose in a number of assays. The paper was pre-treated with 20mM EDTA, pH 8.0, pre-ruled into 2.5x2.5cm squares, and cut into strips of 2x4 squares. The unreacted substrate was washed off the paper by suspending the strips in a 5l beaker, and by allowing tap water to flow through it at a rate of 1 l/minute for 45-60 minutes. The strips were washed with ethanol in order to decrease their fragility. The individual squares were cut out and assayed for radioactivity in 10ml of Instagel. Less than 1% of the unreacted substrate remained on the paper, while less than 10% of the product was lost. Quenching of radioactivity by the PEI-cellulose and the paper was 10%. All these factors were taken into consideration when the enzyme activity was calculated. The data was analysed by linear regression, on a Tektronix 4050 computer, by the method of least squares (Bevington 1969). The reaction was regarded as linear when a correlation of greater than 0.99 was obtained. All the assays were performed in duplicate, and the activities given are the mean of 1-3 sets of assays. The magnesium concentration was kept constant at 14mM, which was ten times greater than the highest PP-ribose-P concentration used (Giacomello and Salerno 1978). The unit of enzyme activity (U), was defined as that which catalysed the formation of one micromole of product per minute.

4.3.2. Data analysis

The values for the various kinetic parameters of the control and variant enzyme, were obtained by the reiterative method of Wilkinson (1961) by fitting the data directly to the appropriate rate equations. Reciprocal velocities were ploted graphically against the reciprocals of the substrate or inhibitor concentrations. These curves were used to obtain estimates of the kinetic parameters, which are required in the reiterative method. The figures show curves with the best fit to the data. The data obtained for the curves following hyperbolic kinetics were fitted to equation 1, those for substrate inhibition were fitted to equation 2, and those for double inhibition were fitted to equation 3.

$$v = VA \times (K+A)^{-1} \qquad (1)$$

$$v = VA \times (K+A+A^2/K_i)^{-1} \qquad (2)$$

$$v = V_o \times (1+I/K_i+J/K_j+IJ/\beta K_i K_j)^{-1} \qquad (3)$$

In equation 3, I and J are inhibitors of the enzyme activity, K_i and K_j are the dissociation constants for I and J, V_o is the enzyme activity in the absence of of the inhibitors, and ß is the interaction constant for the inhibitors on the enzyme. In the other equations, K is the Michaelis constant or the apparent Michaelis constant, V is the maximum velocity and K_i is the dissociation constant for the second substrate molecule.

Note: For origin of equation 3 see Cleland (1979) and Ullr et al (1974)

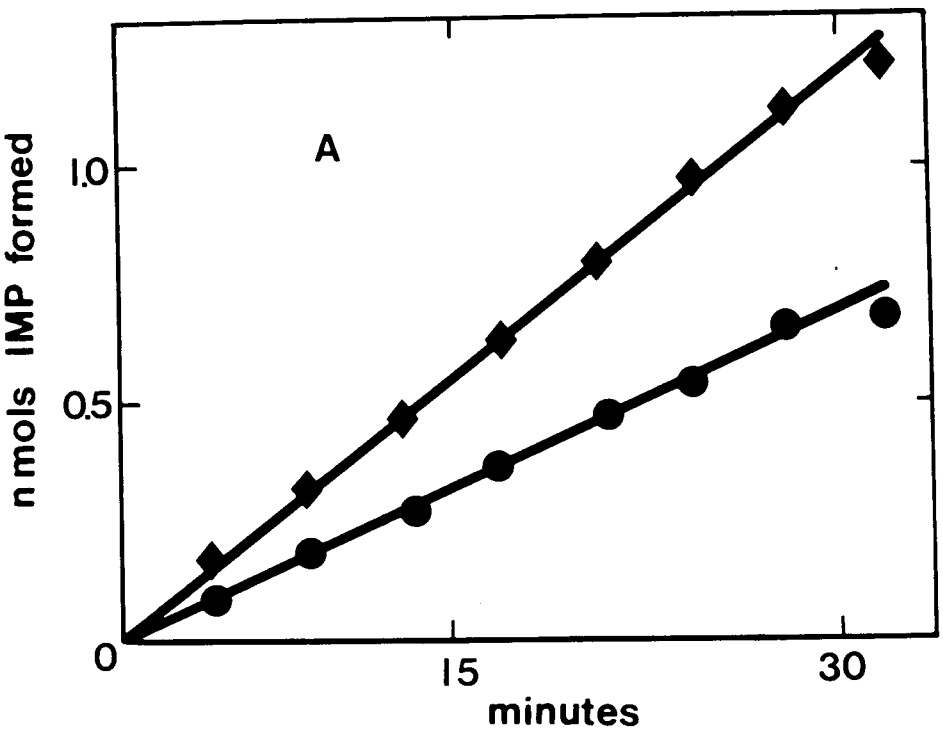
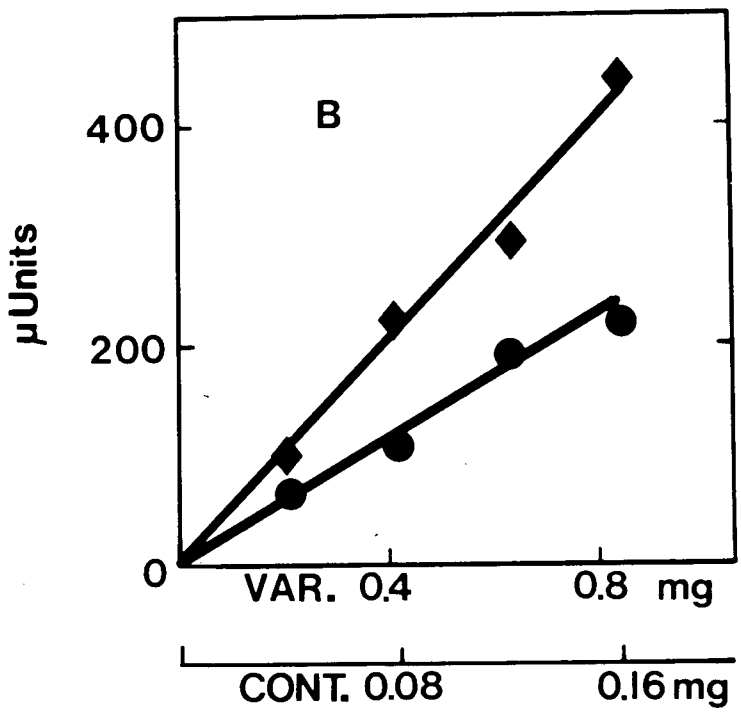
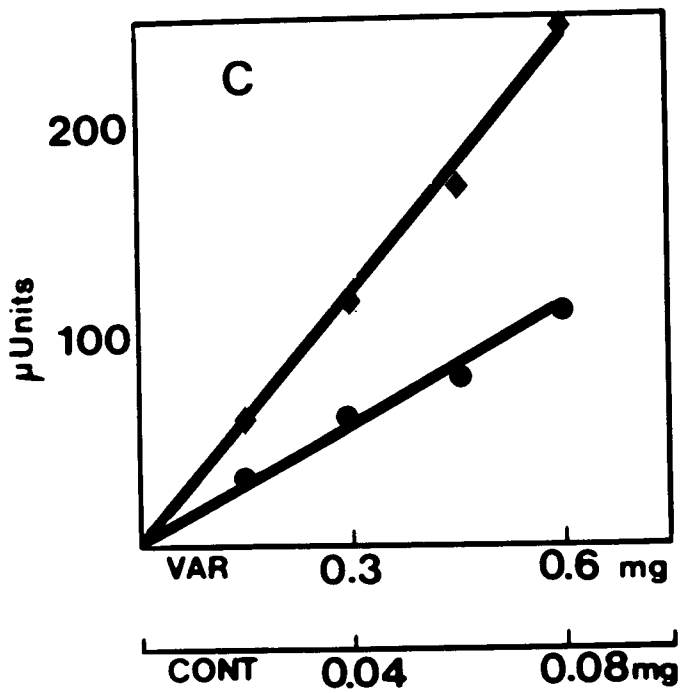


Fig. 3. Linearity of the variant HPRT activity versus time and protein concentration. Enzyme activity was determined at 350µM hypoxanthine or 20µM guanine, and 1.4mM PP-ribose-P. A) The amount of IMP formed was determined from aliquots of 20µl taken from the assay mixture at 4 minute intervals. The total assay volume was 200µl and the reaction was started with 40µl of enzyme extract. B) Hypoxanthine as substrate: enzyme activity was determined in aliquots of enzyme activity containing 0.22 to 0.88 and 0.08 and 0.16 mg protein for the variant and control, respectively. C) Guanine as substrate: enzyme activity was determined in aliquots of enzyme activity containing 0.3 to .0.6 and 0.04 to 0.08 mg protein for the variant and control, respectively. All other conditions were as described under 'Methods'. (●), control; (◆), variant.


B
400
200
0
µUnits
VAR. 0.4
0.8 mg
CONT. 0.08
0.16 mg


C
200
100
µUnits
VAR
0.3
0.6 mg
CONT
0.04
0.08mg

4.4. Results

The time dependence of formation of the product, IMP, in the assay of the variant HPRT is shown in Fig. 3a. The rate of formation of IMP was constant for 30 minutes. The initial hypoxanthine concentration was 350μM, of which 34% was converted to product within 30 minutes. The formation of IMP by the control enzyme was also constant over this period. In Fig. 3b, the effect of protein concentration on the variant enzyme activity is shown. Both control and variant enzyme activities increased linearly with an increase in the protein concentration. At the substrate concentration used, the control enzyme preparation contained 4 times the activity of the variant enzyme preparation. Similarly, the activities of the variant and control enzymes were linear when plotted against protein concentration with guanine as the purine base substrate (Fig. 3c).

4.4.1. Comparison of the variant and control enzyme activities

4.4.1.1. Hypoxanthine as the varied substrate.

The activities of variant and control HPRT, assayed at a constant PP-ribose-P concentration and at varied hypoxanthine concentrations are shown in Fig. 4a. The control enzyme activity

demonstrated a hyperbolic dependence on the hypoxanthine concentration, while the variant enzyme activity reached its greatest activity at 70μM of hypoxanthine. Thereafter it showed a progressive decrease as the hypoxanthine concentration was increased. At 350μM of hypoxanthine the variant activity was approximately 50% of the maximal activity obtained. The double reciprocal plots of the enzyme activities **versus** hypoxanthine concentration, demonstrated that the control enzyme activity followed Michaelis-Menten kinetics, while the variant followed those for substrate inhibition. The data were fitted to the appropriate rate equations, and the values for the various kinetic parameters were obtained (Table 2). The values for the $K_{m(app)}$ are 10± 2μM and 34± 16μM for the control and variant enzyme activities, respectively, while those for the $V_{max(app)}$ are 1779± 39μU/mg prot., and 1937± 427μU/mg prot., respectively. The variant enzyme has a $K_{i(app)}$ for hypoxanthine of 118± 28μM.

4.4.1.2. Guanine as the varied substrate.

The variant enzyme also demonstrated substrate inhibition with guanine as the purine substrate (Fig. 4b). The enzyme activity reached a maximum value at a guanine concentration of 6μM, with about 70% of the activity remaining at 25μM of guanine. The values for the $K_{m(app)}$, $V_{max(app)}$, and $K_{i(app)}$, are 2.6μM, 1179± 295μU/mg prot., and 28± 9μM, respectively (Table 2). The control enzyme activity showed a hyperbolic relationship to the guanine concentration, and the values obtained for the $K_{m(app)}$, and $V_{max(app)}$ are 3± 0.6μM and 1298± 72μU/mg prot., respectively.

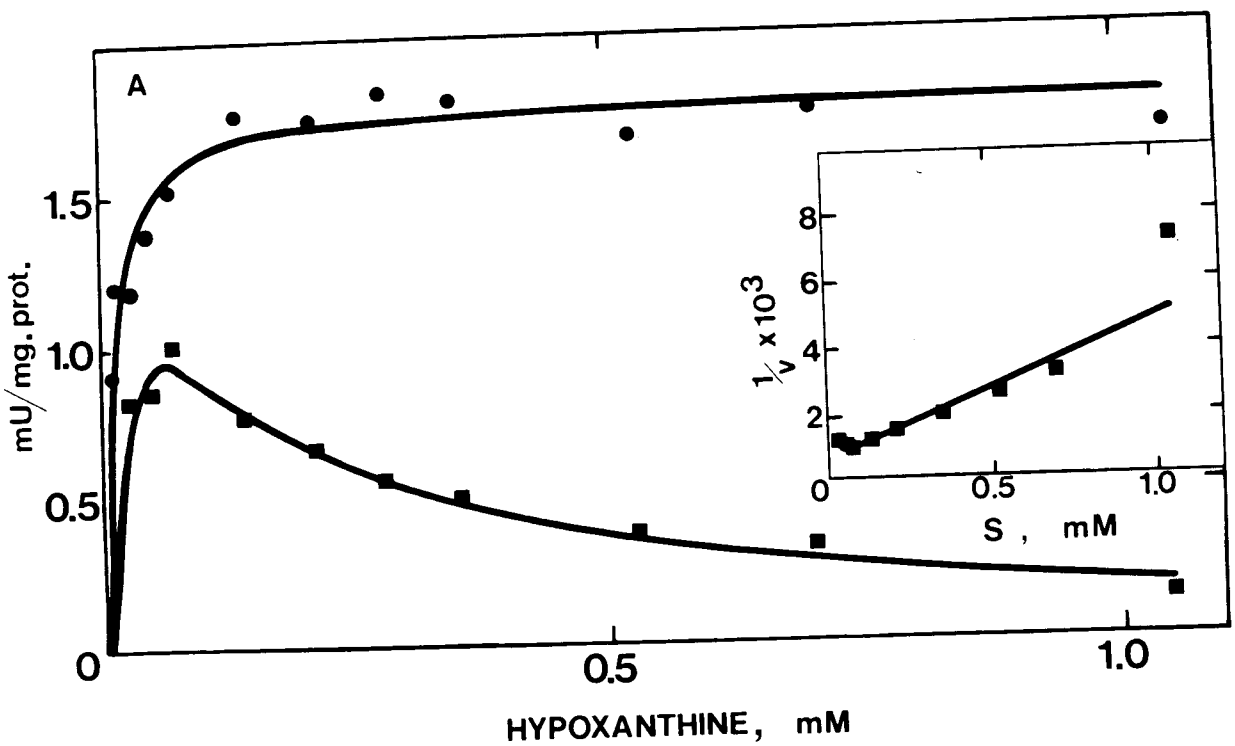


Fig. 4a. Effect of varied hypoxanthine concentration on variant and control HPRT activity. Enzyme activity was determined at a constant PP-ribose-P concentration of 1.4mM. All other conditions were as described under 'Methods'.
(●), control; (■), variant.

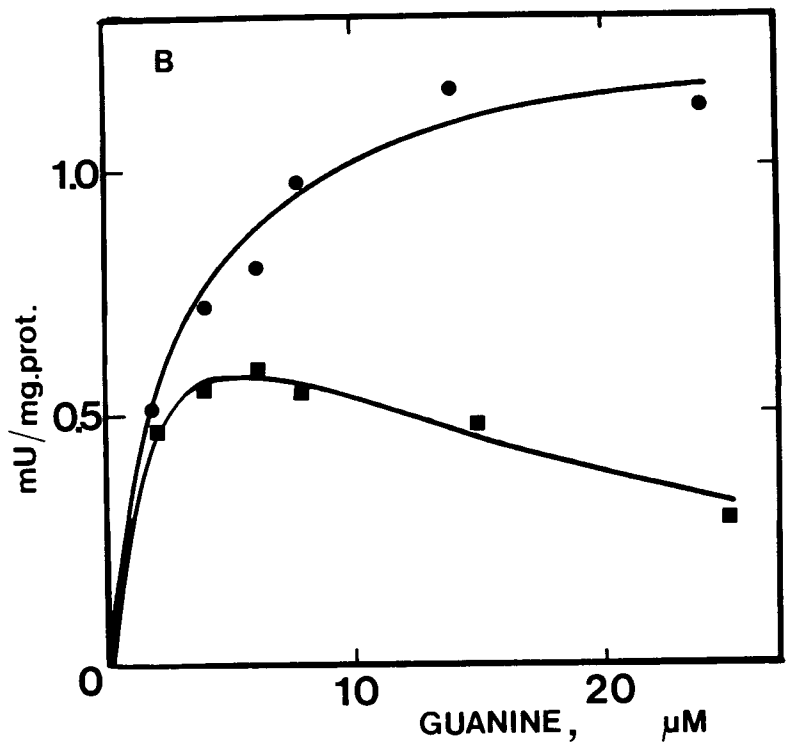


Fig. 4b. Effect of varied guanine concentration on variant and control HPRT activity. Enzyme activity was determined at a constant PP-ribose-P concentration of 1.4mM. All other conditions were as described under 'Methods'.
(●), control; (■), variant.

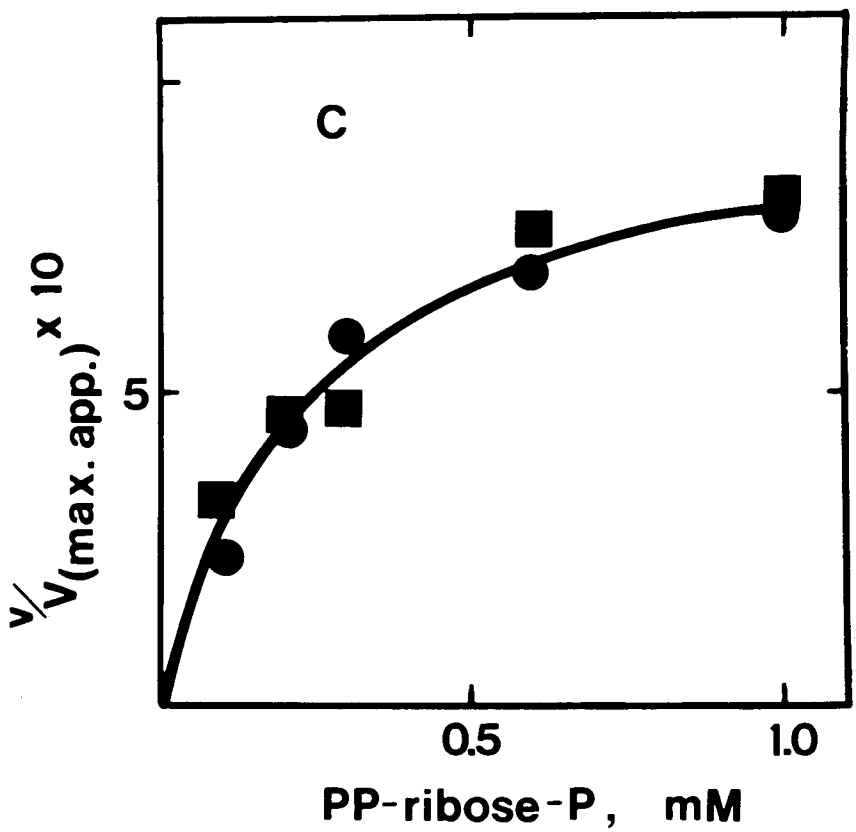


Fig. 4c. Effect of varied PP-ribose-P concentration on variant and control HPRT activity. Enzyme activity was determined at a constant hypoxanthine concentration of 350μM. All other conditions were as described under 'Methods'.
(●), control; (■), variant.

4.4.1.3. PP-ribose-P as the varied substrate.

The variant and control enzyme activities were determined at a fixed hypoxanthine concentration of 350μM, with PP-ribose-P as the varied substrate (Fig. 4c). No substrate inhibition by PP-ribose-P could be demonstrated for the variant enzyme. The low activity of the variant was due to inhibition by hypoxanthine, which was held at the relatively high concentration of 350μM to avoid any significant change in the base concentration during the assay. The data were fitted to equation 1, and values were obtained for the $K_{m(app)}$, and $V_{max(app)}$. The respective values for the variant enzyme are 244± 54μM and 180± 20μU/mg prot.,and those for the control enzyme activity, are 251± 54μM and 1399± 110μU/mg protein (Table 2).

4.4.2. Determination of the true K_m, true V_{max}, and true K_i of the variant enzyme activity for hypoxanthine

The values for the 'true' kinetic parameters of the variant enzyme were obtained by assaying the enzyme activity with PP-ribose-P as the variable substrate, at different, fixed concentrations of hypoxanthine (Fig. 5a). By fitting the data to equation 1, values for the $V_{max(app)}$ at each hypoxanthine concentration, could be obtained. These values were fitted to equation 2, and the values for the true K_m, true V_{max}, and true K_i for hypoxanthine, were obtained. This is illustrated in Fig. 5b, where the reciprocal of the $V_{max(app)}$ value for PP-ribose-P is

plotted against the reciprocal of the hypoxanthine concentration. A curve typical of substrate inhibition by hypoxanthine was obtained. The values for the true K_m, true V_{max} and true K_i, are 26± μM, 1761± 382μU/mg prot., and 80± 20μM, respectively (Table 2).

4.4.3. Determination of the true K_m, true V_{max}, and true K_i of the variant enzyme for guanine

The values for the true K_m, true V_{max}, and true K_i for guanine were obtained as described for hypoxanthine. The double reciprocal plots of enzyme activity **versus** PP-ribose-P concentration are shown in Fig. 6a. The values for the $V_{max(app)}$ for PP-ribose-P, obtained at each guanine concentration were fitted to equation 2, and the values for the true K_m, true V_{max} and true K_i, were obtained (Fig 6b). They are 1.87±0.9μM, 1152± 219μU/mg prot., and 27.5± 8.9μM, respectively (Table 2).

4.4.4. Determination of the true K_m and true V_{max} of the variant enzyme for PP-ribose-P

The variant enzyme activity was assayed at a number of fixed concentrations of PP-ribose-P with hypoxanthine as the variable substrate (Fig 7), and the data was fitted to equation 2, in order that the values for the $V_{max(app)}$ for hypoxanthine could be obtained. These values were fitted to equation 1 (Fig. 7, inset), and the values for the true K_m and true V_{max} for PP-ribose-P were obtained. These

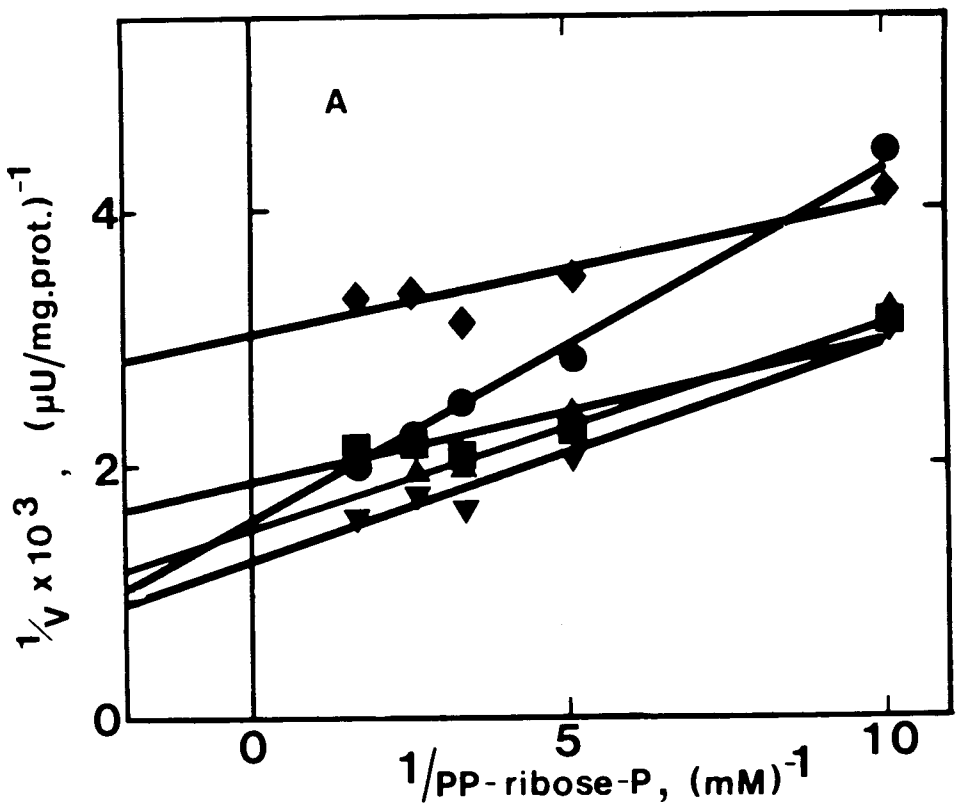


Fig. 5a. Variant HPRT activity at different fixed concentrations of hypoxanthine and varying PP-ribose-P concentration. The hypoxanthine concentrations were: (●), 18μM; (▼), 45μM; (▲), 120μM; (■), 180μM; (◆), 300μM.

