THE DEVELOPMENT, DISTRIBUTION AND PROPERTIES OF PURINE PHOSPHORIBOSYLTRANSFERASES IN MAMMALS

BY

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A THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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DECLARATION

I declare that this thesis is my own composition, and that the work herein has been carried out by myself and that it has not been submitted in any previous application for a higher degree.

This thesis describes the results of research carried out in the Department of Paediatric Biochemistry, the Royal Hospital for Sick Children, and the University of Edinburgh, under the supervision of Dr R.A. Wadsworth between October 1969 and October 1972.

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SUMMARY

The object of the work described in this thesis has been to determine the tissue distribution, development, and properties of the purine phosphoribosyltransferases (PRTases), especially in man but also in some other animals.

A major part of the work was directed to the study of hypoxanthine guanine phosphoribosyltransferase EC 2.4.2.8 (HGPRTase) in man in view of its importance in human metabolism as shown by pathological abnormalities resulting from its deficiency.

As existing assay methods would tend to give falsely low results, an assay method was developed for the purine PRTases from tissues, which incorporated thymidine triphosphate as a selective inhibitor of 5'-nucleotidase EC 3.1.3.5, to prevent the hydrolysis of the PRTase reaction products.

The specific activity of HGPRTase and adenine phosphoribosyltransferase EC 2.4.2.7 (APRTase) was determined in tissues from foetuses, children and adults. In the central nervous system and,

unexpectedly, in the testis, HGPRTase activity increased during childhood to reach adult levels which were several times higher than those found in the foetus and in other tissues in the adult. The activity of HGPRTase in the other tissues studied remained constant or increased only slightly during development. In central nervous system, APRTase activity decreased during the period between foetal and neonatal life, while liver APRTase activity increased during childhood. In the adult, liver activity was about three times that found in foetal liver.

Since it was difficult to obtain human tissues between the ages of 2 years and 60 years, a study was carried out on the developmental changes in rat tissue HGPRTase activity. Complex changes in activity were detected, being most marked in testis, liver, and cerebral cortex. However, some evidence was obtained for a rise in testicular HGPRTase occurring during sexual maturation. Low activities of HGPRTase were detected in the testis, liver, and cerebral cortex of rats at 16 months of age which, for the P.V.G. strain of rats used, was very old age.

The electrophoretic properties of the purine PRTases were investigated using a newly developed detection technique and four isoenzyme bands were detected for both HGPRTase and APRTase.

HGPRTase from several human tissues was studied by determining the thermal denaturation rate constants and the results suggested the possible presence of a tissue specific testicular HGPRTase.

Autoradiographic localisation of testicular HGPRTase showed that the majority of the activity was in the basal layers of the epithelium of the seminiferous tubule.

As hypoxanthine is the major purine released by erythrocytes, and the uptake of hypoxanthine into erythrocytes is dependent on HGPRTase, the possible effect of differing tissue enzyme activities on the uptake of purines by tissues from erythrocytes, was investigated in the rabbit using prelabelled rabbit erythrocytes. The results demonstrated that erythrocyte purines were transported to tissues and incorporated into cellular nucleic acids. The uptake of purines by tissues from the erythrocytes did not bear a simple direct relationship to the tissue HGPRTase activity.

In conclusion, the developmental changes detected in HGPRTase activity in the central nervous system may provide an explanation for the delayed onset of the neurological symptoms found in children with the Lesch-Nyhan syndrome and would appear to be broadly related to the development of function of some parts of the central nervous system. The unexpectedly high HGPRTase activity located in the seminiferous tubule of the testis, and the developmental changes in activity found to occur in this tissue, suggest a role for HGPRTase in spermatogenesis which may be important.

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SUMMARY

SECTION I

GENERAL INTRODUCTION: PATHWAYS OF PURINE NUCLEOTIDE METABOLISM

1.1 HISTORICAL

The existence of the purine phosphoribosyltransferases (PRTases) was first demonstrated by Buchanan and co-workers (Williams and Buchanan, 1953; Remy, Remy and Buchanan, 1955; Korn, Remy and Buchanan, 1955), who observed the synthesis of adenylic acid and inosinic acid from the free bases adenine and hypoxanthine. Using pigeon and beef liver homogenates, these workers showed that the synthesis of AMP and IMP from adenine or hypoxanthine in the presence of ribose-5-phosphate and ATP, was direct and not via adenosine or inosine.

The enzymes catalysing these reactions were characterised, and, in view of their apparently peripheral role in purine metabolism, were designated as 'salvage' pathways for the reutilisation of free purine bases (Kornberg, 1957). These salvage reactions thus conserved the intact purine ring system, synthesised at the expense of five ATP requiring steps in the *de novo* biosynthetic pathway.

The term 'salvage pathway' is perhaps unfortunate as it could imply a lack of importance. The full significance of the purine phosphoribosyltransferases was

not fully realised until the discovery that children with the Lesch-Nyhan syndrome had a congenital deficiency of hypoxanthine guanine phosphoribosyl-transferase (HGPRTase); these children suffered from a massive hyperuricaemia causing atypical gout, along with bizarre neurological symptoms. Massive increases in de novo purine biosynthesis were later demonstrated (Rosenbloom, 1968).

1.2 THE LESCH-NYHAN SYNDROME

In 1964, Michael Lesch and William Nyhan reported, in a paper entitled "A Familial Disorder of Uric Acid Metabolism and Central Nervous System Function" (Lesch and Nyhan, 1964), the clinical features of the disorder to be known as the Lesch-Nyhan syndrome. Children with this disorder showed developmental retardation, cerebral palsy, choreoathetosis, and hyperuricaemia. The behavioral features of these children included physical and verbal aggression, and compulsive self-mutilation. The self-mutilation was extreme and involved partial amputation of the finger and the complete removal of the tissue of the upper lips (Fig. 1). These children had to be restrained by the use of large padded gloves on the hands, and the removal of the teeth.