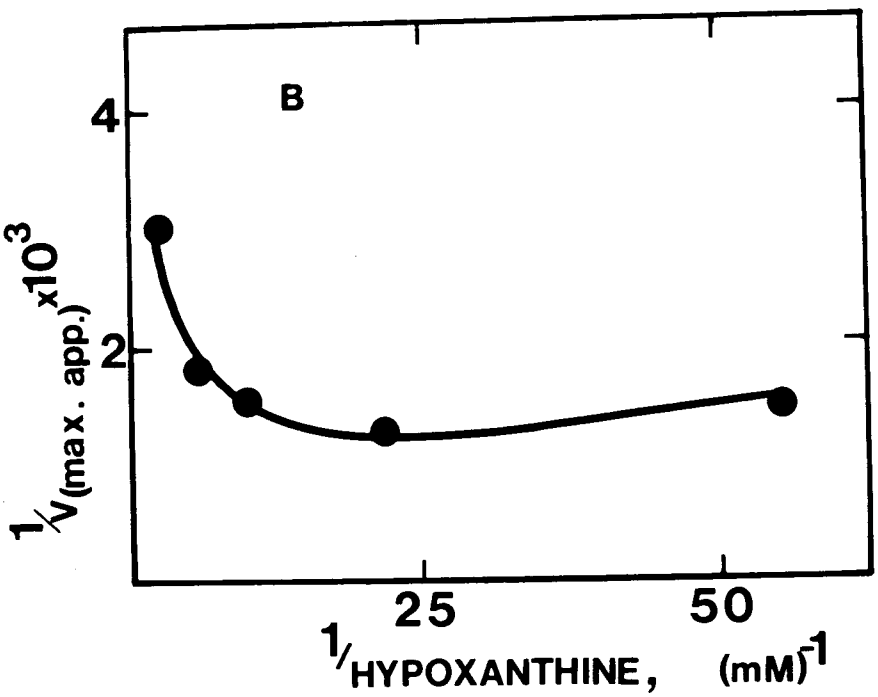


Fig. 5b. Replot of the values for $V_{max(app)}$ for PP-ribose-P against the reciprocal of the hypoxanthine concentration. The value for the $V_{max(app)}$ for PP-ribose-P at each hypoxanthine concentration was obtained by fitting the data shown in Fig. 5a, to equation 1.

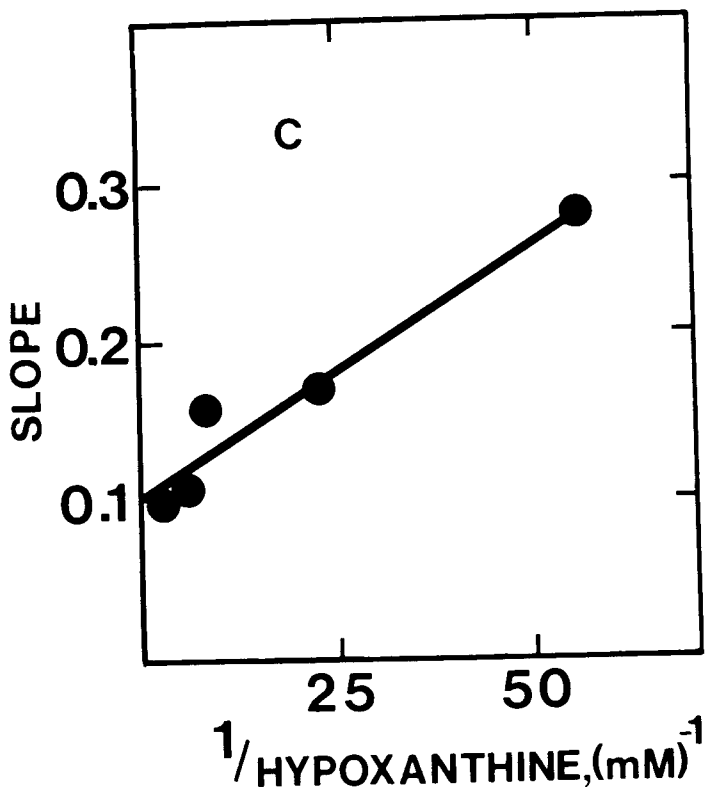


Fig. 5c. Replot of the slopes of the double reciprocal plots of the variant enzyme activity and PP-ribose-P concentration against the reciprocal of the hypoxanthine concentration. The slopes were calculated from the values for $V_{max(app)}$ and $K_{m(app)}$, obtained by fitting the data shown in Fig. 5a, to equation 1.

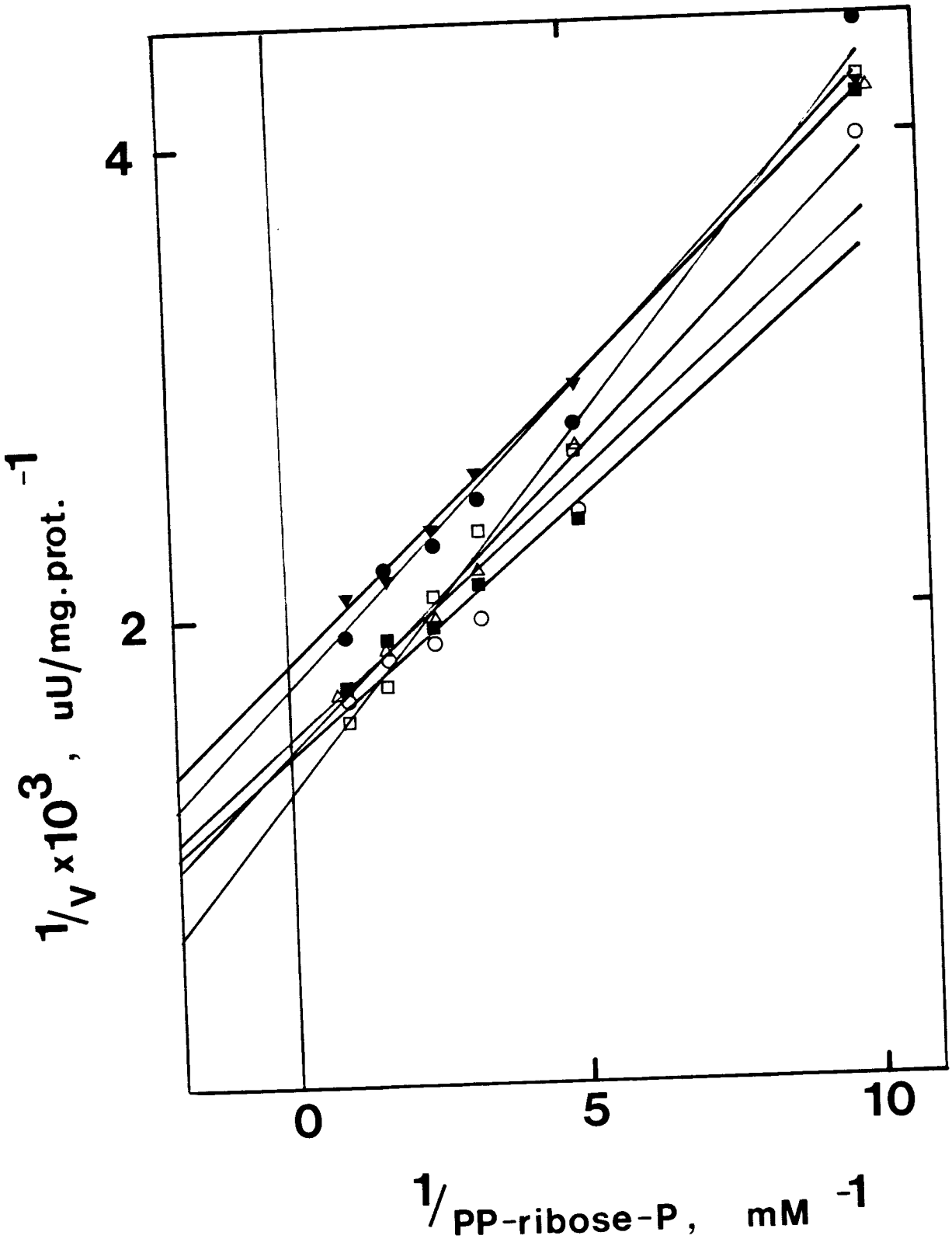


Fig. 6a. Variant HPRT activity at different fixed concentrations of guanine and varying PP-ribose-P concentration. The guanine concentrations were: (●), 2μM; (△), 4μM; (■), 6μM; (□), 8μM; (○), 15μM; (▼), 25μM.

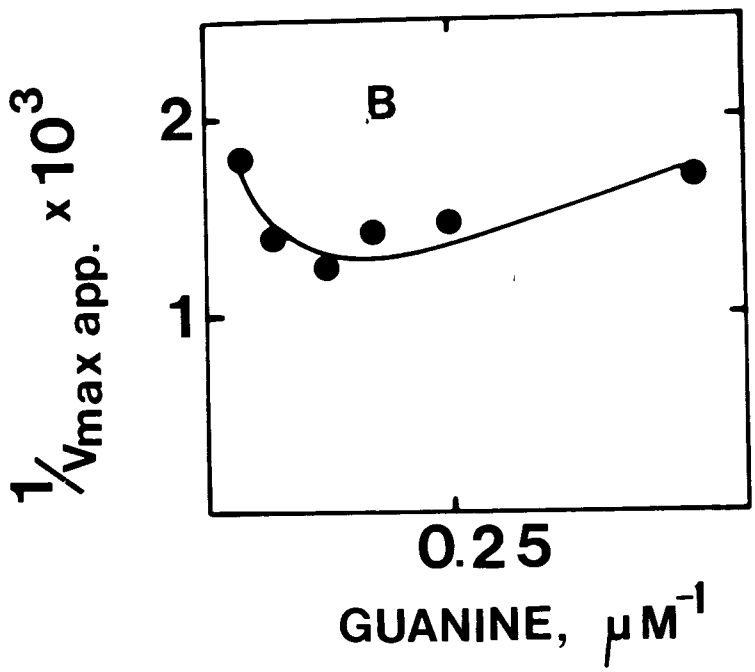


Fig. 6b. The replot of the values for $V_{max(app)}$ for PP-ribose-P against the reciprocal of the guanine concentration. The value for the $V_{max(app)}$ for PP-ribose-P at each guanine concentration was obtained by fitting the data shown in Fig. 6a to equation 1.

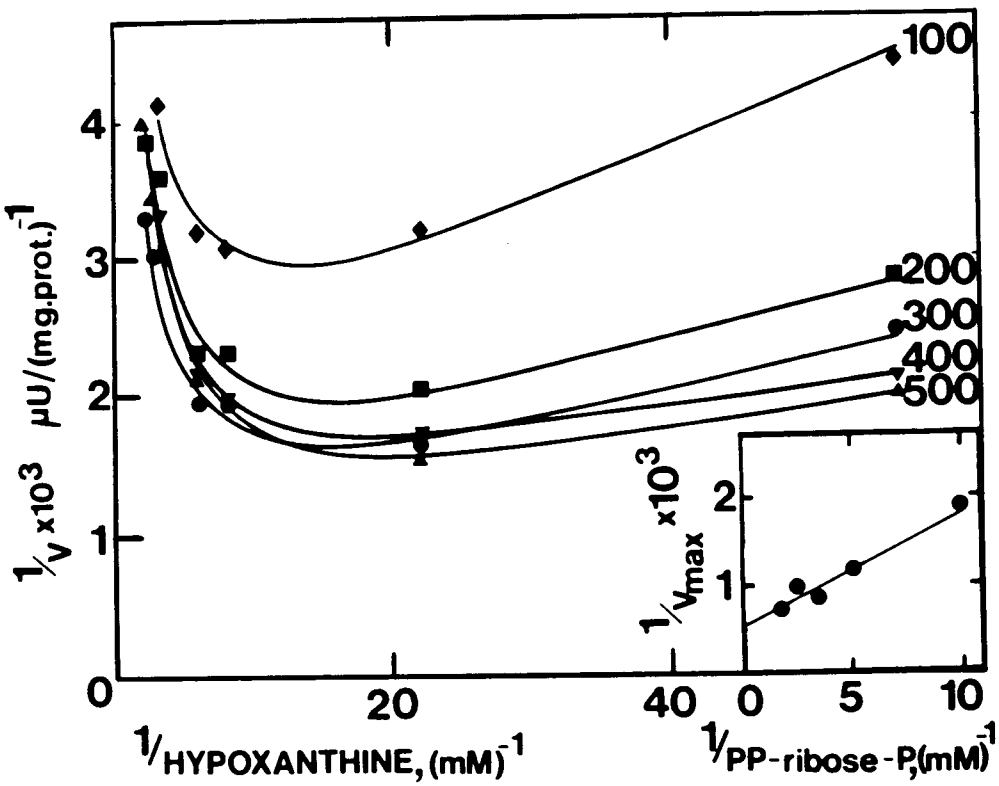


Fig. 7. Variant HPRT activity at different fixed concentrations of PP-ribose-P and varying hypoxanthine concentrations. The PP-ribose-P concentrations were: (◆), 100µM; (■), 200µM; (●), 300µM; (▼), 400µM; (▲), 600µM. All other conditions were as described under 'Methods'. The figures opposite the curves indicate the PP-ribose-P concentration (µM). The inset shows the reciprocal of the $V_{max(app)}$ versus the reciprocal of the PP-ribose-P concentration.

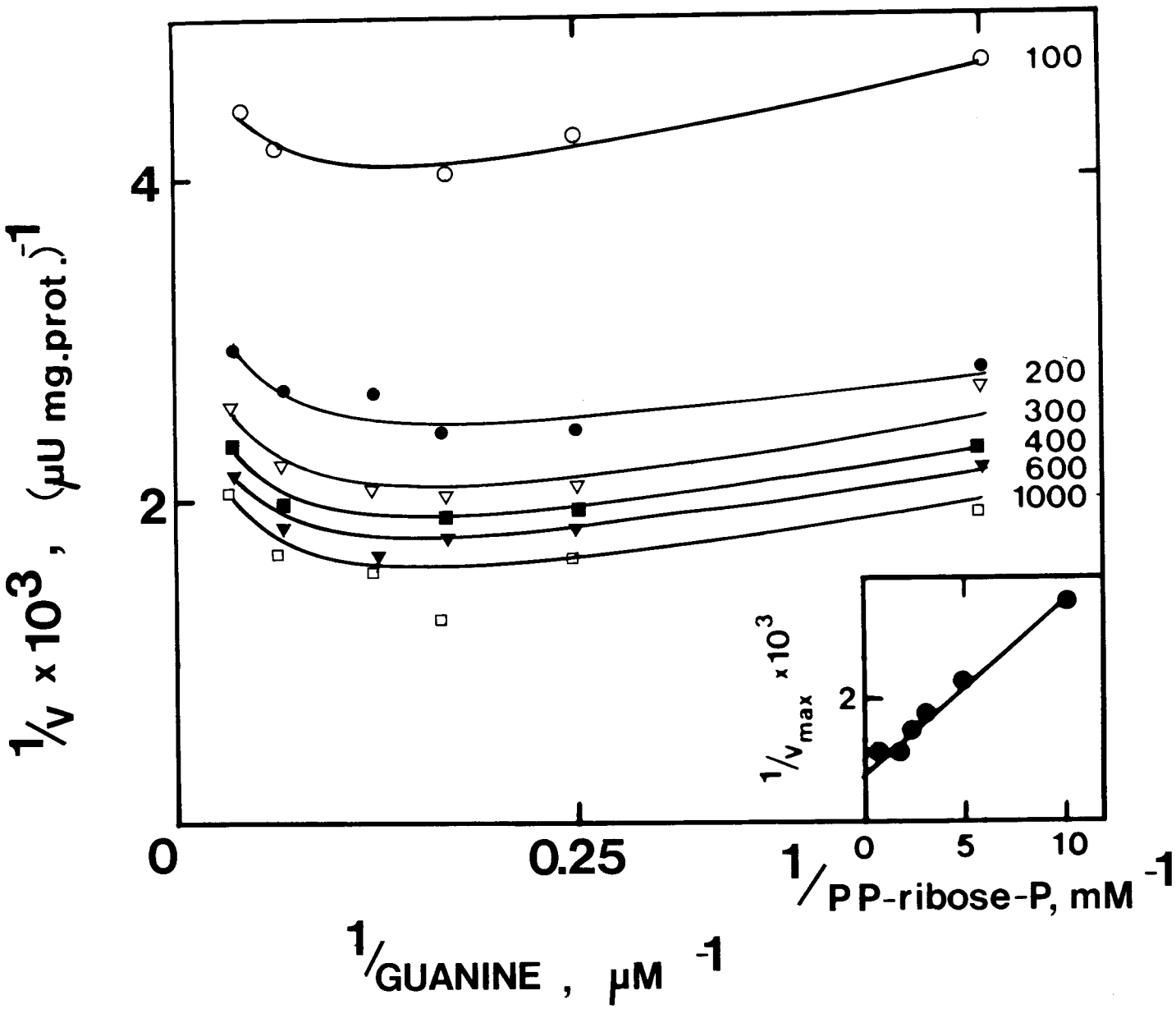


Fig. 8. Variant HPRT activity at different fixed concentrations of PP-ribose-P and varying guanine concentrations. The PP-ribose-P concentrations were: (○), 100μM; (●), 200μM; (▽), 300μM; (■), 400μM; (▼), 600μM; (□), 1000μM. All other conditions were as described under 'Methods'. The figures opposite the curves indicate the PP-ribose-P concentration (μM). The inset shows the reciprocal of the $V_{max(app)}$ versus the reciprocal of the PP-ribose-P concentration.

Table 2

The kinetic parameters obtained for the variant and control HPRT activities, assayed by the kinetic assay of Wohlhueter (1975).

A) Hypoxanthine as the purine substrate

	Hypoxanthine		PP-ribose-P	
	Variant	Control	Variant	Control
$K_{m(app)}$ µM	34± 16	10± 2	244± 75	251± 54
$V_{max(app)}$ µU/mg.prot	1937± 427	1779± 39	180± 20	1399± 110
$Ki_{(app)}$ µM	118± 28	-	-	-
$K_{m(true)}$ µM	26± 10	N.D.	227± 116	N.D.
$V_{max(true)}$ µU/mg.prot	1761± 382	N.D.	1779± 371	N.D.
$Ki_{(true)}$	80± 20	-	-	-

N.D.= not determined

Table 2

The kinetic parameters obtained for the variant and control HPRT activities, assayed by the kinetic assay of Wohlhueter (1975).

B) Guanine as the purine substrate

	Guanine		PP-ribose-P	
	Variant	Control	Variant	Control
$K_{m(app)}$ µM	2.6± 1.3	3.0± 0.6	N.D.	N.D.
$V_{max(app)}$ µU/mg.prot	1179± 295	1298± 72	N.D.	N.D.
$Ki_{(app)}$ µM	28± 9	-	-	-
$K_{m(true)}$ µM	1.9± 0.9	N.D.	271± 22	N.D.
$V_{max(true)}$ µU/mg.prot	1152± 219	N.D.	1108± 35	N.D.
$Ki_{(true)}$ µM	27.5± 8.9	-	-	-

N.D.= not determined

are 227± 116μM and 1779± 371μU/mg prot., respectively (Table 2). The true V_{max} and true K_m for PP-ribose-P with guanine as the purine substrate were obtained, as is illustrated in Fig 8. The values for the true V_{max} and true K_m are 1108± 35μU/mg prot., and 271± 22μM, respectively (Table 2).

4.4.5. Investigation of the mechanism of substrate inhibition of the variant HPRT activity

The inhibition of the variant HPRT activity is shown as a plot of the reciprocal of enzyme activity **versus** the concentration of hypoxanthine (Fig. 4a, inset). The inhibition of enzyme activity was proportional to the hypoxanthine concentration above 100μM. This pattern of inhibition has been termed linear or total substrate inhibition (Cleland 1979, Uhr **et al**, 1974). Further information about the nature of the substrate inhibition can be obtained from the curves shown in Fig. 5a, where the reciprocals of enzyme activity, assayed at different fixed concentrations of the inhibitory substrate, hypoxanthine, were plotted against the reciprocals of the non-inhibitory substrate, PP-ribose-P. The slopes of the curves decreased with increasing hypoxanthine concentration, whereas their intercepts on the ordinate increased after showing an initial decrease in value. A replot of the slopes **versus** the reciprocals of the hypoxanthine concentrations, showed a linear relationship (Fig. 5c), while the intercept replot gave a curve typical of substrate inhibition by hypoxanthine (Fig. 5b). This pattern of inhibition has been termed uncompetitive substrate inhibition (Cleland 1979, Uhr **et al**, 1974), and is characteristic of an ordered sequential reaction mechanism, where

the non-inhibitory substrate binds before the inhibitory substrate i.e. PP-ribose-P binds before hypoxanthine. Two mechanisms have been proposed to explain uncompetitive substrate inhibition (Cleland 1979, Northop and Cleland 1974). Firstly, the inhibitory substrate may bind to the central complexes formed during the reaction and inhibit catalysis or product release. Secondly, the inhibitory substrate may interact with an enzyme-product intermediate and prevent the release of the product from the enzyme. Double inhibition experiments may be used to distinguish between these possibilities (Cleland 1979, Northop and Cleland 1974). In the particular case of the variant HPRT, hypoxanthine could interact with the central complexes, enzyme-hypoxanthine-PP-ribose-P or enzyme-IMP-PP_i. Alternatively hypoxanthine could interact with an enzyme-PP_i intermediate to give a dead-end complex. Double inhibition assays of the variant enzyme were performed at hypoxanthine concentrations of between 150 and 350μM. These concentrations of hypoxanthine are well above the K_i of 80$\pm$ 20μM. When the product PP_i was used as a second inhibitor, plots of the reciprocals of enzyme activities **versus** inhibitory substrate concentration showed a series of straight lines, the slopes of which increased with increasing concentrations of PP_i (Fig. 9a). The data were fitted to equation 3, and a value for ß of 0.69 was obtained. ß is the interaction constant of the inhibitors; the product PP_i and the substrate hypoxanthine. A value for ß of unity is expected if the two inhibitors acted independently. Higher values indicate repulsive interaction. The actual value obtained for ß, indicates positive interactions between PP_i and hypoxanthine (Northop and Cleland 1974, Yonetani and Theorell 1964), resulting in observed inhibition higher than that expected by simple summation.