Few, if any, of the behavioral symptoms were present

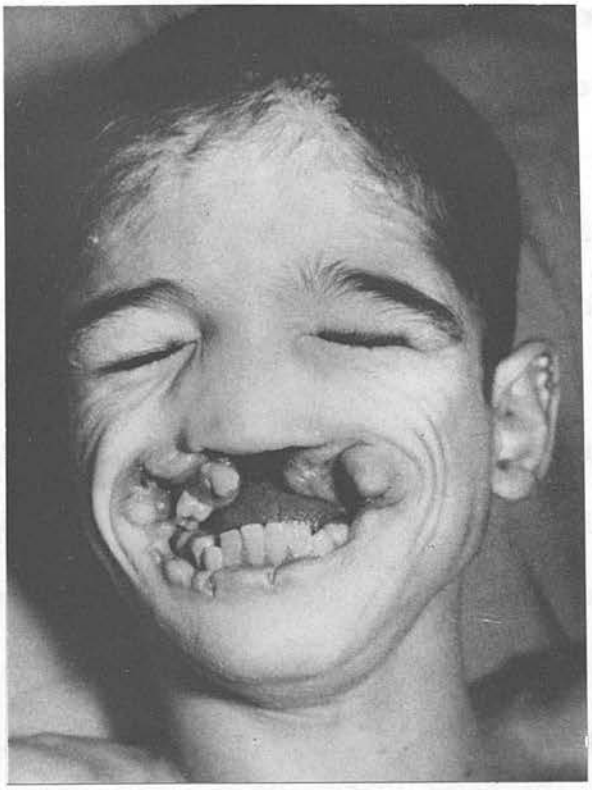


Fig. 1

The mutilated upper lip and ear tophi in a child with the Lesch-Nyhan syndrome (age 14 years). From W.L. Nyhan (1968) Fed. Proc. 27, 1030

at birth, and during the first year the children appeared to develop and grow normally. Before the age of two, however, the neurological and behavioral symptoms usually began to develop. Dizmang and Cheatham (1970), have made a psychiatric study of some of these children, and found they had a compulsion to inflict pain; early coincidental pain often triggered the compulsion and determined the site of self-mutilation.

It would appear that HGPRTase plays an important role in the central nervous system, and a detailed study of the distribution and developmental changes of HGPRTase activity in the central nervous system may enable a fuller understanding of the biochemical pathology of this disorder.

Biochemical studies on children with the Lesch-Nyhan syndrome revealed serum uric acid levels of 10 mg/100 ml (Lesch and Nyhan, 1964) compared to values for normal children of 4.0 mg/100 ml (Harkness and Nicol, 1969). Urinary uric acid excretion in these children was of the order of 50 mg/kg/day, compared to values of 10.3 mg/kg/day for normal children (Lesch and Nyhan, 1964). Due to the presence of high levels of serum uric acid, these children also showed the clinical features of gout. Although arthritic processes take

several years of hyperuricaemia to develop, three of the children examined by Lesch and Nyhan (1964) had episodes of acute arthritis. In one of the children, an anti-inflammatory response was obtained on treatment with colchicine. Many Lesch-Nyhan patients presented with haematuria, uric acid crystalluria, and with colic and abdominal pain. On examination these children were found to have multiple tophi in the ears.

Some aspects of uric acid metabolism in children with this syndrome were studied by the administration of isotopically labelled glycine. This amino acid was chosen for this study as it is incorporated intact into the 4,5, and 7 positions of the purine ring system. Children with the Lesch-Nyhan syndrome converted 2.25% of the glycine into urinary urate compared to 0.12% incorporation in control children. This represented an increase in de novo purine biosynthesis by a factor of twenty (Lesch and Nyhan, 1964). Studies on the concentrations of total oxypurines (xanthine + hypoxanthine) in the cerebrospinal fluid (C.S.F.) of Lesch-Nyhan patients showed that oxypurine concentrations were about five times those of control children. The C.S.F. xanthine concentrations were similar in control and Lesch-Nyhan children, the increase in total oxypurines

being due to increased hypoxanthine concentrations (Rosenbloom et al., 1970a)

The line of transmission of the syndrome was found to be through the female to the male and was shown to be an X linked recessive trait (Nyhan et al., 1967).

The enzymological basis of the Lesch-Nyhan syndrome was discovered in 1967 when Seegmiller, Rosenbloom and Kelley (1967) showed that tissue extracts from Lesch-Nyhan patients were almost completely lacking in hypoxanthine guanine phosphoribosyltransferase activity. The enzyme defect has subsequently been demonstrated in skin fibroblasts (Rosenbloom et al., 1968) and brain and liver obtained at necropsy (Rosenbloom et al., 1967).

Sorensen (1970) has reported residual activities of 0.05 - 0.64% of normal in erythrocyte lysates from one patient.

Partial deficiencies of HGPRTase activity of 0.01 - 17.0% of normal have been reported for erythrocyte lysates from members of families with hyperuricaemia and gout by Kelley et al. (1967) and Kelley et al. (1969).

The erythrocyte HGPRTase from these patients with partial HGPRTase deficiencies has been shown to be more thermolabile than normal in two families, and more thermostable in one family (Kelley et al., 1969).

A recent and very elegant study by Rubin *et al.* (1971) has demonstrated that the genetic defect in the Lesch-Nyhan syndrome results in the production of normal amounts of immunologically identifiable but catalytically incompetent HGPRTase protein. Similar results were obtained in a study of fourteen Lesch-Nyhan patients' erythrocyte HGPRTase by Arnold, Meade and Kelley (1972).

Thus, evidence is emerging that the deficiency in the Lesch-Nyhan syndrome is probably the result of a point mutation in a structural gene, and not the result of a deletion of the structural gene or a defect in a regulatory gene.

As many of the symptoms of the Lesch-Nyhan syndrome are also found in other diseases of childhood (see section 3.2) the only definitive method of diagnosis is the demonstration of lack of HGPRTase activity in erythrocyte lysates.

Abnormal Adenine Phosphoribosyltransferase Activity

Increased erythrocyte adenine phosphoribosyltransferase (APRTase) activity has been reported in patients with HGPRTase deficiency by Kelley *et al.* (1969) and Greene, Boyle and Seegmiller (1970) have suggested that this is due to stabilisation of the APRTase protein by 5-phosphoribosyl-1-pyrophosphate.

Increases of up to twice normal erythrocyte APRTase activity have been reported (Seegmiller et al., 1967), and decreases to 20% of normal have been reported in four healthy individuals (Henderson et al., 1968b)

Differences in the heat stability of erythrocyte APRTase have been reported, and about 15% of a randomly selected population were found to possess alleles which were expressed as an unusually heat resistant APRTase (Henderson et al., 1968a).