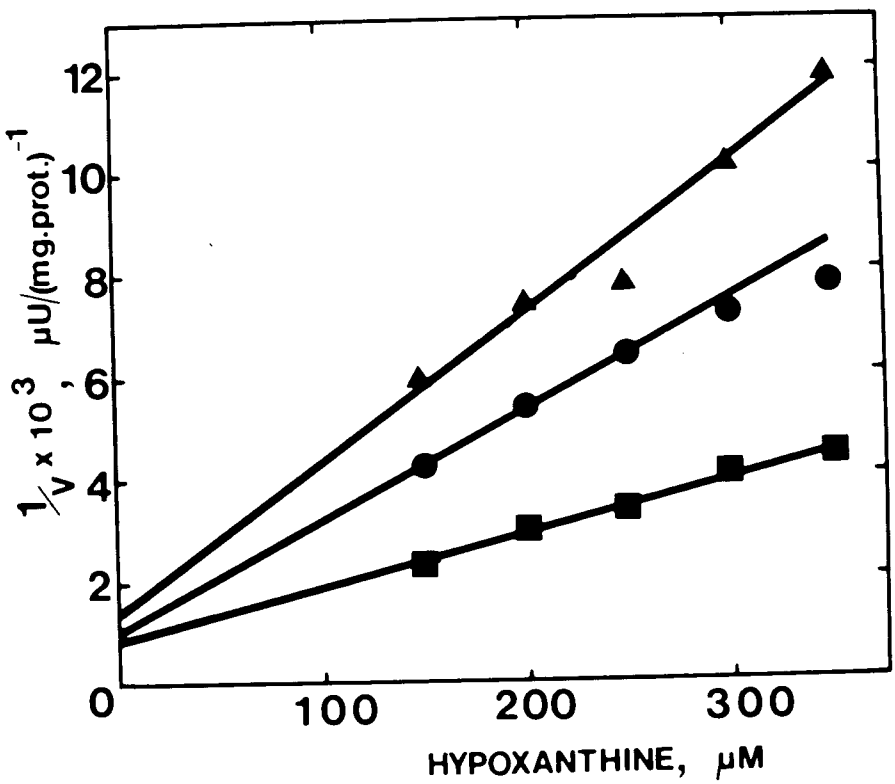


Fig. 9a. Double inhibition of variant HPRT activity by hypoxanthine and pyrophosphate. Enzyme activity was determined at hypoxanthine concentrations well above the K_i for hypoxanthine and at different fixed concentrations of pyrophosphate. The pyrophosphate concentrations were: (■), nil; (●), 0.5mM; (▲), 1mM. The PP-ribose-P concentration was 1.4mM. All other conditions were as described under 'Methods'.

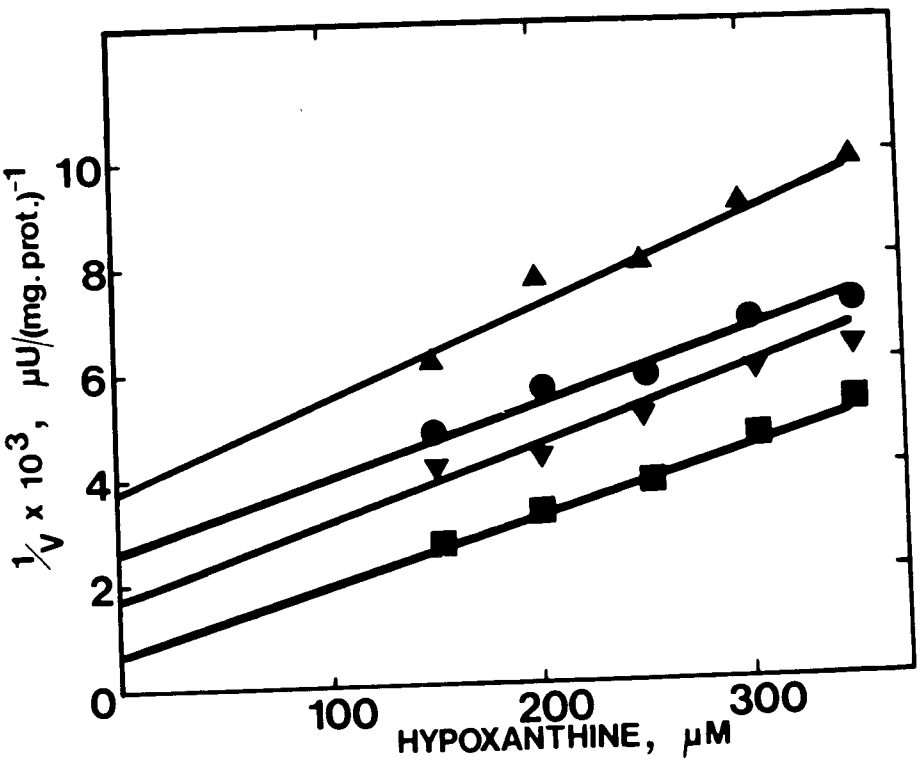


Fig. 9b. Double inhibition of variant HPRT activity by hypoxanthine and IMP. Enzyme activity was determined at hypoxanthine concentrations well above the K_i for hypoxanthine and at different fixed concentrations of IMP. The IMP concentrations were: (■), nil; (▼), 50µM; (●), 100µM; (▲), 200µM. The PP-ribose-P concentration was 1.4mM. All other conditions were as described under 'Methods'.

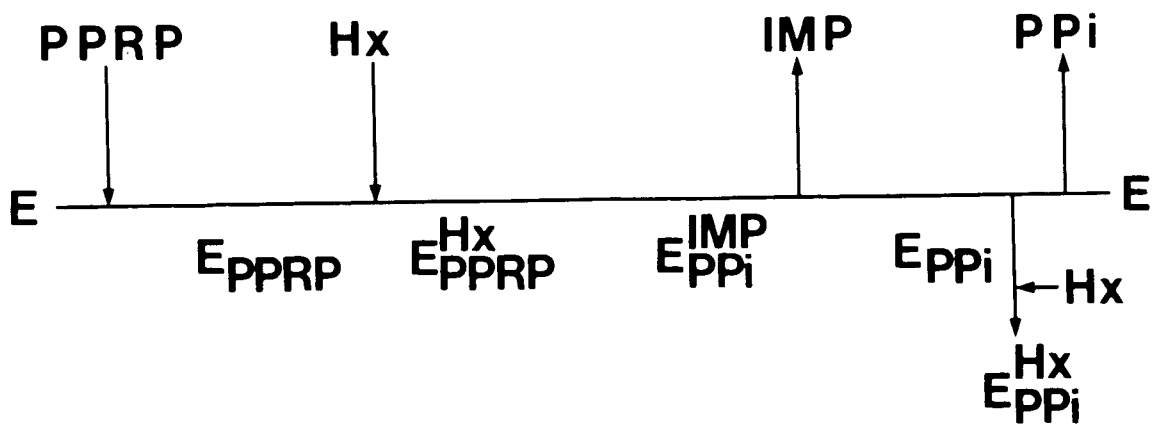


Fig. 10. The proposed reaction mechanism for the variant HPRT at high concentrations of hypoxanthine.

When IMP was used as the second inhibitor, a series of almost parallel lines was obtained (Fig. 9b). The value of ß in this case was greater than 6, showing that IMP and hypoxanthine interacted repulsively, which is to be expected if they bound at the same site. Thus the double inhibition experiments demonstrate that the inhibitory substrate, hypoxanthine binds to an enzyme-PP_i intermediate to form an enzyme-hypoxanthine-PP_i complex, which is responsible for the loss of enzyme activity at high hypoxanthine concentrations (Fig. 10).

4.5. Discussion

The values of the $K_{m(app)}$ and $V_{max(app)}$ obtained for both the variant and control HPRT activities with each of the substrates are similar (Table 2). These values, and those obtained for the true K_m, true V_{max} of the variant enzyme, are in close agreement with those published for human erythrocyte HPRT (Henderson **et al** 1968, Krenitsky and Papaioannou 1968, Benke **et al** 1973). Thus the low activity obtained for the variant at high purine base concentration, unlike many of the previously described low activity variants, was not due to changes in the kinetic parameters, or to any methodological factors which would influence the activity of the control enzyme as well. Instead, the low activity was caused by total substrate inhibition (Cleland 1979) of the enzyme activity by the purine base substrates (Fig. 4a, inset). The kinetics of the enzyme activity and its dependence on

the concentration of PP-ribose-P at different fixed concentrations of hypoxanthine (Fig. 5a, b, c), is compatible with uncompetitive substrate inhibition and is associated with an ordered sequential reaction mechanism (Cleland 1979). The positive interaction between the inhibitory substrate hypoxanthine, and PP_i, inferred from a ß value of less than one, is compatible with hypoxanthine interacting with an enzyme-PP_i intermediate. A proposed reaction mechanism for the variant HPRT is represented diagramatically in Fig. 10. The binding of the substrates is ordered sequential, with PP-ribose-P binding before hypoxanthine. After the release of IMP, hypoxanthine binds to the enzyme pyrophosphate intermediate. Pyrophosphate is unable to dissociate from this complex and a dead-end complex is formed. The plot of the reciprocal activity **versus** hypoxanthine concentration (Fig. 4a, inset) is characteristic of that associated with the formation of a dead-end complex (Cleland 1979). In the case of the normal enzyme, hypoxanthine is unable to bind to the enzyme-pyrophosphate intermediate. A random, sequential release of products as proposed for the normal enzyme by Giacomello and Salerno (1978), would not preclude this mechanism of inhibition, as an enzyme pyrophosphate intermediate would still be formed. It is interesting to note that the binding of the substrates to the variant enzyme is ordered sequential at ratios of magnesium to PP-ribose-P of greater than five to one, at which the normal enzyme has been reported to have a ping-pong mechanism (Krenitsky and Papaioannou 1968). Substrate inhibition has not been reported as a cause of decreased activity in assays for HPRT, nor, to my knowledge, has substrate inhibition been associated with a decrease in enzyme activity in any other metabolic disorder.

There are several possible mechanisms for the aquisition of substrate inhibition in the variant enzyme:

1) a mutation in the coding sequence, or defective post-translational modification of the mRNA, for the enzyme;

2) altered post-translational modification of the enzyme;

3) deficiency or absence of an effector molecule required for normal HPRT activity. The molecular defect in $HPRT_{London}$ has been defined as an amino acid substitution (serine-to-leucine) in position 109 (Wilson **et al** 1983). Wilson **et al** (1982), using isoelectric focusing, have shown differences in post-translational modification of HPRT from red blood cells and from lymphoblasts in a number of different patients. Hora **et al** (1982) have presented evidence of a non sex-linked HPRT deficiency, which implies a defect separate from the HPRT gene on the X-chromosome. Wohlhueter (1975), has demonstrated different kinetics for HPRT in post-microsomal supernatants from rat livers and hepatoma cell cultures. It is interesting to note that the rat liver enzyme appeared to show substrate inhibition by hypoxanthine. Cultured cells from the patient T.K. showed approximately 10% of the relative incorporation of ^{14}C-labelled purine substrates to ^{3}H-thymidine into acid precipitable material, as compared to control cell lines (11.5). This indicates a deficient intracellular activity of the variant HPRT. Whether this reflects intracellular behavior similar to that observed in vitro, is not known, but if this is the case, these results imply an intracellular hypoxanthine concentration of greater than 300μM at the vicinity of the active site.

Detailed protein structural studies will be required to define the molecular basis of the substrate inhibition observed in vitro, therefore the term 'variant enzyme ' has been used throughout as an operational definition.

5. THE CONTINUOUS SPECTROPHOTOMETRIC ASSAY OF HPRT ACTIVITY

5.1. Materials

The enzymes, pyruvate kinase (200U/mg, 2mg/ml), lactate dehydrogenase (300U/mg, 5mg/ml), and guanosine-5'-monophosphate kinase (10U/mg, 2mg/ml), as well as GMP and NADH, were obtained from Boehringer Mannheim, while ATP, guanine, phosphoenolpyruvate and PP-ribose-P (sodium salt) were obtained from Sigma. All other chemicals were of analytical grade.

5.2. Enzyme extracts

The enzyme extracts used in this assay were prepared in a similar manner to those used in the multiple end point assay. Alternatively, the partially purified variant HPRT activity was used.

5.3. Methods

In this assay the HPRT activity is linked via a marker enzyme system to the oxidation of NADH. (Giacomello and Salerno 1977). The GMP that is formed by the activity of HPRT, is converted to GDP by the action of guanosine-5'-monophosphate kinase, with ATP as co-substrate. The GDP is phosphorylated to form GTP, and the ATP regenerated by the activity of pyruvate kinase on the

dinucleotides and on phosphoenolpyruvate. This reaction also results in the formation of pyruvate, which is reduced to lactate by the lactate dehydrogenase activity, with the oxidation of NADH to NAD^+, the rate of which can be monitored at 340nm. Both the GDP and ADP act as substrates for pyruvate kinase, therefore the NADH is oxidised stoichiometricaly at a ratio of 2:1 for each mole of GMP that is formed. The assays were performed at 37°C, and each assay contained the following in a final volume of 1ml: 50mM tris-HCl, pH 7.5, 40mM $MgSO_4$, 1.4mM PP-ribose-P, 130mM KCl, 160µM NADH, 2U of pyruvate kinase activity, 0.1U of guanosine-5'-monophosphate kinase activity, 7.5U of lactate dehydrogenase activity, 10µl of the enzyme extract. The assay mixture was brought to 37°C for 5 minutes in the cuvette chamber before the reaction was started by adding guanine at the appropriate concentration. There was a lag period of approximately 2 minutes before the reaction became linear. When dialysed haemolysates were used as the source of the enzyme activity, the reaction was monitored in a Varian Techtron Model 635 spectrophotometer with a Corning Recorder 840, whereas when the partialy purified enzyme activity was assayed, a Beckman DU-2 spectrophotometer interfaced with a Data General Micronova computer, was used. Both these systems gave similar results, With the first, however, the results were calculated from the tracing of the change in extinction, whereas with the second the change in extinction was obtained directly. The data were fitted to the appropriate rate equations as was described for the multiple end point assay.

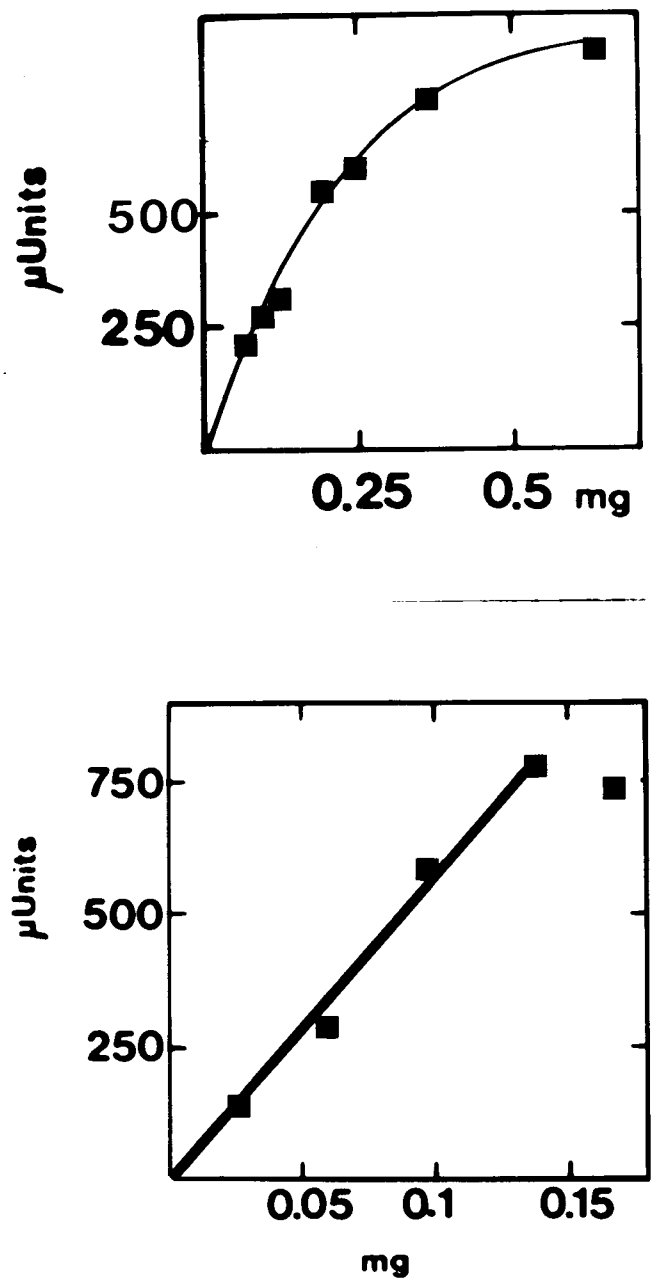


Fig. 11. Continuous spectrophotometric assay: activity of the variant HPRT versus protein concentration. A) Variant HPRT activity in samples of dialysed haemolysate, containing 0.1 to 0.65 mg protein per assay. B) Variant HPRT activity in samples of the partially purified protein, containing 0.03 to 0.16 mg protein per assay.

5.4. Results

5.4.1. Haemolysates

The continuous spectrophotometric assay (Giacomello and Salerno 1977), was used in the initial characterisation of the variant HPRT activity. The enzyme activity in dialysed haemolysates was not linear with protein concentration (Fig. 11a), while a linear relationship was obtained when the enzyme activity was partialy purified (Fig. 11b). The low activity obtained in haemolysates with this assay, led to the assumption that the $V_{max(app)}$ for the variant enzyme with guanine as the purine base substrate, was lower than that of the control enzyme. However, the protein content per assay was twice that in the control enzyme activity assays, a factor that could explain the discrepancy in the V_{max} values. The kinetic parameters for the control and variant enzyme activities, are compared in Table 3. The values for the $K_{m(app)}$ for the control and variant activities are in close agreement with each other, 2.0μM and 1.7μM, respectively. These values are similar to those obtained with the multiple end point kinetic assay, 3.0μM and 2.6μM, respectively (Table 2). A value of 68μM was obtained for the $K_{i(app)}$ for guanine, when the variant enzyme activity was assayed by the continuous spectrophotometric assay, and 28μM, when the multiple end-point assay was used.

Table 3

Kinetic parameters obtained for the variant and control HPRT activities, assayed by the continuous spectrophotometric assay (Giacomello and Salerno 1977)

Enzyme activity	$V_{max(app)}$ μU/mg.prot	$K_{m(app)}$ μM	$Ki_{(app)}$ μM
Variant	229± 26	1.7± 0.5	68± 16
Control	2104± 46	2.0± 0.2	-

5.4.2. Discussion

Similar values for the kinetic parameters of the variant and control HPRT activities for guanine were obtained by the continuous specrophotometric assay and the multiple end-point assay of Wohlhueter (1975), (Tables 2 & 3). Therefore the results obtained with the multiple end-point assay, were reproducible using a second method, and were accepted with a greater amount of certainty.

5.4.3. Effect of partial purification of the variant enzyme on the degree of substrate inhibition obtained with guanine

The degree of substrate inhibition of the variant enzyme activity, was determined from the percentage loss in activity at 120μM guanine compared with that at 20μM guanine. For the partialy purified activity it varied between 12% and 28%, while values of 25% and 33% were obtained for the activity in haemolysates. These findings show that the degree of inhibition in both enzyme preparations was similar. The enzyme activity in haemolysates was more stable than that in the partially purified form and could be used for the further characterisation of the variant enzyme. Similarly the results obtained by Henderson **et al** (1968), with the HPRT activity in dialysed haemolysates, were similar to those obtained by Krenitsky and Papaioannou (1968), with a partially purifed HPRT preparation.

6. THE pH DEPENDENCE OF HPRT ACTIVITY

6.1. Methods

The pH dependence of the variant and control HPRT activities, was determined for pH ranging from pH 7.2 to pH 10. The buffer used was tris-HCl (25mM), and glycine (25mM), at the appropriate pH. The magnesium concentration was kept constant at 5mM, and the PP-ribose-P concentration was 1.4mM. The purine base substrate was ^{14}C-hypoxanthine (350µM, 5.5 dpm/pmol), in a final assay volume of 100µl. The reaction was started by adding 20µl of enzyme extract and the amount of ^{14}C-IMP formed after 4 minutes was determined. An aliquot of 20µl of the reaction mixture was placed on DE81 paper, the unreacted hypoxanthine removed by washing with tap water and the radioactivity, remaining on the paper, was measured.

6.2 Results and Discussion

Both enzyme activities had a broad pH optimum (Fig. 12). The maximal activity for the variant enzyme was found between a pH of 8.4 and 9.2, while that for the control enzyme was between pH 8.4 and pH 10. Because of the low enzyme activity below pH 7.5, the enzyme assays were performed at pH 7.8 in the kinetic studies on the variant HPRT. A similar, broad pH optimum for erythrocyte HPRT activity has been reported (Krenitsky **et al** 1968).

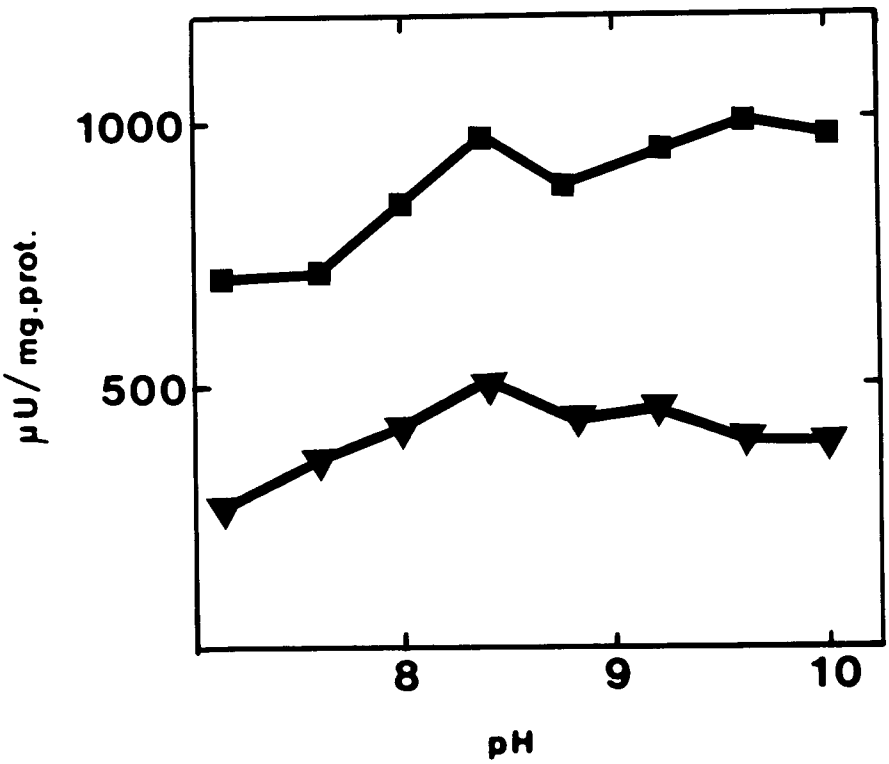


Fig. 12. The pH dependence of the variant and control HPRT activities. Enzyme activity was determined in a tris-glycine buffer at the appropriate pH, as has been described under 'Methods'. (■) control, (▼) variant.

7. MAGNESIUM DEPENDENCE OF HPRT ACTIVITY

7.1. Methods

The effect of the magnesium concentration, at a constant concentration of PP-ribose-P, on the variant and control enzyme activities, was measured by an end point assay. The assay mixture contained the following in a final volume of 100µl: 50mM tris-HCl, pH 7.8, 1mM PP-ribose-P, 350µM ^{14}C-hypoxanthine (5.5 dpm/pmol), and $MgSO_4$ at the appropriate concentration. The reaction was started with 20µl of enzyme extract and the amount of IMP formed after 4 minutes, was determined. The curve of enzyme activity **versus** magnesium concentration was normalised by dividing the enzyme activity at each magnesium concentration by the activity at the optimal magnesium concentration.

7.2. Results and Discussion

The curves obtained for the variant and control enzyme activities were similar, the maximal activity occurring at magnesium concentrations of between 25-100mM (Fig. 13). Dimagnesium PP-ribose-P is the true substrate for the enzyme (Salerno and Giacomello 1981). The HPRT assays, used in the characterisation of the variant enzyme, were performed at magnesium concentrations of 10-20 times higher than the PP-ribose-P concentrations.

The inhibition of enzyme activity at magnesium concentrations of greater than 100mM is thought to be due to the competition of free magnesium ions with dimagnesium PP-ribose-P for binding sites on the enzyme (Salerno and Giacomello 1981).

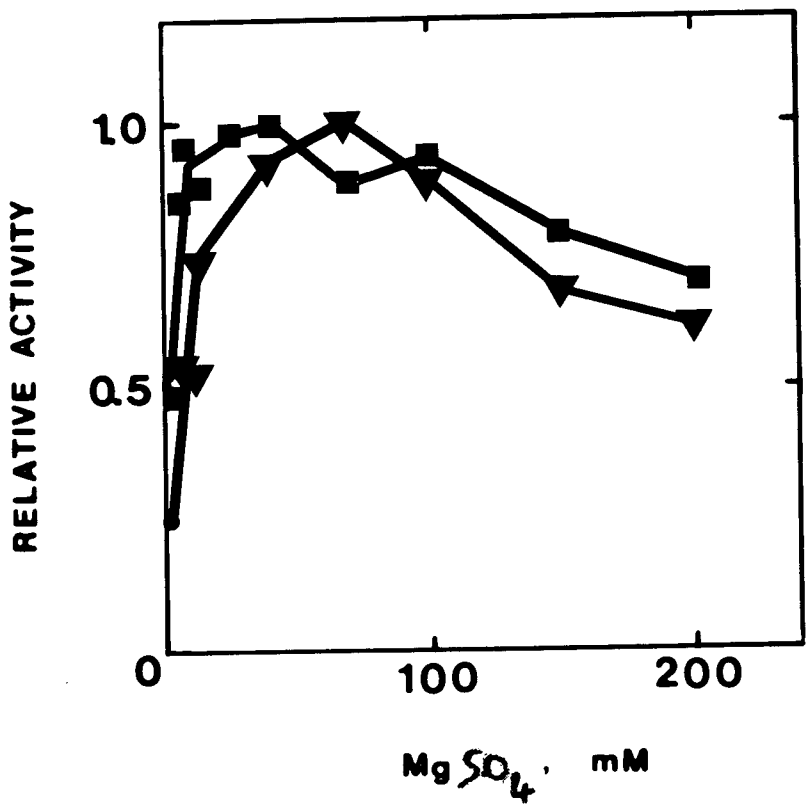


Fig. 13. Effect of magnesium concentration on the variant and control HPRT activity. The enzyme activities were determined at a fixed concentration of PP-ribose-P of 1mM, and $MgSO_4$ concentrations of between 1 to 200mM. All other conditions were as described under 'Methods'. (■) control, (▼) variant.

8. EFFECT OF MIXING HAEMOLYSATES ON HPRT ACTIVITY

8.1. Methods

Haemolysates obtained from the patient T.K. and from a normal individual were diluted with 50mM tris-HCI, pH 7.8, to give approximately 200μU of HPRT activity at 300μM hypoxanthine, per 20μl of haemolysate. Equal aliquots of each haemolysate were mixed and preincubated on ice for 30 minutes and the HPRT activity was assayed.

8.2. Results and Discussion

The HPRT activity predicted in the mixture of haemolysates was 396μU/mg prot., and values of 383 and 405μU/mg prot. were obtained (Table 4) . This demonstrated that there was no inhibition of the control HPRT activity, nor was there activation of the variant enzyme activity under these conditions. Although the mixture contained more than three times the haemolysate protein from T.K. than from the control, the HPRT activities contributed by both enzymes, was equal at the hypoxanthine concentration used. Therefore any HPRT inhibiting factor in the T.K. haemolysates should have been detected. Bakay and Nyhan (1972), have demonstrated the activiation of a number of variant HPRT activities by the enzyme activities from normal individuals. However, activation of the variant activities was only observed if the enzyme preparations

were preincubated under denaturing conditions or passed through a column.

Table 4

Effect on HPRT activity of mixing haemolysates from the patient T.K., and a from a normal individual. The HPRT activity in a mixture of haemolysates from T.K. and a normal individual, was assayed after the mixture had been preincubated at 0°C for 30 minutes.

Enzyme	HPRT activity (μU/mg prot.)		Protein (mg/assay)
	Measured	Predicted	
Variant	283	-	.75
	289	-	
Control	986	-	.22
	977	-	
Mixture	383	396	.56
	405		

9. PARTIAL PURIFICATION OF HPRT

9.1. Method

The HPRT activities from haemolysates, obtained from T.K. and a normal individual, were partially purified on a DEAE anion exchange column (Agarwal **et al** 1978). DEAE-cellulose (Whatman) with the fines removed, was poured into a column, 30 x 1.5cm, and equilibrated with K_2HPO_4 (1mM, pH 7.4). The haemolysates were prepared by lysing washed red blood cells with cold, distilled water (1:1), and the stroma removed by centrifugation. The supernatant was diluted with K_2HPO_4 (1mM, pH 7.4), and applied to the column at a rate of 1.2ml/minute. The column was washed with 4 volumes of the buffer. The bound protein was eluted off the column with a salt gradient of 1mM to 500mM of K_2HPO_4, pH 7.4, at a flow rate of 25ml/hr, and fractions of 5ml were collected. The HPRT activity in each fraction was determined by the $LaCl_3$ precipitation assay (Johnson **et al** 1977) and all fractions containing HPRT activity were pooled. The pooled fractions were stirred with hydroxyapatite (2g/100ml) for two hours, and filtered on a Büchner funnel. The filtrate was brought to 60% saturation with $(NH_4)_2SO_4$ and the protein was precipitated overnight. The precipitate was redissolved in 2ml of 50mM tris-HCl, pH 7.4, and dialysed against two changes of 2 litres of 50mM tris-HCl, pH7.4, containing DTT (0.8mM). The HPRT activity in this dialysate is referred to as 'partially purified'.

9.1 Results

The protein bound to the column eluted in three major peaks; the HPRT activity of both the variant and control enzymes eluted before the first protein peak, immediately after the gradient was started. The specific activity of the variant enzyme increased from 198 to 3600μU, an 18 fold purification. However only 8% of the enzyme activity was recovered, while there was a 60% recovery of the control enzyme.

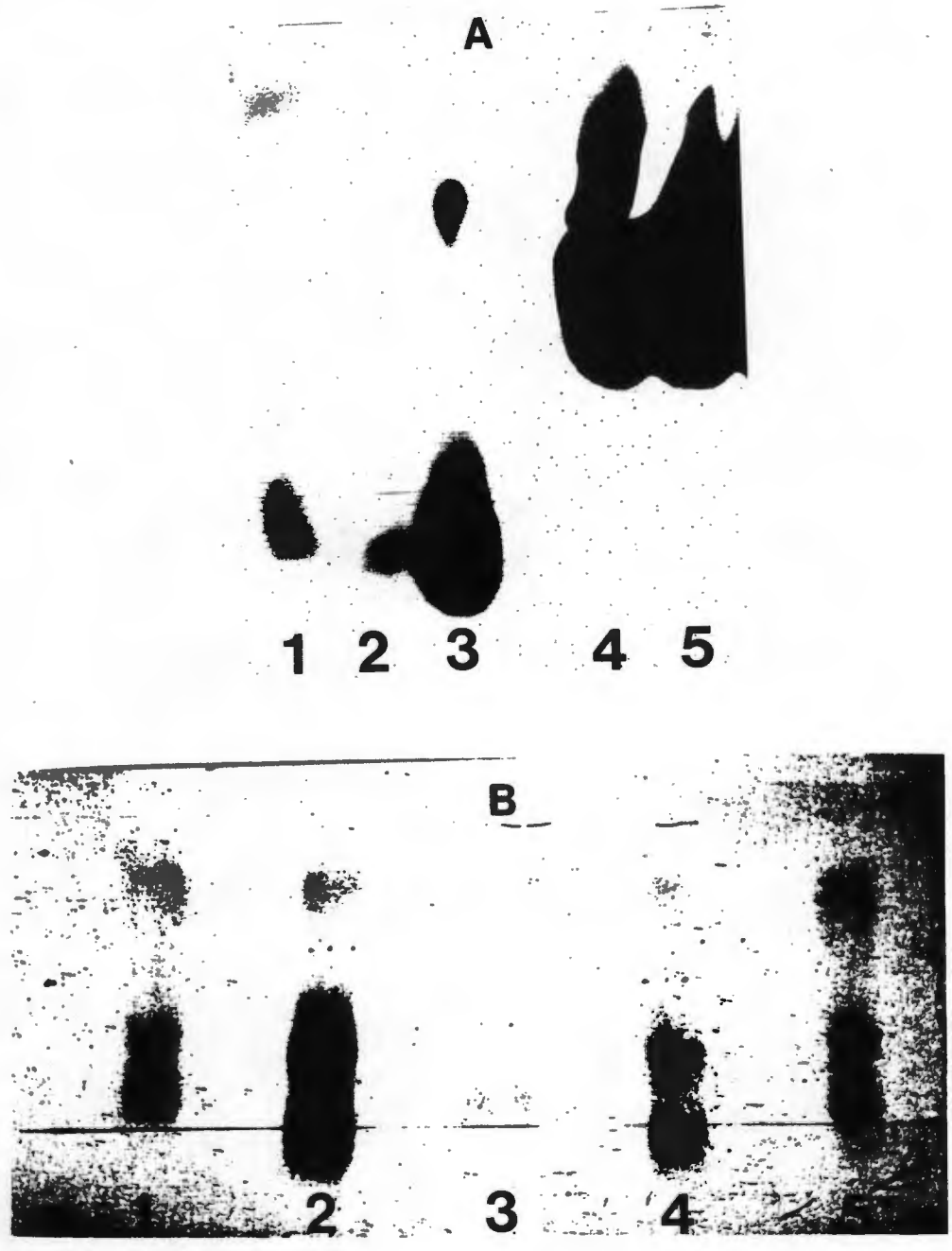


Fig. 14. Autoradiographs of polyacrylamide gel electropherograms of haemolysates from patient T.K., three Lesch-Nyhan Syndrome patients and normal controls. HPRT activity was detected using ^{14}C-labelled hypoxanthine and by selectively precipitating the product, IMP, with $LaCl_3$.
A) 9% polyacrylamide gel: lanes 1 and 2, two brothers with the Lesch-Nyhan Syndrome; lane 3, a third, unrelated, Lesh-Nyhan Syndrome patient; lane 4, control; lane 5, T.K.
B) 5-20% polyacrylamide gel: lanes 1 and 5, T.K.; lanes 2 and 4, normal control; lane 3, partially purified HPRT activity from T.K.

10. ELECTROPHORESIS OF RED BLOOD CELL HPRT

10.1. Methods

Haemolysates, prepared from the patient T.K. and a normal individual, were diluted 1:10 with cold, distilled water and were mixed with an equal volume of 50% glycerol, 1M tris-HCl, pH 7.4, and bromophenol blue and a sample of 100µl was applied to the gel. Undiluted haemolysates from three patients with the Lesch-Nyhan Syndrome (blood by courtesy of Prof. T. Jenkins, Dept. of Genetics, University of the Witwatersrand), were treated in a similar manner. The samples were electrophoresed for 20 hours at 20 mA and 150 V, at 4°C. The HPRT activity present in the gels, was detected by placing a sheet of DE81 paper on the gel, and adding 10ml of staining buffer, and incubating at 37°C for 1 hour. The staining buffer consisted of 50mM tris-HCl, pH 7.4, 4mM $MgSO_4$, 1.4mM PP-ribose-P, and 14µM ^{14}C-hypoxanthine (55mCi/mmol). The ^{14}C-IMP that was formed in the gel, was precipitated with $LaCl_3$ (0.1M) for 2 hours. The unreacted ^{14}C-hypoxanthine was washed out of the gel overnight. The gel was dried between two layers of dialysis membrane, and the ^{14}C-IMP was detected by autoradiography at -20°C for 72 hours. The ^{14}C-IMP bound to the DE81 paper, was detected in a similar manner after the unreacted substrate had been washed off the paper with 25 litres of distilled water. The autoradiographs obtained by these methods were of a similar quality.

10.2. Results and Discussion

The variant and control HPRT activity migrated for the same distance during the electrophoresis in a 9% polyacrylamide gel (Fig. 14a), whereas the activity from patients with the Lesch-Nyhan Syndrome migrated further. In addition the activity from one of these patients, unrelated to the other two, had a slower migrating component. The rapid migration of HPRT activity from patients with the Lesch-Nyhan Syndrome has previously been described (Bakay and Nyhan 1972). When electrophoresed in a 5-20% gradient gel the variant HPRT activity migrated more slowly than the control activity (Fig. 14b). Both the variant and control activities consisted of at least three components, while in the partialy purified form of the variant activity only the fastest moving component was visible. The heterogeneity of the HPRT activity in haemolysates has been described before (Arnold and Kelley 1971, Bakay and Nyhan 1972). The electrophoretic mobility of the variant and control enzyme activities differed in a polyacrylamide gradient gel, where differences in molecular size may be expected to play a greater role in determining the rate of migration of the protein, than the charge.

SECTION 2

11. EXPERIMENTS WITH CULTURED CELLS

11.1. Materials

The following radioactive substrates were obtained from The Radiochemical Centre, Amersham: [8-^{14}C]-hypoxanthine (55mCi/mmol), [G-^{3}H]-hypoxanthine (1.2Ci/mmol), [8-^{14}C]-guanine sulphate (51mCi/mmol), [8-^{3}H]-guanine sulphate (7Ci/mmol), [^{14}CG]-formic acid sodium salt (60.3mCi/mmol), [8-^{14}C]-adenine (296mCi/mmol), [methyl-^{3}H]-thymidine (47Ci/mmol), and L-[4,5-^{3}H]-leucine (52Ci/mmol). The biochemicals hypoxanthine, guanine, adenine and thymidine, as well as the purine analogues 6-thioguanine and 8-azaguanine, were obtained from Sigma. Mycophenolic acid was a gift from Lilly Research Laboratories. Amethopterin sodium (Methotrexate), was obtained from Lederle Laboratories. The 'purine-free' F10 was a gift from Flow Laboratories, while the other media and the foetal calf serum were obtained from Gibco Europe or Flow Laboratories. The transformed lymphoblast cell lines, L106 and L107, were obtained from The Human Genetic Mutant Cell Repository, New Jersey.

11.2 Methods

11.2.1. General

All cells were grown in a Hotpack CO_2 incubator at 37°C with a relative humidity of 90% and CO_2 concentration of 4%. The medium used was Ham's F10, supplemented with 15% foetal calf serum. Long term cultures were maintained in the absence of antibiotics to minimise the risk of mycoplasma contamination and were screened for such contamination every 3 to 4 months. Antibiotics, when used, were sodium benzyl penicillin (30µg/ml), streptomycin sulphate (50µg/ml) and neomycin sulphate (50µg/ml).

11.2.2. Establishment of EBV-transformed lymphoblast cultures

Permanent B-lymphoblast cell lines were established from the patient's peripheral blood by incubating the mononuclear cells, obtained by a modification of the method of Boyum (1968), with medium containing Epstein-Barr Virus (EBV). Five to ten millilitres of heparinised or EDTA blood was placed on 7ml of Ficol-Paque (Pharmicia) in a 15ml tube and centrifuged at 1000g for 15 minutes at room temperature. About 2ml of the fluid at the plasma/Ficol-interface, containing mostly mononuclear cells and platelets, was removed. The cells were pelleted and washed once with 1ml of medium. This wash removed most of the contaminating Ficol-Paque, as well as a large number of the platelets. The cells were resuspended in 3ml of EBV-medium and incubated in 25cm^2 tissue culture flasks. The incubated cells consisted of a mixture

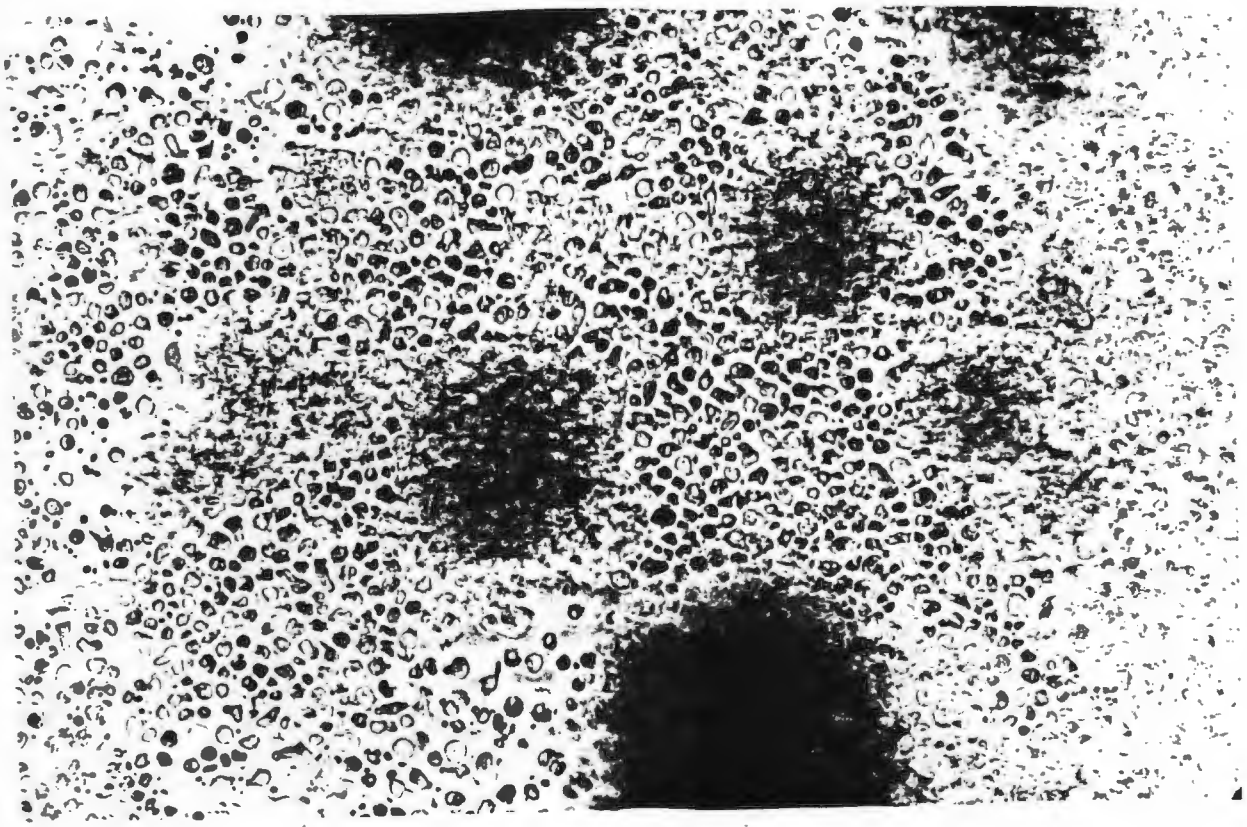


Fig. 15. Appearance of transformed lymphoblasts in a stock culture. The cells can be seen growing singly or in colonies (Magnified 100x).

of B-lymphocytes, T-lymphocytes and macrophages. The two latter cell-types survived for approximately 2 weeks in culture, while the transformed B-lymphoblasts could be seen growing in suspension, singly or in colonies of 3 cells or more, after 7 to 30 days. No fresh medium was added during the first week of incubation, after that aliquots of 2ml of medium were added at 3 day intervals until the transformed cells divided rapidly. Aliquots of medium were removed and replaced with fresh medium. When the transformed lymphoblasts reached a density of $100\text{-}400x10^3$/ml, half of the cells were transferred to other flasks. Care was taken to avoid over-diluting the cells, as this inhibited their growth rate. The medium of the rapidly growing cells was changed on alternate days. The cells were resuspended by gentle agitation, and half of the medium was replaced by an equal volume of fresh medium. The cell density was maintained at between $100\text{-}400x10^3$/ml. The appearance of cultured, transformed lymphoblasts is shown in Fig. 15.

11.2.3. Preparation of medium containing EBV.

Marmoset B95.8 cells were a gift from Dr. L. Wilson, Dept. of Immunology, U. C. T. These leukocytes secrete large amounts of EBV into their growth medium (Miller and Lipman 1973). Medium for transforming B-cells was prepared as follows: B95.8 cells were grown in large tissue culture flasks until they were confluent (unlike human transformed B-cells, they adhere to the culture flasks). The medium was replaced with 15ml of fresh medium and the cells were grown in this medium for a week. The medium was then

centrifuged at 3000 rpm for 10 minutes and filtered through a Millipore filter (0.45μ pore size) to remove any marmoset cells. The EBV-containing medium was diluted with an equal volume of fresh medium and used to transform the human, peripheral B-cells.

11.2.4. Establishment of fibroblast cultures.

A sterile, skin biopsy (3x3mm), was taken from the forearm of the patient, placed into culture medium, and transported to the tissue culture laboratory. The biopsy was cut up into smaller sections (less than 1x1mm). These were placed under glass coverslips in 35x10mm petri dishes containing 2ml of medium and antibiotics. After 7-14 days the first fibroblasts appeared, a week later they had increased in number sufficiently for the coverslip to be removed. The cells were dispersed by trypsinisation, resuspended in fresh medium, and transferred to a second petri dish. The dispersion was necessary to minimise the contact inhibition of the fibroblasts. When the cells reached confluence they were trypsinised and transferred to a $25cm^2$ tissue culture flask. This was defined as the first passage. Cells were passaged when confluent (3:1 split ratio). The medium was changed every 3-4 days. Early passage cells were frozen in fresh medium, containing 10% glycerol and stored in liquid nitrogen until needed.

11.2.5. Microscopy.

The visual appearance of the cells was monitored daily, using phase contrast, with an inverted microscope (Nikon).

11.2.6. Determination of lymphoblast cell number in stock cultures

The number of lymphoblasts was determined by counting samples of the cells in aliquots of medium, diluted 1/100 or 1/200 with 'Isoton' (Coulter Electronics, S.A.), on a Coulter Counter Model Z (Coulter Electronics, Inc. Hialeah, Florida). The settings were selected to exclude the small vesicles, formed by the cells from being counted.

11.2.7. Protein determination

The protein concentration in the fibroblast culture wells was determined by a modification of the method of Lowry **et al** (1951), with BSA as the standard.

11.2.8. Lymphoblast DNA extraction

Lymphoblast DNA was extracted by the method of Kunkel **et al** (1977). The cells were washed three times with 1ml of cold, 0.9% NaCl and resuspended in 1ml of lysis buffer (0.32M sucrose, 5mM $MgCl_2$, 1% triton X-100 and 10mM tris-HCl, pH7.6). After lysis the insoluble material was pelleted and resuspended in 200µl of cold saline/EDTA (75mM NaCl, 25mM EDTA, pH8.0), and 20µl of 10% SDS was added. Ten microlitres of pronase (10mg/ml) was added and the

samples incubated for 1 hour at 37°C. After digestion, 20µl of herring sperm DNA was added as a cold carrier, and 10µl of 5M sodium perchlorate. Two hundred microlitres of phenol was mixed with the samples. After centrifugation, the upper aquous layer was extracted twice with 200µl of chloroform/octanol (24:1), and the DNA was precipitated overnight with 500µl of absolute ethanol at -20°C. The precipitated DNA was redissolved in 100µl of tris/EDTA (10mM tris-HCl and 1mM EDTA, pH7.8), at 37°C for 15 minutes. All extractions were carried out at room temperature in 2ml Eppendorf tubes. Centrifugation was done in an Eppendorf Centrifuge 5412, for 3 minutes.

11.3. Designation of the lymphoblast cell lines.

The origin of the various, transformed B-lymphoblasts and their designation was as follows:

Patient T.K.	L45
Patient with the Lesch-Nyhan Syndrome	L106
Mother of the patient with the Lesch-Nyhan Syndrome	L107

Cell lines used as controls were acquired from patients with disorders not affecting their purine metabolism, for example L59 was aquired from a patient with the Sickle Cell Trait

11.4. Selective media experiments.

11.4.1. Transformed lymphoblasts.

11.4.1.1. Method

Lymphoblasts for selective media experiments were taken from stock cultures. The number of cells per millilitre of the stock cultures was determined, and aliquots containing approximately 150 000 cells, were centrifuged in culture tubes (Falcon 2054 with a cap). The cells were resuspended in 0.5ml of medium, containing the desired selective agent. The initial number of cells was checked by counting the cells in 0.1ml of the medium. The medium was changed every 3-5 days, and the number of cells was determined as has been described. These cultures were generally continued for two weeks. Reference cultures of each cell line, maintained under the same conditions with the same medium changes, were used to determine cell growth in the absence of selective agents. Initially all experiments were performed in medium containing hypoxanthine and thymidine, but were repeated in F10 medium, without these constituents, when this became available. Their presence or absence was not found to influence the actions of the selective agents. Undialysed, foetal calf serum was used in all these experiments, as it was found that the lymphoblasts did not grow well in medium containing dialysed serum. Each experiment was performed in duplicate.