The kinetic constants for both substrates for APRTase from individuals with high or low heat stabilities were investigated by Henderson et al. (1968b) and abnormal Michealis constants were found for these mutant APRTases.

The inheritance of the APRTase gene is thought to be autosomal.

1.3 HYPOXANTHINE GUANINE PHOSPHORIBOSYLTRANSFERASE EC 2.4.2.8

HGPRTase catalyses the direct reaction of the purine bases hypoxanthine, guanine and to a lesser extent, xanthine, with 5-phosphoribosyl-1-pyrophosphate (PRPP), to form the corresponding 5-mononucleotide and pyrophosphate. The enzyme is widely distributed in living organisms. HGPRTase has been found in all tissues in man which have been examined (Rosenbloom et al.,

1967; section 3.4) with the exception of spermatozoa. It has been detected in brewers' yeast (Miller and Bieber, 1968), rats, rabbits, (section 3.5; section 3.7), mice (Murray, 1966), monkey tissues (Krenitsky, 1969), and S. typhimurium (Adye and Gotts, 1966). In bacteria, there is evidence for separate enzymes for hypoxanthine, guanine, and xanthine (Kalle and Gotts, 1961).

Evidence that a single protein is involved for the human enzyme is provided by the same rate of loss of activity towards both hypoxanthine and guanine during heat denaturation (Kelley et al., 1967b). Henderson et al. (1968c) has obtained kinetic evidence that a single enzyme from human erythrocytes reacts with both hypoxanthine and guanine. In addition, loss of activity with regard to both substrates is found in the Lesch-Nyhan syndrome.

Erythrocyte HGPRTase has been purified to homogeneity by Arnold and Kelley (1971), and shown to have a Stokes radius of 36Å with a molecular weight of 68,000. Arnold and Kelley (1971) concluded that HGPRTase was composed of two subunits with the same molecular weight and net charge. Interaction between the subunits has been suggested by Bakay et al. (1972) to be a possible mechanism affecting the total catalytic

activity of the enzyme complex.

A pH optimum of 7.2 - 7.8 has been reported for HGPRTase and the enzyme requires divalent cations for optimal activity. Maximal activity is obtained at 5 mM magnesium concentrations, while increasing concentrations of magnesium are inhibitory (Krenitsky, Papaioannou and Elion, 1969). The inhibition observed at high magnesium concentration is compatible with the true substrate being mono-magnesium-PRPP, while the di-magnesium-PRPP complex is inactive (Gadd and Henderson, 1970a). HGPRTase is strongly inhibited by the sulphydryl group binder p-chloromercuribenzoate, implicating an -SH group at or near the active site.

The kinetic constants for erythrocyte HGPRTase have been determined by initial velocity and product inhibition studies. Apparent Michaelis constants for hypoxanthine, guanine, and PRPP for HGPRTase are reported by Henderson (1968) to be 10, 4, and 200 μM respectively.

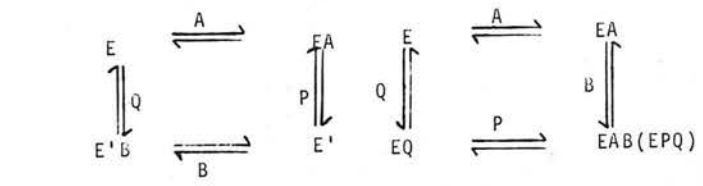
Double reciprocal plots of initial velocity versus varying concentrations of the first substrate at a series of fixed concentrations of the second substrate, yield a series of parallel lines consistent with a uni-uni-uni-uni ping pong mechanism (Cleland, 1963).

This is shown in mechanism I, Fig. 2. However, product inhibition and IMP-hypoxanthine isotope exchange studies suggested an ordered mechanism proceding via a ternary complex of the enzyme and both substrates (Henderson et al., 1968a), mechanism II, Fig. 2.

These mechanisms have been challenged by Krenitsky and Papaioannou (1969) on the grounds that the parallel initial velocity plots are unchanged, even at substrate concentrations approaching the enzyme-substrate dissociation constant. In order to reconcile these two mechanisms, Krenitsky and Papaioannou (1969) suggested a tentative mechanism involving alternate reaction sequences with both binary and ternary enzyme-substrate complexes.

1.4 ADENINE PHOSPHORIBOSYLTRANSFERASE EC 2.4.2.7

Adenine phosphoribosyltransferase (APRTase) is also widely distributed, and has been studied from several sources including brewers' yeast (Kornberg, Lieberman and Simms, 1955), beef liver (Flaks, Erwin and Buchanan, 1957), and monkey liver (Krenitsky, 1969). APRTase activity has been detected in human erythrocytes and several other human tissues studied (Rosenbloom et al., 1967; section 3.4).



MECHANISMS I II

The abbreviations used are A,B,C,P and Q, PP-ribose-P, guanine, hypoxantnine, pyrophosphate and guanylate; E and E', free enzyme and enzyme-riuose 5-phosphate complex respectively.

J. biol Chem. 243, 2514.

Fig. 2

Suggested possible mechanisms for catalysis by HGPRTase

The tissue distribution of human APRTase activity (section 3.4) differs greatly from that of HGPRTase activities, and there would appear to be little functional metabolic relationship between the two enzymes apart from the similarities in their reaction type.

APRTase from human erythrocytes has been purified 6730-fold to a specific activity of 2.5 μmoles/minute/mg protein, and was homogeneous as determined by polyacrylamide gel electrophoresis and ultracentrifugation (Greene, Boyle and Seegmiller, 1969), and was estimated to have a molecular weight of 37,000. APRTase from human erythrocytes is considerably more heat labile than is HGPRTase (Henderson et al., 1969; section 3.2), and has a broad pH optimum at about pH 7.0 with Tris/HCl buffers (Blair, 1967).

APRTase from human erythrocytes was, in contrast to HGPRTase, found to be resistant to p-chloromercuri-benzoate (Greene, Boyle and Seegmiller, 1969).

The apparent Michaelis constants for APRTase from the human erythrocyte are reported by Henderson (1968) to be 1 and 6 μM respectively for adenine and PRPP. An intermediate in de novo purine biosynthesis, 5-aminoimidazole,-4-carboxamide has also been reported as a substrate for APRTase, but the apparent Km value is

about one thousand times greater than that for adenine (Krenitsky et al., 1969)

The equilibrium for the reaction of APRTase favours AMP formation with an equilibrium constant of 290 (Hori and Henderson, 1966), but the back reaction can be shown to occur by the use of pyrophosphate dependent isotope exchange between AMP and adenine. Inhibition of APRTase by both products of the reaction has been reported by Hori and Henderson (1966) and inhibition by other nucleotide mono and triphosphates has been reported.

The binding requirements for adenine to APRTase from Erlich ascites tumour cells have been studied by Gadd and Henderson (1970a) and the removal of a proton from N-9 was shown to be the rate limiting step for the reaction (Gadd and Henderson, 1970b).

The evidence regarding the reaction mechanism for APRTase is contradictory and similar to that for the HGPRTase reaction. However, from studies on the dependence of AMP-adenine isotope exchange on pyrophosphate the evidence would appear to favour an ordered mechanism with the formation of a ternary complex (Komberg, Lieberman and Simms, 1955).

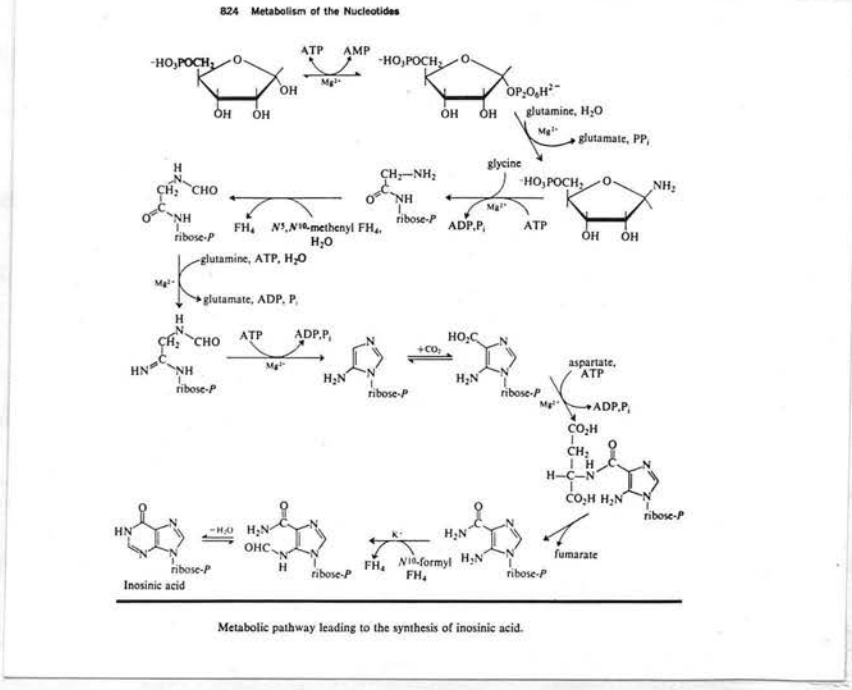
The metabolic role of APRTase remains an enigma, as no known enzymic mechanism exists in mammals for the

formation of its substrate, adenine (Murray, 1971). As small quantities of free adenine are found in the urine (Ayvayian and Skaup, 1965), it is possible that this arises via the 'back' reaction of APRTase.

1.5 DE NOVO PURINE BIOSYNTHESIS

The enzyme involved in the first reaction in de novo purine biosynthesis, the formation of 5-phosphoribosyl-1-amine from PRPP and glutamine (reaction 2, Fig. 3), is thought to be the pacemaker enzyme of this pathway. This enzyme, phosphoribosylpyrophosphate amidotransferase EC 2.4.2.14, has been purified from rat liver and exhibits sigmoid kinetics with respect to the binding of PRPP (Wyngaarden and Ashton, 1959). This suggests that the enzyme is subject to allosteric regulation; the purine nucleotide end-products of the pathway, AMP, IMP, and GMP, are strong feedback inhibitors of PRPP-amidotransferase and are competitive with respect to PRPP (Wyngaarden and Ashton, 1959). Detailed studies on the precise nature of the feedback inhibition of this enzyme by purine nucleotides are however conflicting (Caskey, Ashton and Wyngaarden, 1964), and appear to depend on the source of the enzyme and the degree of purification obtained.

The Km for PRPP-amidotransferase with PRPP is 0.2 - 0.4 mM (Hill and Bennett, 1969; Wyngaarden and



Metabolic pathway leading to the synthesis of inosinic acid.

Fig. 3

De Novo purine biosynthetic pathway. From Biological Chemistry, Mahler and Cordes p. 824

Ashton, 1959), while the normal PRPP concentration found in human erythrocytes is 1 - 5 μM and concentrations of up to 130 μM have been detected in cultured fibroblasts (Kelley, Fox and Wyngaarden, 1970). These intracellular PRPP concentrations are considerably lower than the Km for PRPP-amidotransferase.

The phosphoribosylamine produced in the first reaction is metabolised to IMP by nine further enzymic reactions, four of which are ATP dependent.

The IMP produced is converted to AMP and GMP via adenylosuccinate and XMP respectively.

Estimates of de novo purine nucleotide biosynthesis in human tissues are unavailable due to the extreme lability of the enzymes involved, but studies carried out using rat tissues by Howard, Kerson and Appel (1970) have shown that the capacity of the liver for de novo synthesis exceeds that of brain by a factor of four.

1.6 INTERCONVERSIONS OF PURINE RIBONUCLEOTIDES AND DERIVATIVES

The interconversions of purine ribonucleotides and their derivatives are shown in Fig. 4.