11.4.1.2. Evaluation of cell viability

The viability of the lymphoblasts in selecive media was demonstrated by their ability to grow in such media. However, the incorporation of ^{3}H-thymidine into TCA precipitable material was used as an additional index of lymphoblast viability. After the lymphoblasts had been cultured in the selective medium for 10-14 days the number of cells per tube was calculated from a sample of 0.1ml of the medium. The remaining cells were pelleted and resuspended in 0.5ml of fresh medium, without any selective agents, and cultured overnight in the presence of 1 µCi/ml of ^{3}H-thymidine. The incorporation of tritium label into DNA was determined by precipitating the DNA with 5% TCA onto GF/C circles as described for the double lable experiments (see 11.5). The amount of tritium incorporated into the precipitable material was found to correlate with the cell number.

11.4.1.3. Results

11.4.1.3.1. HAT-medium

HAT-medium consists of hypoxanthine, aminopterin and thymidine. Aminopterin inhibits **de novo** purine synthesis, as well as the formation of thymidine from dUDP, and therefore makes cells dependent on the salvage of hypoxanthine and thymidine for their purine and pyrimidine requirements (Littlefield 1964). The salvage of

Table 5

The effect of HAT-medium on transformed lyphoblast growth. The % change in the number of cells, cultured for 13 days in the presence of the various components of HAT-medium, is shown for L45 and for a control cell line.

Medium	% Change in cell-number	
	L59	L45 (T.K.)
F10	+500	+650
F10+ Aminopterin (10μM)	+200	- 75
F10+ Aminopterin (10μM) + Thymidine (400μM)	+475	- 75
F10+ Aminopterin (10μM) + Hypoxanthine (200μM)	+200	- 75
F10+ Aminopterin (10μM) + Hypoxanthine (200μM) + Thymidine (400μM)	+425	- 75
F10+ Aminopterin (10μM) + Guanine (100μM)	+125	- 75
F10+ Aminopterin (10μM) + Guanine (100μM) + Thymidine (400μM)	+175	- 60

Table 6

The growth of transformed lymphoblasts in HAT-medium.
The % change in the number of cells, cultured for 12 days in F10 medium with additional hypoxanthine (200μM), and thymidine (400μM), in the presence of aminopterin, is shown for L45 and as well as number of control cell lines.

Cell Line	Medium			
	Purine Containing		Purine Free	
	F10	F10+ HAT	F10	F10+ HAT
L45 (T.K.)	+375	- 75	+350	- 50
L106 (HPRT$^-$)	+300	- 75	+850	- 40
L59	+550	+425	-	-
L57	-	-	+100	+ 60
L107	+375	+375	+550	+475

The 'purine-free' medium is prepared without hypoxanthine and thymidine, while the 'purine-containing' medium contains 30μM hypoxanthine and 3μM thymidine.

Table 7

The effect of HAT-medium on ^{3}H-thymidine uptake by transformed lymphoblasts. Lymphoblasts were cultured in HAT-medium for 11 days. They were then cultured for 17 hours in the presence of 1μCi/ml of ^{3}H-thymidine and the incorporation label into TCA precipitable materal was determined.

Cell Line	dpm x10^2		Cell Number x10^3	
	F10	F10+HAT	F10	F10+ HAT
L45 (T.K.)	140	0.9	151	31
L46	270	486	316	340
L43	170	212	134	110
L31	508	831	324	275

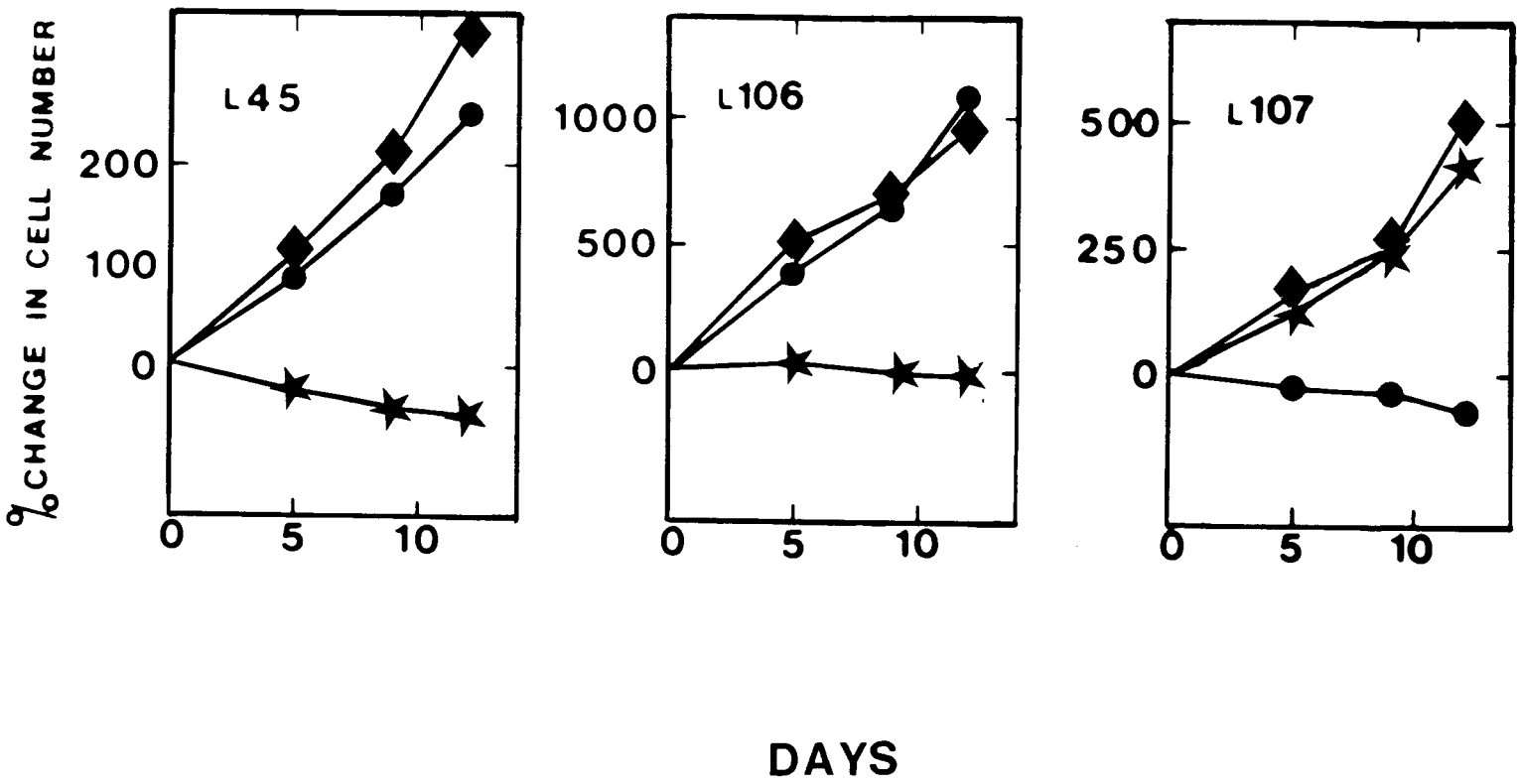


Fig. 16. Effect of selective media on the growth of transformed lymphoblasts. Transformed lymphoblasts were cultured for 14 days in medium containing: (◆), no additions; (★), HAT; (●), thioguanine. L45, cells from T.K.; L106, cells from a patient with the Lesch-Nyhan Syndrome; L107, cells from the mother of the patient with the Lesch-Nyhan Syndrome.

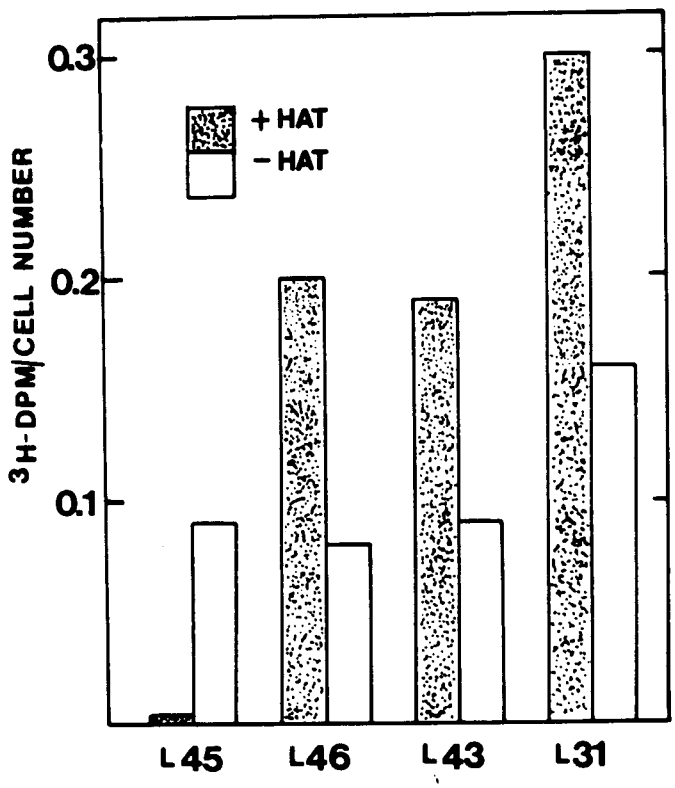


Fig. 17. Incorporation of ^{3}H-thymidine into the TCA precipitable material of transformed lymphoblasts after they had been cultured in HAT-medium for 14 days. The cells were resuspended in medium containing ^{3}H-thymidine (1µCi/ml) and the radioactivity incorporated into TCA precipitable material after 17 hours, was determined. Reference cultures of each cell line were maintained under similar culture conditions without HAT-medium. L45, cells from T.K.; L46, L43, and L31, cells from normal controls.

hypoxanthine is dependent on a normal HPRT activity, and that of thymidine is dependent on thymidine kinase activity. A deficiency in either of these enzyme activities results in the failure of the cells to grow in HAT-Medium. The effect of culturing lymphoblasts in the presence of aminopterin is shown in Table 5. The rate of increase in the number of the control lyphoblasts over a period of 13 days, was reduced, while an actual reduction in the number of the L45 lymphoblasts was observed. The effect of aminopterin on the control cell line was reversed by the presence of hypoxanthine and thymidine in the medium, while that on the L45 cells was unchanged. The F10 medium used in this experiment was not entirely free of hypoxanthine, the addition of thymidine alone was therefore adequate to reverse the effects of the aminopterin on the control cell line. The effects of culturing the L45 and L106 lymphoblasts in HAT-medium, are shown in Table 6 and in Fig. 16. While neither of these cell lines was able to grow, the growth of the control cell lines was maintained at a similar rate to that of the cells cultured in the absence of HAT-medium. The viability of the lymphoblasts after being cultured in HAT-medium, was also determined by their ability to incorporate ^{3}H-thymidine into TCA precipitable material. The control cells grown in HAT-medium, showed an increased ability to incorporate ^{3}H-thymidine into TCA precipitable material when compared with cells cultured in the absence of HAT-medium (Table 7 and Fig. 17). The L45 cells, however, showed a marked reduction in the ^{3}H-thymidine uptake after being cultured in HAT-medium. The absence of an enhanced ^{3}H-thymidine uptake, and reduction in the number of cells, demonstrated the inability of the L45 lymphoblasts to survive in HAT-medium.

11.4.1.3.2. GAMA-medium

GAMA-medium consists of guanine (200μM), adenine (100μM), mycophenolic acid (6μM), and azaserine (10μM), and may be used as an alternative to HAT-medium (Liskay and Patterson 1979). Azaserine blocks **de novo** purine synthesis, while mycophenolic acid inhibits the formation of GMP from IMP by inhibiting the activity of IMP dehydrogenase (Franklin and Cook 1969). In the presence of these inhibitors, cells are made dependent on the salvage of adenine by APRT for the formation of adenine nucleotides, and on the salvage of guanine by HPRT for GMP formation. Therefore both adenine and guanine must be present in the medium containing azaserine and mycophenolic acid for the cells to survive. If hypoxanthine were to be substituted for adenine in the medium, the cells would have to form adenine nucleotides from IMP, and would be dependent on the salvage activity of HPRT alone. The effects of the various components of GAMA-medium on the growth of transformed lymphoblasts is shown in Table 8. Both azaserine and mycophenolic acid reduced the number of the L45 and of the control cells over an 8 day culture period. While the effect of mycophenolic acid on the growth of the control cell line was partialy reversed by guanine, this was not so for the L45 lymphoblasts. The effects of azaserine on the growth of the cells was not reduced by the presence of hypoxanthine, guanine, or adenine. Both cell lines failed to grow in the complete GAMA-medium, as well as when hypoxanthine was substituted for adenine. The failure of the complete GAMA-medium to differentiate between the

Table 8

The effect of GAMA-medium on transformed lymphoblast growth. The % change in the number of cells, cultured for 8 days in the presence of the various components of GAMA-medium, is shown for L45 and for a control cell line.

Medium (F10)	% Change in cell number	
	L45 (T.K.)	L57
No additions	+80	+180
+Azaserine (10μM)	-60	-40
+Mycophenolic acid (6μM)	-60	-50
+Mycophenolic acid (6μM) +Guanine (200μM)	-60	+60
+Mycophenolic acid (6μM) +Hypoxanthine (200μM)	-55	-50
+Mycophenolic acid (6μM) +Adenine (100μM)	-70	-60
+Mycophenolic acid (6μM) +Azaserine (10μM)	-60	-60
+Azaserine (10μM) +Guanine (200μM)	-60	-50
+Azaserine (10μM) +Hypoxanthine (200μM)	-60	-10
+Azaserine (10μM) +Adenine (100μM)	+10	-10
+Guanine (200μM) +Adenine (100μM) +Mycophenolic acid (6μM) +Azaserine (10μM) (GAMA)	-70	-25

Table 9

A) The reversablilty of the effects of azaserine by adenine, hypoxanthine and guanine. The % change in the number of cells, cultured for 11 days in the presence of azaserine and various combinations of purine bases, is shown for L45 and for a control cell line.

Medium (F10)		% Change in cell number	
		L45 (T.K.)	L57
No additions		+500	+500
+Azaserine	(10μM)	+200	+ 50
+Guanine	(200μM)		
+Adenine	(100μM)		
+Azaserine	(10μM)	- 75	+150
+Guanine	(200μM)		
+Hypoxanthine	(200μM)		
+Azaserine	(10μM)	+125	+ 25
+Hypoxanthine	(200μM)		
+Adenine	(100μM)		

Table 9

B) The effect of the concentration of azaserine on the growth of a transformed lymphoblast cell line. The % change in the number of cells, cultured for 8 days in the presence of azaserine or azaserine with hypoxanthine (200μM) and adenine (100μM), was determined.

Azaserine concentration	% Change in number of cells grown in F10 medium containing:	
	Azaserine (only)	Azaserine, Hypoxanthine & Adenine
Nil	+350	-
10μM	- 40	- 40
5μM	+100	+100
1μM	+250	+250
0.5μM	+350	+300
0.1μM	+375	+325

Table 10

The substitution of aminopterin and thymidine for azaserine in GAMA selective medium. The % change in the number of cells cultured for 11 days in the presence of mycophenolic acid (6μM), and either azaserine (10mg/ml) or aminopterin (10μM) and thymidine (400μM), was determined in the presence of various combinations of the purine bases, guanine (200μM), adenine (100μM), and hypoxanthine (200μM).

Medium (F10)	% Change in number of cells			
	L45 (T.K.)	L106 ($HPRT^-$)	L57	L59
No additions	+500	+225	+500	+400
+Guanine +Adenine +Mycophenolic acid +Azaserine	- 75	- 65	+75	- 10
+Guanine +Hypoxanthine +Mycophenolic acid +Azaserine	- 75	-	+125	-
+Guanine +Adenine +Mycophenolic acid +Aminopterin +Thymidine	- 75	- 65	+425	+350
+Guanine +Hypoxanthine +Mycophenolic acid +Aminopterin +Thymidine	- 75	-	+450	-

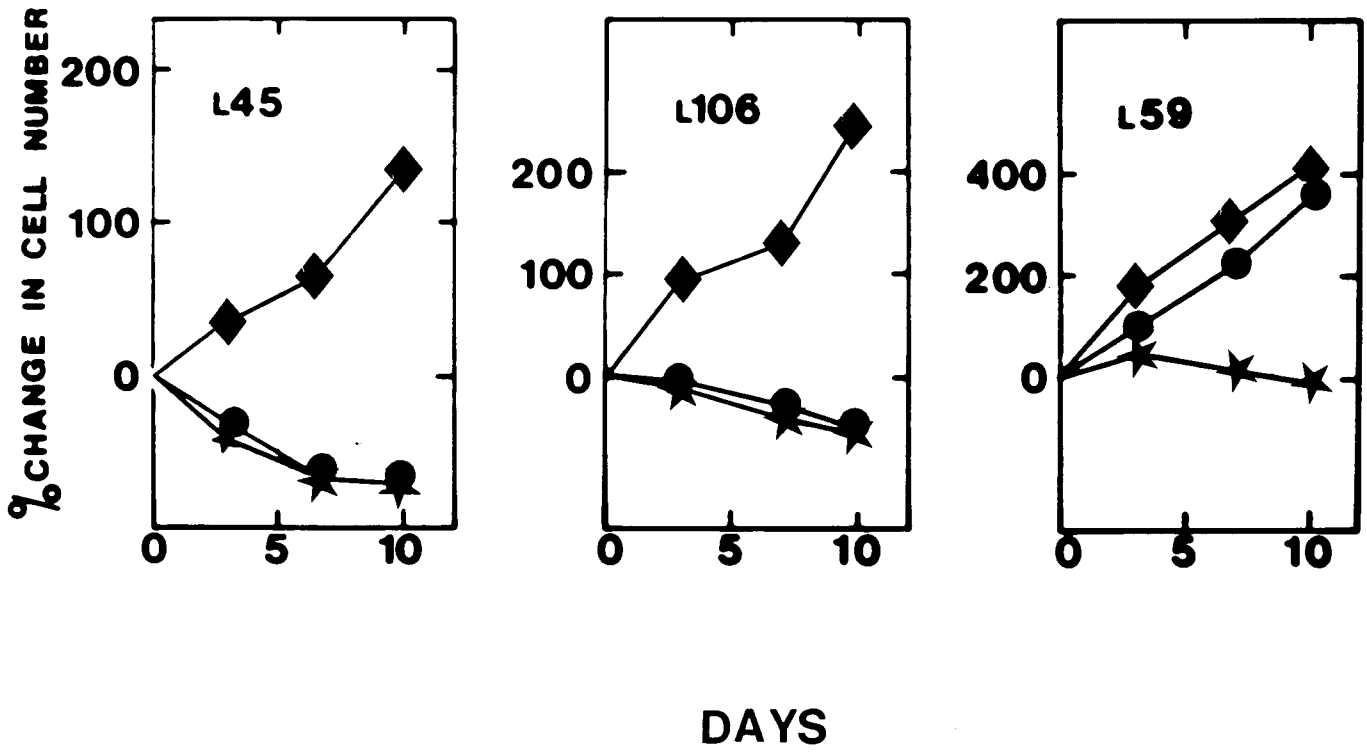


Fig. 18. Effect of GAMA-medium and modified GAMA-medium, on the growth of transformed lymphoblasts. (◆), no additions; (★), GAMA-medium; (●), modified GAMA-medium, in which azaserine was replaced with aminopterin. L45, cells from T.K.; L106, cells from a patient with the Lesch-Nyhan Syndrome; L59, cells from a normal control.

L45 and control lymphoblasts was further investigated. Lymphoblasts were cultured in the presence of azaserine and various combinations of the bases; hypoxanthine, guanine, and adenine (Table 9, a). The effects of azaserine could not be fully reversed in either cell line. The effect of the concentration of azaserine on the growth of the lymphoblasts is shown in Table 9, b. The rate of growth of the lymphoblasts was reduced at concentrations of above 0.5μM of azaserine. The presence of hypoxanthine and adenine in the culture medium could not reverse the effects of azaserine at any concentration. The toxicity of azaserine to the lymphoblasts therefore appears to be due to other effects of the inhibitor, and not only due to its inhibition of **de novo** purine synthesis. When aminopterin was substituted for azaserine, and the medium supplemented with thymidine, the growth of the control cell line was restored to normal by a combination of guanine and hypoxanthine, or of guanine and adenine (Table 10 & Fig. 18). Both the L45 and L106 lymphoblasts failed to grow in these modified media. Therefore, while the modified 'GAMA-medium' could differentiate between the control cell lines and the HPRT-deficient cell lines, GAMA-medium could not.

11.4.1.3.3. Purine analogues

The purine base analogues, thioguanine (6-thioguanine) and azaguanine (8-azaguanine) are converted to their respective nucleotides by the activity of HPRT (Kelley and Wyngaarden 1983). These analogue nucleotides are able to inhibit RNA and DNA synthesis. In cells lacking HPRT activity, these toxic nucleotides

Table 11

The effects of thioguanine and azaguanine on transformed lymphoblast growth. The % change in the number of cells, cultured for 8 days in the presence of thioguanine (10µg/ml) or azaguanine (40µg/ml) was determined. The ability of the cells to incorporate ^{3}H-thymidine into TCA precipitable material was also determined.

Medium (F10)	% Change in cell number			dpm ^{3}H-thymidine incorporated ($x10^{-2}$)		
	L45 (T.K.)	L46	L39	L45	L46	L39
No additions	+134	+266	+142	184	449	178
+Thioguanine	+272	- 10	- 10	142	1.4	7.1
+Azaguanine	+ 98	- 50	- 70	74	1.1	1.2

are not formed, and their growth is unaffected by the presence of the purine analogues in the culture medium. The ability of HPRT-deficient cells to grow in the presence of toxic purine analogues serves to distinguish them from cells containing HPRT activity. When the L45 lymphoblasts were cultured for 8 days in the presence of thioguanine (10ug/ml) or azaguanine (40ug/ml), the number of cells increased, while the number of cells in the control lymphoblast cultures decreased (Table 11). Under the same conditions the control lymphoblasts incorporated less than 1000 dpm of ^{3}H-thymidine into TCA precipitable material. The L45 cells grown in the presence of thioguanine or azaguanine, incorporated 80% and 40% respectively, of the ^{3}H dpm that were incorporated by L45 cells cultured in the absence of the purine analogues i.e. 18 400 dpm (Table 11). The L45 and L106 lymphoblasts demonstrated a similar ability to grow in the presence of the purine analogues (Fig. 16). The resistance of the L45 lymphoblasts to the effects of toxic purine analogues was, therefore a further demonstration of their low intracellular HPRT activity.

11.4.2. Skin fibroblasts

11.4.2.1. Methods

Fibroblasts from stock cultures were passaged into 24 well culture dishes (Costar, 16mm well diameter), at a density 1/3 of that of the stock cultures. The cells were incubated overnight in 2ml of fresh medium, which was replaced with selective medium the

Table 12

A) The effect of selective media on the growth of skin firoblasts from T.K. and from a normal individual. The % increase in the protein content of the culture wells in which the skin fibroblasts were cultured for 10 days, in the presence of HAT-medium or thioguanine (10ug/ml), was determined.

Medium (MEM)	% Increase in protein content	
	T.K.	Control
No additions	430	520
+HAT	110	470
+Thioguanine	330	200

B) The effect of selective medium on the growth of skin fibroblasts from T.K. and from a normal individual. The % change in the protein content of the culture wells in which the skin fibroblasts were cultured for 5 days in the presence of different concentrations of thioguanine, was determined.

Medium (MEM)	% Change in protein content	
	T.K.	Control
+Thioguanine (10ug/ml)	+ 35	-70
+Thioguanine (5ug/ml)	+ 25	-20
+Thioguanine (2ug/ml)	+160	+90

following morning. Enough wells for three time-points (in triplicate), were prepared. The growth of the cells at each time-point was determined as follows: each well was washed three times with 2ml of cold normal saline and the cells were dissolved overnight in 1ml of 1N NaOH and the protein concentration in each well was determined. The ratio of protein concentration on day 10 to that on day 0, was used as an index of cell growth.

11.4.2.2. Results

The ratio of the protein content on day 10 to that on day 0, of the culture wells in which the skin fibroblasts were cultured in the presence of various selective media, is shown in Table 12. These ratios indicate that, while the fibroblasts obtained from T.K. do not grow as well as the control fibroblasts in HAT-medium, they grow better in the presence of thioguanine.