De novo purine ribonucleotide biosynthesis results in the production of IMP, which can be converted to AMP and GMP. The formation of AMP or GMP both require two separate enzymic steps, one of which is energy

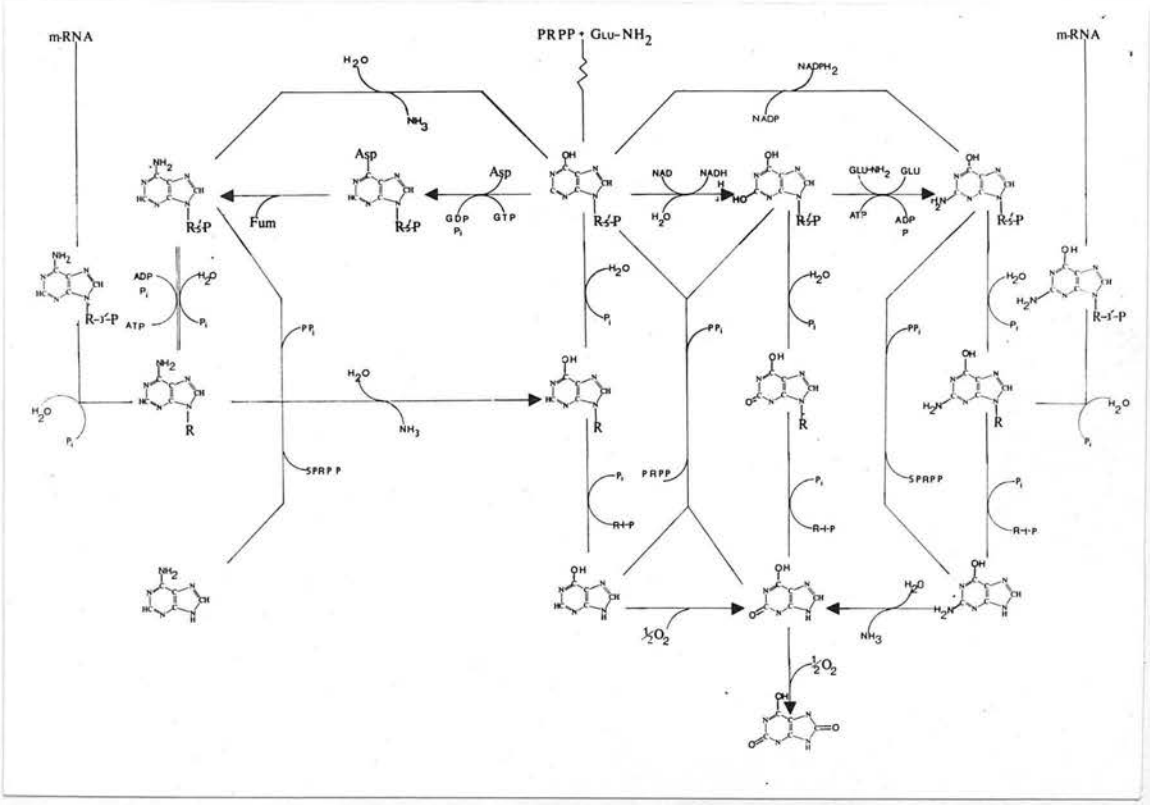


Fig. 4

Interconversions of purine ribonucleotides. Top to bottom; ribonucleotides, ribonucleosides, free bases, urate. Left to right; adenine and derivatives, inosine and derivatives, xanthine and derivatives, guanine and derivatives.

dependent in each case. IMP reacts with aspartate and GTP catalysed by IMP : L-aspartate ligase (GTP) EC 6.3.4.4 to form adenylosuccinate; fumarate is removed from this compound by the action of adenylosuccinate : AMP ligase EC 4.3.2.2 to produce AMP.

IMP also reacts with NAD^+ and water catalysed by IMP : NAD^+ oxidoreductase EC 1.2.1.14 to form XMP; this compound reacts with glutamine and ATP catalysed by XMP : L-glutamine amidoligase (AMP) to generate GMP. This system can be seen to exert a cross-over control whereby the production of AMP requires a GTP driven reaction and the production of GMP requires an ATP driven reaction; the flow through one of the two pathways is controlled by the concentration of a metabolite of alternate branch of the system.

The end products AMP and GMP may be reduced to 2-deoxy AMP and GMP for DNA biosynthesis (Blakley and Vitols, 1968).

5'-Mononucleotides are dephosphorylated by 5'-ribonucleotide phosphohydrolase EC 3.1.3.5 to the corresponding nucleosides. The resultant nucleosides, inosine, xanthosine, and guanosine can be degraded to the corresponding free bases by the action of nucleoside : orthophosphate ribosyltransferase EC 2.4.2.a.

Adenosine is not a substrate for this enzyme (Huennekens, Nurk and Gabrio, 1956a), but a report by Zimmerman, Gersten and Miech (1970) claimed to have detected a slow reaction with this substrate.

3'-Mononucleotides resulting from the hydrolysis of RNA (Sugar and Sierakowska, 1967) can be dephosphorylated by non-specific phosphatases EC 3.1.3.1 and EC 3.1.3.2, to guanosine or adenosine. The resulting guanosine can be degraded to free guanine which can be recycled to GMP by HGPRTase. Adenosine can be phosphorylated to AMP by ATP : adenosine-5'-phosphotransferase EC 2.7.1.20 or may be deaminated to inosine by adenosine aminohydrolase EC 3.5.4.4. The resultant inosine can be degraded to hypoxanthine which can also be recycled by HGPRTase to yield IMP.

Earl Balis (1968) has proposed a cyclical interconversion of purines involving the sequence IMP → AMP → adenosine → inosine → hypoxanthine → IMP. The mechanism adenosine → inosine → hypoxanthine → IMP has been demonstrated in guinea pig brain by Shimizu, Tanaka and Kodama (1972).

The principal pathway of uric acid production is guanine → xanthine → urate (Sorensen, 1970), and

the most probable purine for salvage by HGPRTase is hypoxanthine resulting from the catabolism of adenine derivatives.

Murray, Eliot and Atkinson (1970) have reviewed the enzymology of purine interconversions.

This system of purine interconversion ensures adequate supplies of nucleotide precursors for normal growth by a "self balancing network of enzymic reactions"(Murray, Eliot and Atkinson, 1970).

1.7 ROLE OF 5'-PHOSPHORIBOSYL-1-PYROPHOSPHATE

PRPP is a common substrate for both de novo purine biosynthesis and for salvage synthesis from the free bases hypoxanthine and guanine. It is also a substrate for several other enzymes, including orotate phosphoribosyltransferase EC 2.4.2.10, a key enzyme in pyrimidine nucleotide biosynthesis. Some of the other enzymes involved in the catabolism of PRPP are shown in Fig. 5, and many anti-tumour drugs are also substrates for the enzymes.

The central role of intracellular PRPP concentrations in the regulation of de novo purine biosynthesis is shown by the depletion of PRPP levels in tissue culture cells by orotic acid resulting in decreased de novo purine biosynthesis (Kelley, Fox and

Wyngaarden, 1970), and similar results have been obtained *in vivo* (Kelley *et al.*, 1970).