11.4.3. Discussion

The ability of cells to grow in selective media was used as a measure of their viability in these media. The viability of the lymphoblasts in selective media was also shown by the incorporation of ^{3}H-thymidine into TCA precipitable molecules. Two types of selective media were used: 1) those which required the cells to salvage purines by the activity of HPRT for their

survivial (HAT-, and GAMA-medium), and 2) those in which HPRT converted purine analogues into toxic nucleotides, preventing the growth of the cells (thioguanine and azaguanine). When cultured in these selective media, the L45 transformed lymphoblasts derived from T.K., behaved in a similar manner to the L106 lymphoblasts, which are known to be deficient in HPRT activity. The growth behavior of both of these cell lines was different from that of the control lymphoblasts, which were obtained from normal individuals. The increased incorporation of ^{3}H-thymidine by the control cells cultured in HAT-medium is probably due to the induction of thymidine kinase activity (Fig. 16) and provides a convenient way of distinguishing viable from non-viable cells. The GAMA-medium (Liskay and Patterson 1979), failed to differentiate between the $HPRT^{-}$ and $HPRT^{+}$ cells, since neither of these cell-types would grow in this medium. This was due to the azaserine (serine diazoacetate), which was used to inhibit purine synthesis **de novo**. When the azaserine was replaced by aminopterin in the modified GAMA-medium, the control lymphoblasts grew at a similar rate to that of the cells cultured in the absence of GAMA-medium (Table 10). Azaserine is a glutamine analogue which inhibits the three glutamine utilising steps in the **de novo** purine synthetic pathway (Wyngaarden and Kelley 1983). Since glutamine is a substrate in a number of other reactions, it is possible that azaserine could inhibit some of these reactions in the lymphoblasts. Therefore the growth of the lymphoblasts which are cultured for long periods in the presence of azaserine, is reduced, even though the inhibition of **de novo** purine synthesis is compenstated for by the purine salvage pathways in these cells.

11.5. Double Label Experiments

The method used, is a modification of that of Rozen **et al** (1977). The incorporation of a ^{14}C-labelled substrate of the metabolic pathway under investigation, into the TCA precipitable molecules of the cells, is determined. The incorporation of a ^{3}H-labelled substrate, not of the same pathway, is also determined, and is used as an internal control. The ratio of ^{14}C to ^{3}H in the TCA precipitates obtained from cells with a defective index pathway, is lower than that obtained from the cells with an intact pathway. While this method of investigation does not demonstrate the enzyme defect directly, it does give qualitative information about the 'intactness' of the pathway in which the enzyme functions. An enzyme defect may therefore be indicated by the double label approach, but it would have to be demonstrated by other more direct methods.

11.5.1. Lymphoblasts.

11.5.1.1. Methods

Approximately 200 000 cells were cultured in 0.5ml of fresh medium for 6 hours, before the radioactive substrates were added (generally 1µCi/ml), and the cells were cultured for a further seventeen hours. After incubation the cells were pelleted and washed twice with cold, 0.9% NaCl, and placed on GF/C circles (Whatman

glass microfibre filters, 2.5cm). The filters were dipped into cold, 5% TCA in a 25ml beaker 4 times. The TCA was removed by dipping the circles into beakers containing ethanol and ether, respectively. The circles were placed into counting vials and allowed to dry for 30 minutes, and 10ml of 'Instagel' was added. The radioactivity was counted for 5 minutes in a Beckman LS-250 scintillation counter. The ratio of ^{14}C to ^{3}H was determined after the counts had been corrected for quenching and carry over of ^{14}C into the tritium window. Each ratio was determined from duplicate cultures, except when, for statistical analysis, cultures were made in quadruplicate. In certain cases the cells were not precipitated onto GF/C circles, but mixed in Eppendorf tubes with 0.5ml of cold, 5% TCA. The precipitate was washed with 0.5ml of ethanol, then by ether, and was dissolved in 100µl of 1N NaOH. After being transferred into counting vials the alkali was neutralised with an equal volume of IN HCl and 10ml of 'Instagel', added. The ratio of ^{14}C to ^{3}H was then determined as above. Both of these precipitation techniques gave compatible results.

11.5.1.2. Results

11.5.1.2.1. Hypoxanthine/guanine salvage

The double label approach was used to evaluate the relative flux through the purine salvage pathway to that through the pyrimidine salvage pathway, in intact cells. The incorporation of ^{14}C-purine

Table 13

The relative incorporation of ^{14}C-hypoxanthine to ^{3}H-thymidine into TCA precipitable material. Lymphoblasts were cultured overnight in 0.5 ml of medium containing 1μCi/ml of each of the labelled substrates, and the ratio of ^{14}C to ^{3}H was determined in the TCA precipitable material.

Cell Line	^{14}C dpm	^{3}H dpm	$^{14}C/^{3}H$ (10^2)	Mean± S.D. (10^2)
L45	1157	24940	4.64	
(T.K.)	1141	27209	4.19	4.23± 0.26
	1020	22689	4.49	
	1079	27104	3.98	
L106	444	59627	0.74	
($HPRT^-$)	394	64329	0.61	0.66± 0.06
	528	78369	0.67	
	400	62942	0.63	
L107	34888	77611	44.95	
	35582	72946	48.77	51.0 ± 5.3
	26315	45839	57.4	
	42640	80867	52.72	
L59	79743	96936	82.26	
	87240	140654	62.02	70.32± 10.6
	83436	125096	66.69	
	82529	132848	62.12	

Table 14

The relative incorporation of ^{14}C-guanine to ^{3}H-thymidine into TCA precipitable material . Lymphoblasts were cultured overnight in 0.5 ml of medium containing 1μCi/ml of each of the labelled substrates, and the ratio of ^{14}C to ^{3}H was determined in the TCA precipitable material.

Cell Line	^{14}C dpm	^{3}H dpm	$^{14}C/^{3}H$ ($\times 10^2$)	Mean ± SD. ($\times 10^2$)
L45	965	20716	4.66	
(T.K.)	655	15713	4.17	4.27 ± 0.26
	725	17324	4.18	
	660	16176	4.08	
L106	147	35094	0.42	
($HPRT^-$)	193	35187	0.55	0.41 ± 0.11
	187	49689	0.37	
	120	40365	0.30	
L107	13916	34041	40.88	
	17258	34703	49.73	46.33 ± 4.95
	18780	43182	43.49	
	12907	25190	51.24	
L59	30669	59070	51.91	
	33095	70580	46.89	46.22 ± 4.33
	24800	59420	41.73	
	22824	51430	44.38	

Table 15

The relative incorporation of ^{14}C-hypoxanthine to ^{3}H-leucine into TCA precipitable material. Lymphoblasts were cultured overnight in 0.5ml of medium containing 1μCi/ml of each of the labelled substrates, and the ratio of ^{14}C to ^{3}H was determined in the TCA precipitable material.

Cell Line	^{14}C dpm	^{3}H dpm	$^{14}C/^{3}H$ ($\times 10^2$)	Mean± S.D. ($\times 10^2$)
L45	527	12793	4.17	
(T.K.)	473	11227	4.26	4.06± 0.19
	434	11371	3.87	
	453	11685	3.93	
L106	132	29955	0.49	
($HPRT^-$)	142	31867	0.49	0.51± 0.03
	148	31196	0.52	
	162	32102	0.55	
L57	14532	28937	50.26	
	15501	30550	50.79	51.05± 0.65
	15860	30860	51.44	
	15392	29675	51.7	

Table 16

The relative incorporation of ^{14}C-guanine to ^{3}H-leucine into TCA precipitable material. Lymphoblasts were cultured overnight in 0.5ml of medium containing 1μCi/ml of each of the labelled substrates, and the ratio of ^{14}C to ^{3}H was determined in the TCA precipitable material.

Cell Line	^{14}C dpm	^{3}H dpm	$^{14}C/^{3}H$ ($\times 10^2$)	Mean ± S.D. ($\times 10^2$)
L45 (T.K.)	455	12814	3.6	
	536	14793	3.67	3.1 ± 0.58
	389	14630	2.71	
	350	13950	2.56	
L106 ($HPRT^-$)	36	35191	0.15	
	40	35672	0.16	0.15 ± 0.03
	28	34378	0.13	
	54	39001	0.19	
L57	10481	27865	37.66	
	11264	29086	38.77	38.03 ± 0.62
	11535	30879	37.4	
	12214	31957	38.27	

base into TCA precipitable material, relative to the incorporation of ^{3}H-thymidine, was determined by measuring the ratio of ^{14}C to ^{3}H in the TCA precipitates, prepared from cells cultured in the presence of the labelled substrates. The relative incorporation obtained for ^{14}C-hypoxanthine and ^{3}H-thymidine is shown in Table 13, while that for ^{14}C-guanine and ^{3}H-thymidine is shown in Table 14. These results show that for hypoxanthine the L45 lymphoblasts incorporated between 6-8% of the relative ^{14}C-counts of the control lines, while the cells from a patient with the Lesch-Nyhan Syndrome (L106), incorporated less than 1.3% of the control values. For guanine the respective values were 9% and $<$1% of the control values. In repeat experiments, the incorporation of label into the L45 cells varied between 3-11% for hypoxanthine and 3-17% for guanine, compared to that of the control cells. The amino acid, ^{3}H-leucine, was used as the substrate for an alternative reference pathway. When the incorporation of ^{14}C-labelled bases relative to that of ^{3}H-leucine was determined in TCA precipitable material, the L45 cells were found to incorporate 8% of the relative counts of the control cells for both hypoxanthine and guanine. The values for the L106 cells were 0.4% and 1% respectively (Tables 15 & 16).

11.5.1.2.2. Purine sythesis **de novo**

A high rate of **de novo** purine synthesis is a biochemical feature of cells deficient in HPRT activity when they are cultured in medium containing hypoxanthine (Hershfield and Seegmiller 1975). Therefore the demonstration of a high flux through the **de novo**

synthesis pathway can be regarded as collateral evidence for such an enzyme deficiency. Formate is one of the small, precurser molecules of purines synthesised **de novo**. Radioactive formate has been used as a measure of the rate of **de novo** purine synthesis (Wyngaarden and Kelley 1983, Goron **et al** 1979). The double label technique was used to measure the flux through the **de novo** purine synthesis pathway with ^{14}C-formate, using ^{3}H-thymidine as reference substrate. The exclusive utilisation of a radioactive substrate through a particular metabolic pathway is important in a double label experiment. If the substrate is utilised through more than one pathway, it is possible that the label will be incorporated into the TCA precipitable material through these alternative pathways (vectorial utilisation, Rozen **et al** 1977). It was decided therefore, to measure the ^{14}C to ^{3}H ratio in DNA extracted from the cells to minimise the effects of the vectorial utilisation of formate on the ratio. The ratio of ^{14}C to ^{3}H, in DNA extracted from lymphoblasts grown in the presence of ^{14}C-formate and ^{3}H-thymidine for 17 hours, is shown in Table 17. The ratio of ^{14}C to ^{3}H was 20 times higher in the L45 cells than in the control cells (1.00 cf. 0.05). In repeat experiments the L45 cells had a ratio 8-12 times higher than that obtained in the control lymphoblasts. Azaserine was used in an attempt to modify the ratio of formate incorporation relative to that of thymidine. The effects of azaserine on the ^{14}C to ^{3}H ratio is shown in Table 18. Cells were cultured for 3 hours in the presence of azaserine (10μM), before being resuspended in medium containing ^{14}C-formate, ^{3}H-thymidine and azaserine and cultured for a further 17 hours. The ratio of ^{14}C to ^{3}H was then determined in the DNA extracted from the cells. In both the L45 and the control cell

Table 17

Purine synthesis **de novo**. Lymphoblasts were cultured for 17 hours in the presence of ^{14}C-formate (5μCi/ml), and ^{3}H-thymidine (0.5μCi/ml) and the ratio of ^{14}C to ^{3}H was determined in the extracted DNA.

Cell line	Medium	^{14}C dpm	^{3}H dpm	$^{14}C/^{3}H$
L45	F10	18738	18126	1.03
(T.K.)		17041	17026	1.00
L31	F10	606	12063	0.05
		509	13892	0.04

Table 18

The effect of azaserine on **de novo** purine synthesis. Transformed lymphoblasts were precultured for 3 hours in the presence of azaserine (10μM), and cultured for 17 hours in the presence of ^{14}C-formate (5μCi/ml),and ^{3}H-thymidine (0.5μCi/ml), and the ratio of ^{14}C to ^{3}H was determined in the extracted DNA.

Cell line	Medium	^{14}C dpm	^{3}H dpm	^{14}C/^{3}H	MEAN
L45	F10	14209	45165	0.32	0.31
(T.K.)		14299	49792	0.29	
L45	F10+	1920	5786	0.33	0.37
(T.K.)	azaserine	1747	4350	0.40	
L59	F10	2241	88579	0.03	0.03
		2051	85001	0.03	
L59	F10+	1787	64072	0.03	0.03
	azaserine	2435	80723	0.03	

Table 19

Purine synthesis **de novo**. Transformed lymphoblasts, maintained in purine-free medium, were resuspended in 0.5ml of fresh medium containing 5μCi of ^{14}C-formate and 0.5μCi of ^{3}H-thymidine and cultured for 17 hours. The ratio of ^{14}C to ^{3}H was determined in the DNA extracted from the cells.

Cell line	^{14}C dpm	^{3}H dpm	^{14}C/^{3}H	MEAN
L45	5647	20567	0.28	0.27
(T.K.)	6408	24626	0.26	
L106	9629	29822	0.32	0.31
(HPRT$^-$)	16021	53630	0.30	
L61	484	16243	0.03	0.04
	581	14788	0.04	
L114	1774	61804	0.03	0.03
	1376	44636	0.03	

line the ratio did not change in the presence of azaserine. However, in the L45 cells a 7 fold reduction in the total ^{14}C and ^{3}H incorporation was found. The control line did not show a similar reduction in total counts. The reduction in counts incorporated into DNA in the case of the L45 cells in the presence of azaserine was reproducible; the incorporated counts varied between 1 to 16% of the counts obtained in the absence of azaserine. This demonstrated the dependence of these cells on purines derived **de novo** for their DNA synthesis. The reduction in ^{3}H incorporation was probably secondary to the reduction in DNA synthesis. The ratios obtained with the L45 and L106 lyphoblasts were similar (Table 19). These experiments indicate that ^{14}C-formate incorporation into DNA is a good index of **de novo** purine synthesis, and that the L45 cells have an accelerated rate of purine synthesis **de novo.**

11.5.1.2.3. Adenine salvage

The activity of adenine phosphoribosyl transferase (APRT), has been found to be elevated in red blood cells deficient in HPRT activity. This increased enzyme activity is believed to be due to the higher intracellular concentrations of the substrate PP-ribose-P, which is common to both enzymes . The PP-ribose-P is thought to stablise APRT and reduce its rate of degradation in the cells (Greene **et al** 1970). The double label approach was used to determine if an increase in APRT activity could be demonstrated in transformed lymphoblasts. Lymphoblasts were cultured 4 hours in the presence of ^{14}C-adenine and ^{3}H-thymidine and the ratio of ^{14}C to

Table 20

The relative incorporation of ^{14}C-adenine to ^{3}H-thymidine into TCA precipitable material. Transformed lymphoblasts were cultured in 0.5ml of medium containing 0.12μCi of ^{14}C-adenine and 2μCi of ^{3}H-thymidine for 4 hours and the ratio of ^{14}C to ^{3}H was determined in the TCA precipitable material.

Cell Line	^{14}C dpm	^{3}H dpm	$^{14}C/^{3}H$ (x10)	Mean± S.D. (x10)
	14265	62597	2.28	
L45	13524	59014	2.3	2.3± 0.05
(T.K.)	13481	57118	2.37	
	14047	62588	2.25	
	22543	147472	1.53	
L106	21529	146769	1.47	1.5± 0.05
($HPRT^{-}$)	19741	138896	1.43	
	22488	146485	1.54	
	17342	167137	1.04	
L57	16176	169498	0.96	1.0± 0.05
	17162	170155	1.01	
	16957	158826	1.07	

Table 21

The relative incorporation of ^{14}C-adenine to ^{3}H-thymidine into TCA precipitable material. Transformed lymphoblasts were cultured in 0.5ml of medium containing 0.12μCi of ^{14}C-adenine and 2μCi of ^{3}H-thymidine for 4 hours and the ratio of ^{14}C to ^{3}H was determined in the the TCA precipitable material.

Cell Line	^{14}C dpm	^{3}H dpm	$^{14}C/^{3}H$ (x10)	MEAN (x10)
L45	16254	96267	1.69	1.65
(T.K.)	16961	105785	1.61	
L106	22782	147257	1.55	1.54
($HPRT^{-}$)	20844	137893	1.52	
L107	6674	51972	1.29	1.3
	6650	51545	1.3	
L114	4833	50560	0.96	0.97
	4784	49383	0.97	
L57	7377	32028	2.31	2.34
	7361	31180	2.37	
L61	5995	26571	2.26	2.36
	6633	27047	2.46	

^{3}H, was determined as has been described (Tables 20 & 21). In the first experiment shown, the ratio of ^{14}C to ^{3}H in the L45 cells was higher than that in the L106 and in the control cells (2.3, 1.5 and 1.0 respectively). In the second, the ratio in the L45 cells was close to that in the L106 cells (1.65 and 1.54). These ratios fell within the range of those obtained for the control cells (1.3, 0.97, 2.34 and 2.36). Thus no difference in the APRT activity between the $HPRT^{-}$ lymphoblasts and control lymphoblasts, could be demonstrated. The ratios obtained for one cell line also varied from one experiment to another. This indicates that the flux through APRT was not constant.

11.5.2. Fibroblasts

11.5.2.1. Method

The fibroblasts were placed into $25cm^2$ flasks, and cultured overnight in 5ml of medium. The medium was replaced with 2ml of medium containing the radioactive substrates, and the cells, cultured for 3-4 hours. They were then washed with 5ml of cold, 0.9% saline, trypsinised and transferred onto GF/C circles and treated with TCA, ethanol and ether and the ratio of ^{14}C to ^{3}H was determined as has been described for the lymphoblasts.

Table 22

The relative incorporation of ^{14}C-hypoxanthine or ^{14}C-guanine to ^{3}H-thymidine into TCA precipitable material. Skin fibroblasts were cultured for three hours in 2ml of medium containing 1μCi of each of the labelled substrates, and the ratio of ^{14}C to ^{3}H was determined in the TCA precipitable material.

A) Hypoxanthine as the purine base.

Cell Line from:	^{14}C dpm	^{3}H dpm	$^{14}C/^{3}H$
T.K.	300	8410	0.045
	265	3676	0.072
Control	2800	6079	0.463
	6245	12376	0.505

B) Guanine as the purine base.

Cell line from:	^{14}C dpm	^{3}H dpm	$^{14}C/^{3}H$
T.K.	1936	21380	0.09
	650	15985	0.04
Control	8720	70960	0.12
	8948	81896	0.11
Patient with the Lesch-Nyhan Syndrome	90	26270	0.003
	50	18095	0.003

11.5.2.2. Results

The ability of cultured skin fibroblasts, obtained from T.K. to incorporate ^{14}C-purine bases relative to ^{3}H-thymidine into TCA precipitable material, is compared with that of a control cell line in Table 22. A lower ratio of ^{14}C to ^{3}H than that in the control cell line was obtained for both ^{14}C-hypoxanthine and ^{14}C-guanine; this indicates a low intracellular activity of HPRT in the fibroblasts obtained from T.K.

11.5.3. Discussion

The double label experiments were used to measure the relative flux through the purine metabolising pathways, to that through a reference pathway (Rozen **et al** 1977). The ratio of ^{14}C to ^{3}H which was obtained with ^{14}C-labelled hypoxanthine and guanine was low in both the L45 and L106 lymphoblasts, when either ^{3}H-thymidine or ^{3}H-leucine was used as the substrate for the reference pathway (Tables 13 to 16). This indicates that the utilisation of hypoxanthine and guanine in these cells was lower than that of the control cells. The ^{14}C to ^{3}H ratios obtained for the L45 and L106 lymphoblasts were less than 20% and 2%, respectively, to those found in the control cells. A reduced ulitisation of the purine bases was also found in the skin fibroblasts obtained from T.K. The relative incorporation of ^{14}C-formate to ^{3}H-thymidine into the DNA of the L45 and L106 lymphoblasts was 10-20 times higher than that found in the control

cells (Tables 17 to 19). This showed that the DNA of these cells contained a greater amount of purines derived from **de novo** synthesis than did the control cells; this indicated a higher rate of **de novo** purine synthesis in these cells. The high rate of purine synthesis **de novo** in HPRT deficient cells appears to be a compensatory biochemical change enabling the cells to maintain their nucleotide levels as close to normal as possible (Zoref-Shani and Sperling 1980). The relative flux through the adenine utilising pathway was similar in all cell lines tested (Tables 20 & 21), and there did not apppear to be an elevation in APRT activity in the HPRT deficient cells. Similar findings have been reported for other cultured cells deficient in HPRT activity (Kelley and Wyngaarden 1983). The double label approach demonstrated that the L45 cells resembled the L106 cells, which were derived from a patient with the Lesch-Nyhan Syndrome, more closely than they did the cells derived from individuals with normal purine metabolism.

11.6. Formation and release of ^{14}C-hypoxanthine and ^{14}C-guanine by transformed lymphoblasts cultured in the presence of ^{14}C-formate.

11.6.1. Method

Cells deficient in HPRT activity have been shown to release greater amounts of ^{14}C-hypoxanthine and ^{14}C-guanine into their culture medium than the control cells do when cultured in the presence of ^{14}C-formate (Gordon **et al** 1979). This may be a more

sensitive indicator of a HPRT deficiency, than the incorporation of label into DNA. The release of hypoxanthine and guanine into the medium by the L45 and control lymphoblasts, was determined by analysing the medium from a ^{14}C-formate/^{3}H-thymidine double label experiment for the presence of ^{14}C-labelled purine bases. The medium was deproteinated with 0.5ml of 1N PCA. After centrifugation the supernatant was mixed with 100µl of 5M K_2CO_3, and the precipitate was removed by further centrifugation. The bases were concentrated by freeze-drying the samples twice and dissolving the residues in 500µl and 100µl of buffer (1mM KH_2PO_4, pH3.0), respectively. The bases were separated by HPLC (Spectra-Physics, Model 3500), using a reverse phase column (Spectra-Physics RP-8, 10µm), and a step gradient of methanol (0-10%) in buffer. The rate and pressure of elution were 1ml/minute and 450-550psi, respectively. Formate eluted off the column first, followed by hypoxanthine. The guanine was eluted with the 10% methanol/buffer. Cold hypoxanthine and guanine were added to each sample as carriers, and aliquots of 50µl from each sample were applied to the column. After a delay of 30 seconds, fractions were collected into counting vials for 15 seconds each. After the elution of the hypoxanthine, the buffer containing the 10% methanol was started, and after another 30 second delay, further fractions were collected. The elution was monitored at 260nm. The fractions were mixed with 10ml of 'Instagel' and the radioactivity in the hypoxanthine and guanine peaks was determined. The recovery of the radioactivity placed on the column varied between 83-98%. Initial analysis of the samples by HPLC with a cation exchange column (Whatman SCX, Partisil-10) was attempted, but fail ed to separate the formate and hypoxanthine.

11.6.2. Results

The release of hypoxanthine and guanine into the culture medium by cells grown in the presence of ^{14}C-formate is shown in Table 23. The radioactivity in the hypoxanthine peak was twenty times higher in the medium of the L45 cells than that of the control cells (23 442 cf 1 174 and 1 278 dpm). For guanine no radioactivity above background was found in the media of the control cells, while 1 990 dpm were obtained from the medium of the L45 cells. When the L45 cells were cultured in the presence of azaserine, the counts in the hypoxanthine peak were reduced to 1 610 dpm, and no counts above background were found in the guanine peak. Therefore the L45 cells released more radioactive hypoxanthine and guanine into the culture medium than the control cells did, while the amount of radioactive bases found in the medium of the cells cultured in the presence of azaserine, was markedly reduced. This implies an increased production of radioactive bases from ^{14}C-formate in the L45 cells, and an enhanced purine synthesis **de novo**. However, a proportion of the radioactive bases were released into the medium of the L45 cells due to a lack of salvage and of cycling of the bases in these cells, and not solely due to an increased synthesis **de novo**.