Fox and Kelley (1971) have suggested that as the range of intracellular PRPP concentrations are lower than the Km values for most other PRTase enzymes, this substrate may be generally rate limiting.

Patients with primary gout and partial HGPRTase deficiency were found to have elevated erythrocyte PRPP concentrations by Greene and Seegmiller (1967), and PRPP turnover rates were shown to be increased in three gouty patients (Jones, Ashton and Wyngaarden, 1962).

Greene and Seegmiller (1969) also found elevated PRPP concentrations in erythrocytes from seven patients with the Lesch-Nyhan syndrome.

It is possible that in Lesch-Nyhan patients, due to the lack of HGPRTase, PRPP concentrations rise and AMP and GMP concentrations fall. As a result of the lack of inhibition of PRPP amidotransferase by AMP and GMP and the rise of PRPP concentrations, *de novo* purine biosynthesis can increase. There are, however, some difficulties in this theory; if *de novo* purine biosynthesis increases to compensate for the failure of the salvage pathway, then AMP and GMP concentrations should rise and PRPP concentrations fall

until the normal equilibrium state is reached.

This does not occur in patients with the Lesch-Nyhan syndrome, where de novo synthesis, as estimated by uric acid excretion, is as much as twenty times greater than normal. This may suggest that other factors in addition to increased intracellular PRPP concentrations are operating to increase de novo purine biosynthesis in these patients and to pull the equilibrium of the system towards catabolic processes.

1.8 ROLE OF THE PRTases IN DRUG METABOLISM

Many anti-tumour drugs exert their effects at the nucleotide or nucleotide triphosphate level. As nucleotides do not cross cell membranes to any extent (Davis, 1958), these compounds are administered as the free bases, for example 6-mercaptopurine and 6-thioguanine. These drugs are converted to their active nucleotide form by HGPRTase to 6-thio IMP and 6-thio GMP (Brockman, 1960) and function as analogs of IMP and GMP. Since 6-mercaptopurine has a Km of 11 μM with HGPRTase (Atkinson and Murray, 1965) this drug is a powerful competitive inhibitor of HGPRTase. In this active nucleotide form these drugs inhibit the interconversions of purine nucleotides and the formation

of nucleotides triphosphates, and hence exercise their control on growth by starving the cells of nucleotide triphosphate precursors for nucleic acid synthesis. It is likely that tissues with high HGPRTase activity which depend on hypoxanthine as a source of nucleotides will show a high sensitivity to these drugs.

SECTION II

MATERIALS AND METHODS

2.1 MATERIALS

All general laboratory chemicals were purchased ANALAR grade from British Drug Houses Ltd., Poole, Dorset.

Radiochemicals were purchased from The Radiochemical Centre, Amersham, Buckinghamshire. These were adenine-[8-^{14}C] at $>$ 50 μCi/μmole, hypoxanthine-[8-^{14}C] $>$ 50 μCi/μmole, adenosine-[8-^{14}C] 5'-monophosphate, ammonium salt, 40 - 60 μCi/μmole, and guanosine-[8-^{14}C]5'-monophosphate, ammonium salt 30 - 50 μCi/μmole. The radiochemicals were chromatographically homogeneous using the system described in section 2.14.

The following fine chemicals were purchased from Sigma Chemical Co. Ltd., Kingston, Surrey: adenine, hypoxanthine, guanine, adenosine, guanosine, inosine, AMP, IMP, GMP, 5'PRPP, TTP, Allopurinol, and 6-mercaptopurine. All chemicals were of the purest grade available and were chromatographically homogeneous in the system described in section 2.14 with the exception of adenine. Adenine had a fast moving impurity with an Rf of 0.6. PRPP was 90% pure, the major impurity being ribose-5-phosphate.

The following enzymes were also purchased from Sigma:-

5'-Nucleotidase: EC 3.1.3.5 Grade II from *Crotalus adamanteus* venom.

Deoxyribonuclease: DN-EP. Electrophoretically purified from beef pancreas.

Ribonuclease T_2: from *Aspergillus* mould.

Protease: Type VI from *Streptomyces griseus*

and also

DNA: Type I from calf thymus.

RNA: Type II from *Torula* yeast.

The liquid scintillators, NE 233, and Bray's solution were supplied by Nuclear Enterprises Ltd., Edinburgh.

Bovine serum albumin was supplied by Koch-Light Ltd., Buckinghamshire.

Kodak AR 10, Kodak D 19, Kodafix, and Depex, used in the autoradiography were obtained from Eastman-Kodak Inc., Kirkby, Liverpool.

2.2 PREPARATION OF TISSUES FOR ENZYME ASSAYS

2.2a Red Cell Lysates

Whole fresh venous blood, stored in lithium heparin tubes, was obtained from patients directly or through the Department of Haematology, Royal Hospital

for Sick Children, and from volunteers.

The erythrocytes were separated from the buffy coat of leucocytes and plasma after centrifugation at 3000 g for 5 min. The erythrocytes were washed twice with five volumes of isotonic saline, and separated by centrifuging at 3000 g for 5 min.

A 100 µl sample of packed erythrocytes was lysed in 900 µl of deionised water, mixed and left for 5 min. at 4°C. After this time, the stroma was removed by centrifuging at 10,000 g for 10 min. The lysate was dialysed for 1 h against 1 litre of distilled water to remove endogenous substrates, and a sample of the dialysis residue used for the PRTase assays.

2.2b Tissue Homogenates

All procedures were carried out in a cold room at 4°C.

Approximately 1 g of tissue was washed in 20 ml of 220 mM Tris/HCl buffer pH 7.4, cut into small pieces, and homogenised using a motor driven Teflon homogeniser (Jencons) at 2000 r.p.m. for about 30 sec. Five passes of the homogeniser were used. The resulting homogenate was centrifuged at 26,000 g for 1 h at 4°C. The supernatant was carefully removed by Pasteur pipette, then dialysed for 1 h against

1 litre of distilled water to remove any endogenous substrate. A sample of the dialysis residue was used for enzyme assays.

2.2c Trypsinisation of Tissue Culture Monolayers

Fibroblasts and amnion cells grown in tissue culture were harvested as follows: the tissue culture medium was poured off, and the cell monolayer washed twice with isotonic saline.