Table 23

The purine synthesis **de novo** was measured by the release of ^{14}C-hypoxanthine and ^{14}C-guanine by transformed lymphoblasts grown in the presence of ^{14}C-formate. Lymphoblasts were cultured in the presence of 10μCi/ml of ^{14}C-formate for 17 hours. The release of ^{14}C-hypoxanthine and ^{14}C-guanine into the medium was determined by analysing the deproteinated medium on reverse phase HPLC and by measuring the radioactivity in the hypoxanthine and guanine peaks. Cells grown in the presence of azaserine (10μM), were precultured for three hours in the presence of the inhibitor.

Cell Line	Medium	^{14}C dpm in hypoxanthine	^{14}C dpm in guanine
L45 (T.K.)	F10	23442	1990
L45 (T.K.)	F10+ azaserine	1810	N.D.
L49	F10	1174	N.D.
L59	F10	1278	N.D.

N.D.= Nil detected

11.6.3. Discussion

Normal cells have a similar rate of purine synthesis **de novo** when they are compared with $HPRT^-$ cells in purine-free medium (Gordon **et al** 1979). However, when they are cultured in medium containing hypoxanthine, their rate of purine synthesis **de novo** is reduced, while that of the $HPRT^-$ cells, remains high. Cells deficient in HPRT activity, release more hypoxanthine into the medium than do the control cells (Gordon **et al** 1979). This is due to the lack of cycling of the bases by HPRT activity and to the high rate of purine biosynthesis in these cells. The release of radioactively labelled bases into the medium, by cells cultured in the presence of ^{14}C-formate, provides another indication of a lack of HPRT activity in the cells. The L45 lymphoblasts released twenty times as much radioactivity into the hypoxanthine fraction of their medium than the control cells did when cultured in the presence of ^{14}C-formate (Table 23). This amount of radioactivity was greatly reduced when azaserine was added to the cultures. Therefore the L45 lymphoblasts showed yet another feature of an intracellular HPRT deficiency.

11.7. Relative utilisation of labelled hypoxanthine to labelled guanine.

Assays of the HPRT activity in haemolysates prepared from T.K. by the continuous spectrophotometric assay, indicated that there was a discrepancy between the V_{max} values obtained for hypoxanthine

Table 24

Comparison of the salvage of hypoxanthine and guanine in intact lymphocytes. Transformed lymphocytes were cultured for 17 hours in the presence of labelled hypoxanthine and guanine and the ratio of ^{14}C to ^{3}H was determined in the TCA precipitable material.

A) Cells cultured with ^{14}C-hypoxanthine (0.25μCi/ml), and ^{3}H-guanine, (1μCi/ml).

Cell Line	^{14}C dpm	^{3}H dpm	$^{14}C/^{3}H$	Mean
L45	437	4074	0.108	0.108
(T.K.)	494	4585	0.108	
L49	264	2510	0.106	0.108
	294	2657	0.111	
L59	7642	71160	0.108	0.112
	6319	55061	0.115	

B) Cells cultured with ^{14}C-guanine (0.25μCi/ml), and ^{3}H-hypoxanthine, (1μCi/ml).

Cell line	^{14}C dpm	^{3}H dpm	$^{14}C/^{3}H$	Mean
L45	223	5190	0.044	0.042
(T.K.)	173	4296	0.041	
L49	244	3586	0.069	0.068
	234	3457	0.068	
L59	5720	94124	0.061	0.057
	5160	97705	0.057	

and guanine. The former was similar to that of the control activity, while that for guanine was much lower than that of the control activity. To determine if these findings could be confirmed in intact cells, L45 lymphoblasts were cultured in the presence of labelled hypoxanthine and guanine and the ratio of ^{14}C to ^{3}H was determined in the TCA precipitable material. When ^{14}C-hypoxanthine and ^{3}H-guanine were used, ratios of 0.1 were obtained for all the cell lines (Table 24, a). When the labels were reversed, ratios of 0.04, 0.068, and 0.05 were obtained for the L45 and the two control cell lines, respectively (Table 24, b). These results indicate that there is no major defect in the relative utilisation of hypoxanthine and guanine by the L45 lymphoblasts.

11.8. HPRT activity in transformed lymphoblast post-microsomal supernatants

11.8.1. Method

The HPRT activity was determined in lymphoblast post-microsomal supernatants, prepared by a method similar to that of Wilson **et al** (1982). Approximately ten million cells were pelleted and washed twice with 1ml of 0.9% saline in 1ml Eppendorf tubes. They were resuspended in 500µl of 50mM tris-HCl, pH7.8, 150mM NaCl, and lysed by six cycles of freeze-thawing. Cells were frozen in an

Table 25

HPRT activity in post-microsomal supernatants of transformed lymphoblasts. The HPRT activity was determined in post-microsomal supernatants prepared from transformed lymphoblasts from the patient T.K. and normal controls. The enzyme activity was assayed at different concentrations of hypoxanthine and guanine.

A) Hypoxanthine as substrate.

Hypoxanthine (μM)	HPRT activity (μU/mg protein)		
	L45	L145	L141
60	797	9183	13122
150	766	10466	15242
400	1111	20933	23482

B) Guanine as substrate

Guanine (μU)	HPRT activity (μU/mg protein)		
	L45	L145	L141
6	967	17027	7818
15	1112	20833	15261
30	1319	21655	18214

ethanol/dry-ice mixture and thawed in a water-bath at 37°C. The intact cells together with most of the cellular debris from the lysed cells, were removed by centrifugation for 1 minute in an Eppendorf Centrifuge (5412). The supernatant was centrifuged for 30 minutes at 100 000g in a Beckman Airfuge. The enzyme activity was determined in the supernatant, and the latter was diluted with the buffer to give approximately 50μU of enzyme activity per 20μl aliquot. Enzyme activity was determined by the multiple end-point assay (Wohlhueter 1975) in the presence of 1mg/ml BSA.

11.8.2. Results

The HPRT activity in post-microsomal supernatants prepared from the L45 and control lymphoblasts are shown in Table 25. The HPRT activity in the L45 lymphoblasts was substantially lower than that in the control cells over a wide range of substrate concentrations. The L45 lymphoblasts had activities varying between 3-4% of the control activities for hypoxanthine and 5-7% for guanine. The low intracellular activity of HPRT as indicated by the intact cell studies, therefore was substantiated by the low activities obtained in the cell post-microsomal supernatants.

11.8.3. Discussion

The HPRT activity in the post-microsomal supernatants from the cultured lymphoblasts derived from T.K. was low compared with

that from control cells. The activity was less than 10% of that in the control cell supernatants at all purine base concentrations, and is in agreement with that demonstrated in the intact cells by the double label assays.

11.9. Effect of extracellular purine concentration on the relative uptake of hypoxanthine.

11.9.1. Method

To determine whether substrate inhibition of HPRT activity could be demonstrated in the intact cells derived from T.K., double label experiments were performed at different concentrations of hypoxanthine. The relative molar incorporation of hypoxanthine to leucine into TCA precipitable material at each hypoxanthine concentration, was determined. The hypoxanthine concentrations in the medium ranged from 44μM to 544μM.

11.9.2. Results

The relative incorporation of hypoxanthine relative to leucine, into TCA precipitable material, showed a slight increase with an increasing hypoxanthine concentration for the control cell lines. This indicated that the hypoxanthine utilisation was close to

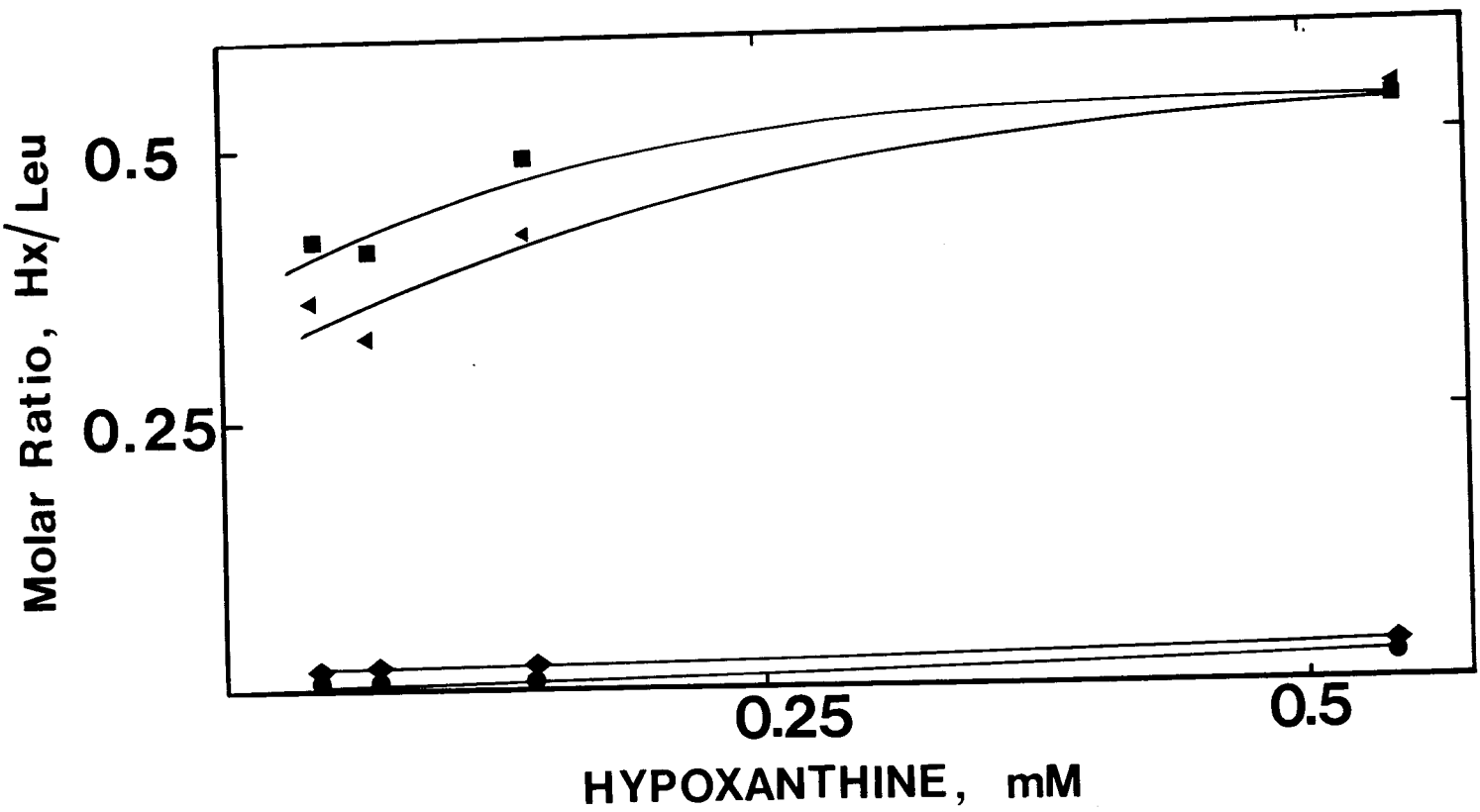


Fig. 19. Effect of the extracellular hypoxanthine concentration on the relative molar incorporation of ^{14}C-hypoxanthine to ^{3}H-leucine into the TCA precipitable material of transformed lymphoblasts. The cells were cultured in the presence of hypoxanthine at concentrations of between 44 to 544μM and the relative incorporation of ^{14}C-hypoxanthine to ^{3}H-leucine into TCA precipitable material, was determined. (●), cells from a patient with the Lesch-Nyhan Syndrome; (◆), cells from T.K.; (◀), and (■), cells from normal controls.

saturation at the lowest concentration used (Fig. 19). The L45 lymphoblasts incorporated 5% of the relative amount of hypoxanthine to leucine, compared with the control cell lines at all the concentrations of hypoxanthine. Therefore no inhibition of hypoxanthine incorporation could be demonstrated. The L106 lymphoblasts showed a relative increase in the incorporation of hypoxanthine from less than 1% to almost 6% of the values obtained for the control cell lines. This may reflect an altered K_{50} for hypoxanthine incorporation or the activation of a non-HPRT dependent system for hypoxanthine uptake in these cells.

11.9.3. Discussion

Studies on several mammalian cell lines have shown that the intracellular and extracellular hypoxanthine are in equilibrium with each other, and that the intracellular concentration of hypoxanthine is similar to that in the culture medium (Murz **et al** 1979). A range of hypoxanthine concentrations in the culture medium was used to ascertain whether a reduction in the flux through the purine salvage pathways in the lymphoblasts, derived from T.K., occurred, and therefore to demonstrate substrate inhibition of the HPRT activity in these cells. However, no change in the incorporation of hypoxanthine in the L45 lymphoblasts, relative to that in the control cell lines, could be shown. It may be that the intracellular concentration of hypoxanthine did not increase sufficiently in the L45 cells for substrate inhibition to be demonstrated, or that the lymphoblast HPRT activity is not susceptible to substrate inhibition.

11.10. Polyethylene glycol-induced cell fusion.

11.10.1. Method

Investigations into the nature of various inherited metabolic diseases have shown that patients presenting with similar biochemical disorders, may have different lesions at a molecular level. This heterogeneity may result in lesions which are complementary to each other. An example of complementary molecular lesions can be found in Methylmalonic Acid Uria. Here deficiencies in methylmalonyl CoA mutase were shown to be due to either apoenzyme defects, or defects in the synthesis of the co-factor, adenosylcobalamin (Rosenberg and Scriver 1980). The fusion of fibroblasts from a patient with an apoenzyme defect, with those from a patient with defective co-factor synthesis, has resulted in hybrid cells with a mutase activity approaching that of control cells (Gravel 1975). To investigate the possibility that the L45 and L106 lymphoblasts had complementary molecluar lesions, it was decided to fuse cells from both lines and to determine if any hybrid cells could survive in HAT-medium. The method used was that of Davidson **et al** (1976). Approximately ten million cells from each of the stock cultures were mixed in a 50ml centrifuge tube and pelleted. The cells were resuspended in 3ml of serum-free medium, transferred to the culture tubes, and were centrifuged at 1500 rpm for 3 minutes. The medium was discarded and the tubes inverted on tissue paper for 1 minute to drain off the last few drops of medium. This was necessary to

prevent dilution of the polyethylene glycol (PEG). After all the medium had been removed, 50% PEG (0.5ml), was added to the cells over a period of 1 minute. While the PEG was added, the tubes were rolled gently to facilitate mixing. After incubation at 37°C for 1 minute, 0.5ml of 20% PEG was added and incubation was continued at room temperature. After 3 minutes the PEG was diluted with 2ml of serum-free medium and the cells were pelleted at 1500 rpm for 3 minutes. The cells were resuspended in 8ml of medium, divided into two 4ml fractions and the cells from each fraction were pelleted. One of the pellets was resuspended in 10ml of HAT-medium and the other in 10ml of medium, and the cells were cultured in $25cm^2$ flasks for 4 weeks. The growth and appearance of the cells was monitored over this period. Reference cultures (isohybrids) of PEG-treated cells were prepared for each of the HPRT-deficient cell lines. Hybrids of control lymphoblasts were also prepared. The PEG and serum-free medium mixture was prepared as follows: 10 grams of PEG (M.W.1500, BDH) was autoclaved at 121°C for 15 minutes. The PEG was allowed to cool to 45°C and mixed with 10ml of pre-warmed, serum-free medium.

11.10.2. Results

The PEG-induced fusion experiments were designed to determine whether complementary biochemical defects existed between the two $HPRT^-$ lymphoblast cell lines, L45 and L106. After the cells from these two lines had been fused, they were divided into two fractions, one for culture in HAT-medium, while the other was maintained as a control culture. The cells in the control

cultures continued to grow rapidly, while those in the HAT-medium did not. No hybrid cell-clones appeared that could survive in HAT-medium. This indicated a lack of hypoxanthine salvage in these cells, and therefore no complementation between the two $HPRT^-$ cell lines. Cells from two control lymphoblast cell lines, fused and treated in a similar manner to the $HPRT^-$ cells, grew well in HAT-medium. The isohybrid $HPRT^-$ cells failed to grow in HAT-medium.

11.10.3. Discussion

Hybridization experiments with $HPRT^-$ and $HPRT^+$ cells have demonstrated that there are at least two cistrons that can affect HPRT activity in cultured cells (Kelley and Wyngaarden 1983). It is possible that defects in either of these cistrons may be responsible for a lack of HPRT activity. Hybrid cells prepared from two different $HPRT^-$ cell lines, may develop adequate HPRT activity to survive in HAT-medium, if their defects are complementary. However, no such complementary defect appears to exist in the two $HPRT^-$ cell lines tested in this experiment. Similar studies with other $HPRT^-$ cells, may yield $HPRT^+$ hybrids.

SECTION 3

12. PARTIAL HPRT-DEFICIENCY: A CASE REPORT

12.1. Medical history

T.K. has a history of repeated attacks of renal colic, the first occured at an age of 4. He was investigated for haematuria at 13 years of age , and again at 17 years. No records of the findings of these investigations are available. At 18 years he had his first attack of arthritis, which affected his great toes and one of his ankle joints. Since then he has had several attacks of arthritis, which have varied in intensity from mild discomfort in his toes, to severe gout. He has no history of any major illnesses, surgery or accidents. He is the only affected member of his family. He is married, with one daughter, and works as a book binder.

12.2. Physical examination

T.K. is slightly built, weighs 54kg and has no physical deformities (Fig. 20). There are no abnormalities of his cardiovascular, respiratory, gastro-intestinal or musculo-skeletal systems. Neurologicaly he has a symmetrical hyper-reflexia of his knees and ankles, and a fine nystagmus on sustained lateral gaze. His I.Q. is 66. Investigations into his genito-urinary system have shown

Fig. 20. Photograph of patient T.K.

that he has a normal left kidney, and a small, barely functioning right kidney with a bifid collecting system and clubbed calyces. The reduced function of his right kidney is secondary to the congenital abnormalities affecting that kidney, and is not related to his hyperuricaemia(Cassidy et al 1980).

12.3. Laboratory investigations

His serum urate fluctuates between 0.71-8.2 mmol/l (reference range 0.12-0.5 mmol/l), and his urinary urate levels are between 6.0-7.4 mmol/d (reference range 1.5-4.4 mmol/d), when he does not receive treatment. His urate clearance is 11 ml/minute. All his other serum biochemical parameters are within the reference limits for this laboratory. Haemotological investigations have shown no signs of macrocytosis.

12.4. Treatment and clinical course

At present he is being treated with allopurinol, 200mg twice daily, and citro soda, 2 g three times daily. With this treatment, his serum urate remains at about 0.45 mmol/l and his urinary pH is about 6.8. However, he does not take his drugs when he is free of symptoms. He has had occasional attacks of arthritis, about once a year, and has had one attack of renal colic, since he has been on treatment. His failure to take his drugs is due to a complete lack of insight into the nature of his disease. There has been no change in his renal function in the last five

years, with the creatine clearance remaining between 80 and 100 ml/minute. He has had one episode of an asymptomatic elevation in his serum creatine kinase (CK) activity. The CK activity reached a peak of 225 U/l (reference range 0-50 U/l), and remained elevated for 4 months. The MB-isoenzyme was negative on electrophoresis and there were no other signs of cardiac involvement. There was a transient increase in his serum LDH activity to a peak of 388 U/L (reference rage 100-350 U/l), with a normal isoenzyme pattern. He remained entirely asymptomatic during this period. The CK activity returned to normal without any treatment, nor did he stop taking his allopurinol. The cause of the elevated CK activity was never established, but it was assumed to be due to a possible deposition of oxipurinol or of other purine crystals in his muscle tissue (Watts **et al** 1971).

12.5. Discussion

The presentation of T.K. with the complications of hyperuricaemia, is typical of a patient with a partial $HPRT^-$ deficiency (Kelley and Wyngaarden 1983). The high daily urate excretion and normal urate clearance, indicate that he is a urate over-producer, and that his hyperuricaemia is not due to a renal mechanism. The HPRT activity in his red blood cells, is about 25% of the normal mean, and is typical of the activities found in patients with a partial HPRT-deficiency (Kelley and Wyngaarden 1983). His renal function has remained constant over the five years since the diagnosis of a partial HPRT-deficiency was made, and, provided that the episodes of renal

colic can be kept to a minimum, and any secondary bacterial infections prevented, it should not change. His treatment is complicated by his failure to take his drugs, and it is unlikely that anything can be done to improve his compliance.
The cause of the transcient elevation in his serum CK activity remains unknown, but muscle injury due to crystal deposition remains a possiblity. The administration of allopurinol may elevate the intracellular levels of hypoxanthine and guanine in his cells and therefore, increase the degree of HPRT-deficiency by substrate inhibition. However this is a theoretical problem. Since his intracellular HPRT activity is 10% normal, a marked change in purine base concentration would be needed to inhibit the enzyme any further if substrate inhibition is the cause of the low activity, particularly as zero enzyme activity occurs only at an infinite substrate concentration in uncompetetive substrate inhibition (Cleland 1979). The beneficial effects of allopurinol in reducing his serum urate levels and in lowering the frequency of his attacks of gout and renal stones is far greater than the danger of increasing his intracellular purine base concentration.

13. SUMMARY AND GENERAL CONCLUSIONS

The patient T.K. presented with the clinical features, typical of purine overproduction and a partial HPRT deficiency; uric acid lithiasis and gout. The interesting kinetic characteristics of the variant HPRT activity in his haemolysates would have been missed had it been assayed at only one substrate concentration. The variant enzyme activity in haemolysates showed substrate inhibition by the purine base substrates. This was responsible for the low HPRT activity observed **in vitro**, since there were no marked differences in the K_m and V_{max} values between the variant and control enzymes. The substrate inhibition of the variant enzyme resulted from the formation of a dead-end complex between hypoxanthine and an enzyme-PP_i intermediate. Hypoxanthine and IMP can bind at the same site on the variant enzyme-PP_i intermediate; the mechanism that prevents this from happening in the normal enzyme is unknown, but it may be that a conformational change takes place on the release of IMP, preventing the binding of hypoxanthine. The HPRT activities were assayed by a modification of the assay published by Wohlhueter (1975), taking into consideration the pH and magnesium dependence of the enzyme activity. An alternative kinetic assay (Giacomello and Salerno 1977) was used in the initial characterisation of the variant enzyme; similar kinetic parameters for the variant and control enzyme activities were obtained with both assays. The variant enzyme activity was unstable in its partially purified form, and since no difference in the degree of substrate inhibition could be shown between the enzyme activity in haemolysates and the

partially purified activity, the former was used for the kinetic characterisation of the variant. A low HPRT activity was shown in cultured cells from the patient. The double label technique demonstrated a reduced flux through the hypoxanthine/guanine salvage pathway, relative to that through the thymidine or leucine pathways. The rate of purine synthesis **de novo** was higher in the transformed lymphoblasts from T.K. compared with that of control cells. This was shown by the double label approach, and by the release of labelled hypoxanthine and guanine into the medium. The APRT activity in the $HPRT^-$ cells was similar to that in the control cells. The behaviour of cultured cells obtained from T.K. and cells known to be defective in HPRT activity was similar in selective media. This included their inability to grow in HAT-medium or the modified GAMA-medium, and their resistance to the toxic effects of thioguanine and azaguanine. No substrate inhibition could be demonstrated in these cells despite the low HPRT activity, nor could a complementary biochemical defect between the two HPRT deficient cell lines, be shown. The HPRT activity in post-microsomal supernatants, obtained from the transformed lymphoblasts from T.K., was 10% of the control activity.

Although these studies have shown this HPRT variant an interesting and unique enzyme, the molecular defect still has to be defined. This could be due to either a change in the primary structure, or to a secondary modification of the enzyme protein. Therefore protein structural studies, complemented by an analysis of the gene coding for the HPRT variant, have yet to be done.