10 ml of trypsin solution (2.5 mg protein/ml) and 10 ml of 20 mM ethylenediaminetetraacetic acid (E.D.T.A.) were added and left for 20 min. at room temperature with occasional gentle shaking to help detach the cells from the glass.

The cell suspension in trypsin/EDTA was poured off into a centrifuge tube, and an equal volume of foetal calf serum in Ham's F10 tissue culture medium added.

The cells were separated by centrifuging at 10,000 g for 10 min. at 4^{o}C.

The cell pellet was washed by resuspending the cells in 10 ml of 220 mM Tris/HCl buffer pH 7.4 and separated by centrifuging as above.

The washed cell pellet was homogenised as described in the procedure for the preparation of tissue homogenates.

2.3 ASSAY OF PURINE PHOSPHORIBOSYLTRANSFERASES

Hypoxanthine guanine phosphoribosyltransferase (HGPRTase) [IMP : Pyrophosphate phosphoribosyltransferase EC 2.4.2.8] and adenine phosphoribosyltransferase (APRTase) [AMP : Pyrophosphate phosphoribosyltransferase EC 2.4.2.7] activities were assayed by a modified method of Cartier and Hamet (1968). This method involves the use of radiochemical substrate purines, and the separation of the unchanged substrate from the polyanionic product by ion exchange chromatography on diethylaminoethyl (DEAE) paper.

However, this method and others, see Chow, Kawahara, Saunders and Sorensen (1970), Fujimoto, Greene and Seegmiller (1968) and Krenitsky, Papaioannou and Elion (1969) are unsuitable for tissues other than erythrocytes because enzyme assays carried out on crude tissue homogenates are complicated by the product of the system under investigation being a substrate for another enzyme present.

5'-mononucleotides are substrates for the widely distributed 5'-nucleotidase (5'Nase) [EC 3.1.3.5] (see Bodansky and Schwartz, 1968), and hence the products of the PRTase reactions are degraded to nucleosides in the presence of 5'Nase.

Thymidine triphosphate (TTP) is an inhibitor of 5'Nase (Murray and Friedrichs, 1969), and by the addition of this compound to the PRTase assay system, erroneously low results due to product breakdown are largely eliminated. TTP was therefore added to the assay system to a final concentration of 1.5 mM. The inhibitory effects of TTP are maximal at 1.1 mM and remain constant with increasing concentrations (Murray and Friedrichs, 1969).

A further modification from the procedure of Cartier and Hamet (1968) was the scaling down of the reaction volume by a factor of two for economic reasons due to the high cost of 5'-phosphoribosyl-1-pyrophosphate (PRPP).

Assay procedure The radioactive substrates adenine-[8-^{14}C] and hypoxanthine-[8-^{14}C] were diluted for use by the addition of the appropriate unlabelled base to give a specific activity of 0.75 µCi/mM.

All additions and removal of components were made by Hamilton microsyringe: when a large number of determinations were being carried out, the assay medium was prepared in bulk and dispensed into assay tubes to minimise pipetting error.

The assay medium was of the composition shown in Table I.

75 µl of the assay medium were dispensed into glass tubes 50 x 6 mm and preincubated for 5 min. in a water bath at $37^{o}C \pm 1^{o}$. Timing of the reaction commenced following the rapid addition of 25 µl of the enzyme sample. The reaction was allowed to proceed for between five and thirty minutes depending on the tissue being investigated, before being halted by the addition of 25 µl of 100 mM EDTA with subsequent chilling in an ice bath at $3^{o}C$.

30 µl samples of the reaction mixture (total volume 125 µl) were applied to a DEAE paper strip as a 5 mm band at 60 mm from one end. The DEAE paper was 150 x 15 mm and is shown in Fig. 6.

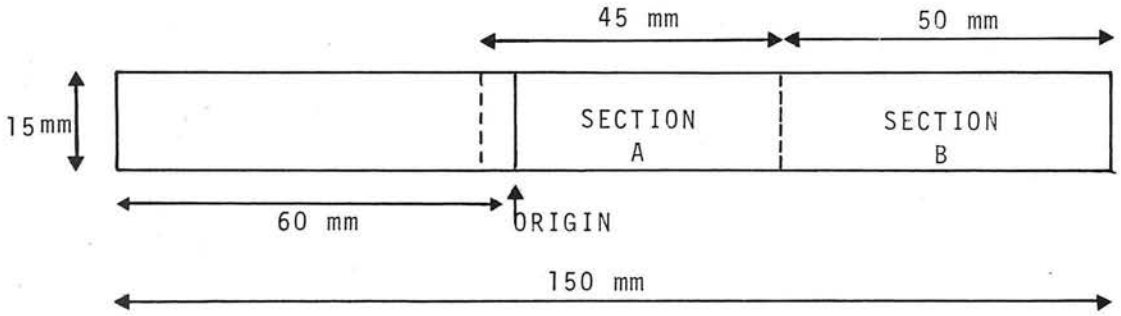
The chromatogram was developed for 100 min. by descending chromatography using a solvent of 95% ethanol; 1M ammonium acetate pH adjusted to 3.8 with acetic acid, 70 : 35 v/v, as described by Paladini and Leloir (1952).

After oven drying the chromatogram for 30 min. it was cut into two sections for counting; section A, -5 to +40 mm (Fig. 6) having bound all the nucleotide product, and section B, +40 to +90 mm binding the faster migrating unreacted substrate. Chromatographic

TABLE I

THE ASSAY MEDIUM FOR THE ESTIMATION OF PRTase ACTIVITY

Reagent	Concn. mM	Vol. added µl	Final concn. mM
Tris/HCl buffer pH 7.4	220	25	55
Magnesium chloride	50	10	5
PRPP - Mg_2 or Na_4	10	10	1
TTP - Na_2	30	5	1.5
Adenine-[8-^{14}C] Hypoxanthine-[8-^{14}C]	2.4	25	0.6
Enzyme	-	25	-



DIMENSIONS OF DEAE PAPER CHROMATOGRAM SHOWING AREAS USED FOR LIQUID SCINTILLATION COUNTING

Fig. 6

reproducibility was checked using mixtures of unlabelled bases and nucleotides which were separated using the above system; the mobilities were determined by visualising the bands using a low pressure ultraviolet lamp maximum emission 254 nm. Dark bands were visible against a slightly fluorescent background.