Note added in proof

A paper based on the kinetic characterisation and investigation into the mechanism of the substrate inhibition of the variant HPRT activity presented in this thesis, has been accepted for publication in the Journal of Biological Chemistry.

REFERENCES

Agarwal, R.P., Agarwal, K.C., & Parks, R.E. (1978) A General method for the isolation of Various Enzymes from Human Erythrocytes.
In: Methods in Enzymology (Colowick S.P., and Kaplan,N.O., eds-in-chief) Vol LI, Purine and pyrimidine nucleotide metabolism (Hoffee P.A., and Jones M.E., eds) pp 581-586 Academic Press, New York

Ali, L.Z., & Sloan, D.L. (1982) Studies of the kinetic mechanism of hypoxanthine-guanine phosphoribosyltransferase from yeast.
J. Biol. Chem. **257**, 1149-1155

Arnold, W.J., & Kelley, W.N. (1971) Human hypoxanthine-guanine phosphoribosyltransferase purification and subunit structure.
J. Biol. Chem. **246**, 7398-7404

Bakay, B., & Nyhan, W.L. (1972) Activation of variants of hypoxanthine-guanine phosphoribosyltransferase by the normal enzyme.
Proc. Natl. Acad. Sci. USA **69**, 2523-2527

Bakay, B., & Nyhan, W.L. (1972) Electrophoretic properties of hypoxanthine-guanine phosphoribosyltransferase in erythrocytes of subjects with Lesch-Nyhan Syndrome.
Biochem. Genet. **6**, 139-147

Balis, M.E., Yip, L.C., Yü, T.F., Gutman, A.B., Cox, R., & Dancis, J. (1974) Unstable HPRTase in subjects with abnormal urinary oxypurine excretion.
In: Purine metabolism in man (Sperling, O., De Vries, A., Wyngaarden, J.B., eds) pp 195-202, Plenum, New York.

Beardmore, T.D., Meade, J.C., & Kelley, W.N. (1973) Increased activity of two enzymes of pyrimidine biosynthesis **de novo** in erythrocytes from patients with the Lesch-Nyhan syndrome
J. Lab. Clin. Med. **81**, 43-52

Becker, M.A., Meyer, L.J., Wood, A.W., & Seegmiller, J.E. (1973) Purine overproduction in man associated with increased PP-ribose-P synthetase activity.
Science **179**, 1123-1126

Benke, P.J., Herrick, N., & Herbert, A. (1973) Hypoxanthine-guanine phosphoribosyl-transferase variant associated with accelerated purine synthesis.
J. Clin. Invest. **52**, 2234-2240.

Bevington, P.R. (1969)
Data reduction and error analysis for the physical sciences. pp 92-118, McGraw-Hill, New York.

Brennand, J., Chinault, A.C., Konecki, D.S., Melton, D.W., & Caskey, C.T. (1982) Cloned cDNA sequences of the hypoxanthine/guanine phosphoribosyltransferase gene from a mouse neuroblastoma cell line found to have amplified genomic sequences.
Proc. Natl. Acad. Sci. USA **79**, 1950-1954

Boyum, A. (1968) Isolation of mononuclear cells & granulocytes from human blood.
Scand. J. Clin. Invest. **21**, Supp. 97, 77-89

Cassidy, M., Gregory, M.C., & Harley, E.H. (1980) Primary overproduction of urate caused by a partial deficiency of hypoxanthine-guanine phosphoribosyltransferase.
S. Afr. Med. J. **57**, 948-950.

Cleland, W.W. (1979) Substrate inhibition.
In: Methods in Enzymology (Colowick, S.P., & Kaplan, N.O., eds-in-chief) Vol 63, Enzyme kinetics & mechanism, Part A, (Purich, P.L., ed) pp 500-513, Academic Press, New York

Craft, J.A., Dean, B.M., Watts, R.W.E., & Westwich, W.J. (1970) Studies on human erythrocyte IMP:pyrophosphate phosphoribosyltransferase.
Eur. J. Biochem. **15**, 367-373

Davidson, R.L., O'Malley, K.A., Wheeler, T.B. (1976) Polyethylene glycol-induced mammalian cell hybridization: Effect of polyethylene glycol molecular weight and concentration.
Somatic Cell Genetics **2**, 271-280.

Debray, H., Cartier, P., Temstet, A., & Cendron, J. (1976) Child's urinary lithiasis revealing a complete deficient in adenine phosphoribosyltransferase.
Pediatr. Res. **10**, 762-766.

Dent, C.E., & Philpot, G.R. (1954) Xanthinuria, an inborn error (or deviation) of metabolism.
Lancet **1**, 182-185

Emmerson, B.T., Thompson, C.J., & Wallace, D.C. (1972) Partial deficiency of hypoxanthine-guanine phosphoribosyltransferase: Intermediate enzyme deficiency in heterozygote red cells.
Ann. Intern. Med. **76**, 285-287

Fishbein, W.N., Armbrustmacher, V.W., & Griffin, J.L. (1978) Myoadenylate deaminase deficiency: A new disease of muscle.
Science **200**, 545-548

Fox, I.H., & Kelley, W.N. (1971) Phosphoribosylphyrophosphate in man: Biochemical and Clinical significance.
Ann. Intern. Med **74**, 424-433

Fox, I.H., & Kelley, W.N. (1972) Human phosphoribosylpyrophosphate synthetase: Kinetic mechanism and end-product inhibition.
J. Biol. Chem. **247**, 2126-2131

Franklin, T.J., & Cook, J.M. (1969) The inhibition of nucleic acid synthesis by mycophenolic acid.
Biochem. J. **113**, 515-524

Ghangas, G.S., & Milman, S. (1975) Radioimmune determination of hypoxanthine phosphoribosyltransferase cross-reacting material in erythrocytes of Lesch-Nyhan patients.
Proc. Natl. Acad. Sci. USA **72**, 4147-4150

Giacomello, A., & Salerno, C. (1977) A continuous spectrophotometric assay of hypoxanthine-guanine phosphoribosyltransferase.
Annalytical Biochem. **79**, 263-267

Giacomello, A., & Salerno, C. (1978) Human hypoxanthine-guanine phosphoribosyltransferase steady state kinetics of the forward and reverse reactions.
J. Biol. Chem. **253**, 6038-6044

Giblett, E.R., Anderson, J.E., Cohen, F., Pollara, P., & Meuwisson, H.J. (1972) Adenine deaminase deficiency in two patients with severely impaired cellular immunity.
Lancet **2**, 1067-1069

Giblett, E.R., Ammann, A.J., Wara, D.W., Sandman, R., & Diamond, L.K. (1975) Nucleoside-phosphorylase deficiency in a child with severely defective T-cell immunity and normal B-cell immunity.
Lancet **1**, 1010-1013

Gordon, R.B., Thompson, L., Johnson, L.A., & Emmerson, B.T. (1979) Regulation of purine **de novo** synthesis in cultured human fibroblasts : The role of P-ribose-PP.
Biochem. Biophys. Acta **562**, 162-176

Gravel, R.A., Mahoney, M.J. Ruddle, F.H., & Rosenburg, L.E. (1975) Genetic complementation in heterokaryons of human fibroblasts defective in cobalamin metabolism.
Proc. Natl. Acad. Sci. USA **72**, 3181-3185

Greene, M.L., Boyle, J.A., & Seegmiller, J.E. (1970) Substrate stabilization: genetically controlled reciprocal relationship of 2 human enzymes.
Science **167**, 887-889

Gutensohn, W., & Jahn, H. (1979) Partial deficiency of hypoxanthine-phosphoribosyl transferase: evidence for a structural mutation in a patient with gout.
Eur. J. Clin. Invest. **9**, 43-47

Hatanaka, M., del Guidice, R., & Long, C. (1975) Adenine formation from adenosine by mycoplasmas: adenosine phosphorylase activity.
Proc. Natl. Acad. Sci USA **72**, 1401-1405

Henderson, J.F. (1968) Kinetic properties of hypoxanthine-guanine and adenine phosphoribosyltransferases.
Fed. Proc. **27**, 1053-1054

Henderson, J.F., Brox, L.W., Kelley, W.N., Rosenbloom, F.M., & Seegmiller, J.E. (1968) Kinetic studies of hypoxanthine-guanine phosphoribosyltransferase.
J. Biol. Chem. **243**, 2514-2522

Henderson, J.F., Dossetor, J.B., Dasgupta, M.K., & Russell, A.S. (1976) Uric acid lithiasis associated with altered kinetics of hypoxanthine-guanine phosphoribosyltransferase.
Clin. Biochem. **9**, 4-8

Hochstadt, J. (1978) Hypoxanthine phosphoribosyltransferase and guanine phosphoribosyltransferase from enteric bacteria.
In: Methods in Enzymology (Colowick, S.P., and Kaplan, N.O., eds-in-chief) Vol I, Purine and pyrimidine nucleotide metabolism, (Hoffee PA, and Jones ME, eds) pp 549-558, Academic Press, New York

Holden, J.A., & Kelley, W.N. (1978) Human hypoxanthine-guanine phosphoribosyltransferase evidence for tetrametic structure.
J. Biol. Chem. **253**, 4459-4463

Holmes, E.W., Mc Donald, J.A., McCord, J.M., Wyngaarden, J.B., & Kelley, W.N. (1973) Human glutamine phosphoribosylpyrophosphate amidotransferase: Kinetic and regulatory properties.
J. Biol. Chem. **248**, 144-150

Holmes, E.W., Wyngaarden, J.B., & Kelley, W.N. (1973) Human glutamine phosphoribosylpyrophosphate amidotransferase: Two molecular forms inter-convertible by purine ribonucleotides and phosphoribosylpyrophosphate.
J. Biol. Chem. **248**, 6035-6040

Hora, K., Kashiwamata, S., Ogawara, N., Ohishi, H., Natsume, R., Yamanaka, T., Hakamada, S., Migazaki, S., and Wahanabe, K. (1982) A female case of the Lesch-Nyhan Syndrome.
Tokoka J. Exp. Med. **137**, 275-282

Hughes, S.H., Wahl, G.M., & Caysecchi, M.R. (1975) Purification and characterisation of mouse hypoxanthine-guanine phosphoribosyltransferase.
J. Biol. Chem. **250**, 120-126

Johns, D.G., & Adamson, R.H. (1976) Enhancement of the biological activity of cordycepin (3'-deoxyadenosine) by the adenosine deaminase inhibitor 2'-deoxyloformycin.
Biochem. Pharmacol. **25**, 1441-1444

Johnson, G.G., Eisenberg, L.R., & Migeon, B.R. (1979) Human and mouse hypoxanthine-guanine phosphoribosyltransferase : Dimers and tetramers.
Science **203**, 174-176

Johnson, L.A., Gordon, R.B., & Emmerson, B.T. (1977) Hypoxanthine-guanine phosphoribosyltransferase: a simple spectrophotometric assay.
Clin. Chim. Acta **80**, 203-207

Jolly, D.J., Esty, A.L., Bernard, H.U., & Friedman, T. (1982) Isolation of a genomic clone partially encoding Human hypoxanthine phosphoribosyltransferase.
Proc. Natl. Acad. Sci. USA **79**, 5038-5041

Kelley, W.N. (1971) Studies on the adenine phosphoribosyltransferase enzyme in human fibroblasts lacking hypoxanthine-guanine phosphoribosyltransferase.
J. Lab. Clin. Med. **77**, 33-38

Kelley, W.N., Greene, M.L., Rosenbloom, F.M., Henderson, J.F., & Seegmiller, J.E. (1969) Hypoxanthine-guanine phosphoribosyl transferase in gout.
Ann. Intern. Med. **70**, 155-206

Kelley, W.N., Rosenbloom, F.M., Henderson, J.F., & Seegmiller, J.E. (1967) A specific enzyme defect in gout associated with overproduction of uric acid.
Proc. Natl. Acad. Sci. USA **57**, 1735-1739

Kelley, W.N., & Wyngaarden, J.B. (1983) Clinical syndromes associated with hypoxanthine-guanine phosphoribosyltransferase deficiency.
In: The metabolic basis of inherited disease, 5th edition (Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L., & Brown, M.S., eds) pp 1115-1143, McGraw-Hill, New York

Kredich, N.M., & Hershfield, M.S. (1983) Immunodeficiency diseases caused by adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency.
In: The metabolic basis of inherited disease, 5th edition (Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L., & Brown, M.S., eds) pp 1157-1183, McGraw-Hill, New York

Krenitsky, T.A., Elion, G.B., Henderson, A.M., & Hitchings, G.H. (1968) Inhibition of human purine nucleoside phosphorylase : Studies with intact erythrocytes and the purified enzyme.
J. Biol. Chem. **243**, 2876-2881

Krenitsky, T.A., & Papaioannou, R. (1969) Human hypoxanthine phosphoribosyltransferase II) Kinetics and chemical modification.
J. Biol. Chem. **244**, 1271-1277

Krenitsky, T.A., Papaioannou, R., & Elion, G.B. (1969) Human hypoxanthine phosphoribosyltransferase I) Purification, properties and specificity.
J. Biol. Chem. **244**, 1263-1270

Kunkel, L.M., Smith, K.D., Boyer, S.H., Borgaonkor, D.S., Wachtel, S.S., Miller, O.J., Berg, W.R., Jones, H.W., & d'Rary, J.M. (1977) Analysis of human Y-chromosome-specific reiterated DNA in chromosome variants.
Proc. Natl. Acad. Sci. U.S.A. **74**, 1245-1249

Lesch, M., & Nyhan, W.L. (1964) A familial disorder of uric acid metabolism and central nervous system function.
Amer. J. Med. **36**, 561-570

Liskay, R.M. & Patterson, D. (1979) A selective medium (GAMA) for the isolation of somatic cell hybrids from $HPRT^-$ and $APRT^-$ mutant cells.
Cytogenet. Cell. Genet. **23**, 61-69

Littlefield, J.W. (1964) Selection of hybrids from matchings of fibroblasts **in vitro** and their presumed recombinants.
Science **145**, 709-710

Lomman, E.J.P., Vogels, G.D., Van Der Zee, S.P.M., Trijbels, J.M.F., & Schoetlen, E.D.A.M. (1971) Concentration of purine nucleotides in erythrocytes of patients with the Lesch Nyhan syndrome before and during oral administration of adenine.
Acta Paediat. Scand. **60**, 642-646

Lowenstein, J., & Tornheim, K. (1971) Ammonia production in muscle : The purine nucleotide cycle.
Science **171**, 397-400

Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951) Protein measurement with the folin phenol reagent.
J. Biol. Chem. **193**, 265-275

Mager, J., Magasanik, B. (1960) Guanosine 5'phosphate reductase and its role in the interconversion of purine nucleotides.
J. Biol. Chem. **235**, 1474-1478

Mapes, J.P., & Krebs, H.A. (1978) Rate limiting factors in urate synthesis and glucoreogenesis in Avian liver.
Biochem. J. **172**, 193-203

Marz, R., Wohlhueter, R.M., & Plagemann, P.G.W. (1979) Purine and pyrimidine transport and phosphoribosylation and their interaction in overall uptake by cultured mammalian cells.
J. Biol. Chem. **254**, 2329-2338

McDonald, J.A., & Kelley, W.N. (1971) Lesch-Nyhan syndrome : altered kinetic properties of mutant enzyme.
Science **171**, 689-691

Miller, G., & Lipman, M. (1973) Release of infectious Epstein-Barr virus by transformed marmoset leukocytes.
Proc. Natl. Acad. Sci. USA **70**, 190-194

Muensch, H., & Yoshida, A. (1977) Purification and characterization of human hypoxanthine/guanine phosphoribosyltransferase.
Eur. J. Biochem. **76**, 107-112

Northrop, D.B., & Cleland, W.W. (1974) The kinetics of pig heart triphosphopyridine nucleotide-isocitrate dehydrogenase II Dead-end and multiple inhibition studies.
J. Biol. Chem. **249**, 2928-2931

Nuki, G., Astrin, K., Brenton, D., Cruikshank, M., Lever, J., & Seegmiller, J.E. (1973) Purine and pyrimidine nucleotide pools in azaserine resistant human lymphoblasts deficient in HGPRT, abstracted.
Am. J. Hum. Genet. **25**, 56A

Nuki, G., Lever, J., & Seegmiller, J.E. (1974) Biochemical characteristics of 8-azaguanine resistant human lymphoblast mutants selected **in vitro**.
In: Purine metabolism in man (Sperling, O., de Vries, A. & Wyngaargen, J.B., eds) pp 255-267, Plenum, New York

Olsen, A.S., & Milman, G. (1977) Human hypoxanthine phosphoribosyltransferase. Purification and properties.
Biochemistry **16**, 2501-2505

Olsen, A.S. & Milman, G. (1978) Hypoxanthinephosphoribosyltransferase from Chinese Hamster Brain and human erythrocytes.
In: Methods in Enzymology (Colowick S.P., and Kaplan,N.O., eds-in-chief) Vol LI, Purine and pyrimidine nucleotide metabolism (Hoffee P.A., and Jones M.E., eds) pp 543-549 Academic Press, New York

Page, T., Bakay, B., Nissenen, E., & Nyhan, W.L. (1981) Hypoxanthine-guanine phosphoribosyltransferase variants: correlation of clinical phenotype with enzyme activity.
J. Inher. Metab. Disease **4**, 203-206

Pehlke, D.M., Mc Donald, J.A., Holmes, E.W., & Kelley, W.N. (1972) Insosinic acid dehydrogenase activity in the Lesch-Nyhan Syndrome.
J. Clin. Invest. **51**, 1398-1404

Reem, G.H., & Friend, C. (1975) Purine metabolism in murine virus-induced erythroleukemia cells during differentiation in vitro.
Proc. Natl. Acd. Sci. USA **72**, 1630-1634

Rosenberg, L.E., & Scriver, C.R. (1980) Disorders of amino acid metabolism.
In: Metabolic Control & Disease (Bondy, P.K., & Rozenberg, L.E., eds) pp 583-776, W.B.Saunders, Philadelphia.

Rosenbloom, F.M., Kelley, W.N., Miller, J., Henderson, J.F., & Seegmiller, J.E. (1967) Inherited disorder of purine metabolism: Correlation between central nervous system dysfunction and biochemical defects.
JAMA **202**, 175-177

Rowe, P.B., McCairns, E., Madsen, G., Sauer, D., & Elliot, H. (1978) **De Novo** purine synthesis in avian liver
J. Biol. Chem. **253**, 7711-7721

Rozen, R., Buhl, S., Mohynddin, F., Caillibot, V., & Scriver, C.R. (1977) Evaluation of metabolic pathway activity in cultured skin fibroblasts and blood leukocytes.
Clin. Chim. Acta **77**, 379-386

Rylance, H.J., Wallace, R.C., & Nuki, G. (1982) Hypoxanthine-guanine phosphoribosyltransferase: assay using high performance liquid chromatography.
Clin. Chim. Acta **121**, 159-165

Salerno, C., & Giacomello, A. (1979) Human hypoxanthine-guanine phosphoribosyltransferase IMP-GMP exchange: stoichiometry and steady state kinetics of the reaction.
J. Biol. Chem. **254**, 10232-10236

Salerno, C., & Giacomello, A. (1981) Human hypoxanthine-guanine phosphoribosyltransferase. The role of magnesium ion in a phosphoribosylpyrophosphate utilizing enzyme.
J. Biol. Chem. **256**, 3671-3673

Seegmiller, J.E., Rosenbloom, F.M., & Kelley, W.N. (1967) Enzyme defect associated with a sex-linked human neurological disorder and excessive purine synthesis.
Science **155**, 1682-1683

Simmonds, H.A., Van Acker, K.J., Cameron, J.S., & Sneddon, W. (1976) The identification of 2,8 dihydroxyadenine, a new component of urinary stones.
Biochem. J. **157**, 485-487

Sperling, O., Boer, P., & de Vries, A. (1974) Properties of erythrocyte purine phosphoribosyltransferases in partial hypoxanthine-guanine phosphoribosyltransferase deficiency.
In: Purine Metabolism in man (Sperling O., de Vries, A., and Wyngaarden J.B., eds) pp 211-213, Plenum, New York.

Sperling, O., Eilam, G., Persky-Brosch, S., & de Vries, A. (1972) Accelerated erythrocyte 5-phosphoribosyl-1-pyrophosphate synthesis. A familial abnormality associated with excessive uric acid production and gout.
Biochem. Med. **6**, 310-316

Steyn, L.M., & Harley, E.H. (1981) Substrate inhibition of HGPRT: Protein structural and kinetic studies of a human variant.
J. Inher. Metab. Dis. **4**, 115-116

Sweetnam, L., Hoch, M.A., Bakay, B., Borden, M., Lesh, P., & Nyhan, W.L. (1978) A distinct human variant of hypoxanthine-guanine phosphoribosyltransferase.
J. Pediatrics **92**, 385:389

Uhr, M.L., Thompson, V.W., & Cleland, W.W. (1974) The kinetics of pig heart triphosphopyridine nucleotide-isocitrate dehydrogenase I initial velocity, substrate and product inhibition, and isotope exchange studies.
J. Biol. Chem. **249**, 2920-2927

Upchurch, K.S., Leyva, A., Arnold, W.J., Holmes, E.W., & Kelley, W.N. (1975) Hypoxanthine phosphoribosyltransferase deficiency: Association of reduced catalytic activity with reduced levels of immunologically detectable enzyme protein.
Proc. Natl. Acad. Sci. USA 72, 4142-4146

Watts, R.W.E., Sneeden, W., & Parker, R.A. (1971) A quantitative study of skeletal-muscle purines and pyrazolo (3,4-d)pyrimidines.
Clin. Sci. **41**, 153-158

Wilkinson, G.N. (1961) Statistical estimation in Enzyme Kinetics.
Biochem. J. **80**, 324-332

Wilson, J.M., Baugher, B.W., Landa, L., & Kelley, W.N. (1981) Human hypoxanthine-guanine phosphoribosyltransferase: Purification and characterization of mutant forms of the enzyme.
J. Biol. Chem. **256**, 10306-10312

Wilson, J.M., Baugher, B.W., Mattes, P.M., Doddona, P.E., & Kelley, W.N. (1982) Human hypoxanthine-guanine phosphoribosyltransferase demonstration of structural variants in lymphoblastoid cells derived from patients with a deficiency of the enzyme.
J. Clin. Invest. **69**, 706-715

Wilson, J.M., Tarr, G.E., & Kelley, W.N. (1983) Human hypoxanthine (guanine) phosphoribosyltransferase: An amino acid substitution in a mutant form of the enzyme isolated from a patient with gout.
Proc. Natl. Acad. Sci. USA **80**, 870-873

Wilson, J.M., Tarr, G.E., Mahoney, W.C., & Kelley, W.N. (1982) Human hypoxanthine-guanine phosphoribosyltransferase complete amino acid sequence of the erythrocyte enzyme.
J. Biol. Chem. **257**, 10978-10985

Wohlhueter, R.M. (1975) Hypoxanthine phosphoribosyltransferase activity in normal, developing, and neoplastic tissues of the rat.
Europ. J. Cancer **II**, 463-472

Wyngaarden, J.B., & Greenland, R.A. (1963) The inhibition of succinoadenylate kinosynthetase of Escherichia coli by adenosine and guanosine 5'-monophosphates.
J. Biol. Chem. **238**, 1054-1057

Wyngaarden, J.B., & Kelley, W.N. (1983) Gout.
In: The metabolic basis of inherited disease, 5th edition (Stanbury J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L., Brown, M.S., eds) pp 1043-1114, McGraw-Hill, New York.

Yonetani, T., & Theorell, H. (1964) Studies on liver alcohol dehydrogenase complezes III Multiple inhibition kinetics in the presence of two competitive inhibitors.
Arch. Biochem. Biophys. **106**, 243-251

Zoref, E., de Vries, A. & Sperling, O. (1975) Mutant feedback-resistant phosphoribosylpyrophosphate synthetase associated with purine over-production and gout.
J. Clin. Invest. **56**, 1093-1099

Zoref-Shani, E., Sperling, O. (1980) Characterization of purine nucleotide metabolism in cultivated fibroblasts with deficiency of HGPRT and with superactivity of phosphoribosylpyrophosphate synthetase.
Enzyme **25**, 413-418