For each chromatographic run, three control chromatograms were developed to correct for activity due to slow running purines. In place of the enzyme sample 25 µl of water was added to an assay tube with 75 µl of the assay medium and 25 µl of 100 mM EDTA. Aliquots of 30 µl from the control assay were applied to the control chromatograms which were developed at the wall edges and centre of the solvent tray.

From these control chromatograms the mean of the counts from section A (Fig. 6) was subtracted from the counts in the assay chromatogram to correct for counts due to slow migrating substrate.

The enzyme activity was calculated as follows:-

Counts from product AMP or IMP
(Section A) = Cp

Counts from substrate Adenine-
[8-^{14}C] or hypoxanthine-[8-^{14}C]
(section b) = Cs

Counts from slow running substrate

(control section A) = Cc

The per cent transformation of substrate is given by

$$\%T = \frac{Cp - Cc}{Cp + Cs} \times \frac{100}{1}$$

Enzyme activity was expressed as nmole of substrate transformed per hour and related to total protein determined as described in section 2.5. Where possible enzyme activities were determined in duplicate.

Example of calculation

Cp = 11,000 Time of incubation = 10 min.

Cs = 89,000 Enzyme sample 5 mg protein/ml

Cc = 500

$$\%T = \frac{11,000 - 500}{11,000 + 89,000} \times \frac{100}{1} = 10.5\%$$

mmoles of substrate in assay medium = 62

$\therefore$ 10.5 x 0.62 = 6.51 nmoles substrate transformed

$\therefore$ $6.51 \times \frac{60}{10} = 39.06$ nmoles/h

Protein in enzyme sample = 5.0 µg/µl.

volume of sample = 25 µl.

$\therefore$ total protein = 125 µg.

nmole substrate transformed/h/mg protein

$$= 39.06 \times \frac{1000}{125} = 312.48$$

Result 312.48 nmole/h/mg protein.

2.4 LIQUID SCINTILLATION COUNTING IN TWO PHASE SYSTEMS

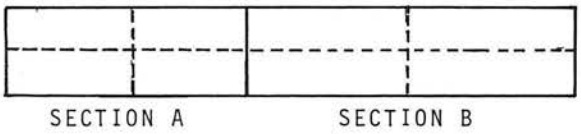
The radioactivity on the separate sections of the DEAE ion exchange paper was counted directly in a two phase system (Davidson, 1961), using a Packard Tricarb model 2002 liquid scintillation counter. Settings for [^{14}C] were as follows, Gain 8%, Window A⟶B with A = 50 and B = 1000.

Each section of DEAE paper was cut into four pieces 7.5 x 25 mm (Fig. 7) and these were placed in parallel walled glass vials 12 x 50 mm to which 3.0 ml of liquid scintillator NE 233 was added. This method was adopted to economise in the use of scintillator, 3.0 ml compared to 10 ml, but also had the advantage of ensuring a regular geometric presentation of the sample to the photomultiplier tubes. The vials were then sealed with plastic caps, and placed in the Packard vials and at least 5000 counts recorded.

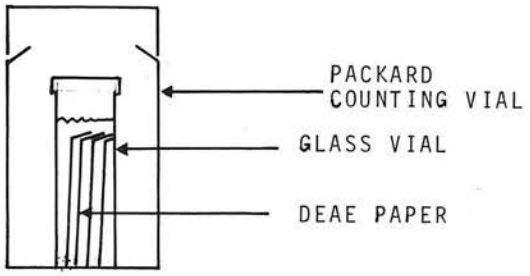
The counting efficiency was determined by the method of internal standardisation (Rogers and Moran, 1966) using hypoxanthine-[8-^{14}C] and found to be 49%. Duplicate samples and single samples counted a number of times were highly reproducible with a coefficient of variation of less than 1%.

2.5 DETERMINATION OF TOTAL PROTEIN

Protein was determined by the Millar (1959)



A



B

(A) METHOD OF CUTTING OF DEAE PAPER FOR LIQUID SCINTILLATION COUNTING

(B) COUNTING VIAL FOR DEAE PAPER

Fig. 7

modification of the method of Lowry et al. (1951).

The copper reagent was prepared for each batch of determinations, and a standard graph prepared of 20, 40, 60, 80 and 100 µg of bovine serum albumin per ml. The optical density was determined at 680 nm using a Pye Unicam SP 500 spectrophotometer.

2.6 ASSAY OF 5'-NUCLEOTIDASE

5'-nucleotidase (5'Nase) EC 3.1.3.5 was assayed under conditions identical to those used for the purine phosphoribosyltransferases except that TTP was omitted. The present conditions of assay were designed to determine the degree of underestimation of measured PRTase activity due to the breakdown of the product nucleotide by 5'Nase. The conditions of assay were not therefore necessarily optimal such as those described by Bodansky and Schwartz (1968) or Belfield and Goldberg (1969).

The radioactive substrates adenosine-[8-^{14}C] monophosphate and inosine-[8-^{14}C] monophosphate were diluted for use by the addition of the appropriate unlabelled nucleotide to a specific activity of 1.0 µCi/mmole.

All additions and removal of components were made using a Hamilton microlitre syringe. When a large number of determinations was being carried out, the

the assay medium was prepared in bulk and 75 µl dispensed to each assay tube. The assay medium contained the components shown in Table II.

The assay tubes 50 x 6 mm containing 75 µl of the assay medium were preincubated in a water bath at $37^{o}C \pm 1^{o}$ for 5 min., and timing of the reaction commenced with the addition of 25 µl of the enzyme sample.

The reaction was allowed to proceed for 15 min. before being halted by the addition of 25 µl of 10% v/v trichloroacetic acid (TCA). The precipitated protein was removed by centrifuging on a bench centrifuge at 1000 g for 5 min. A 30 µl sample of the supernatant was added as a 5 mm band to the origin at 60 mm from one end of a DEAE paper strip. This chromatogram was developed for 100 min. by descending chromatography using a solvent of 95% ethanol; 1M ammonium acetate adjusted to pH 3.8 with acetic acid; 70 : 35 v/v.

After oven drying the chromatogram for 30 min., the strip was cut into two sections for counting: section A (Fig. 6) -5 to +40 mm bound all the unreacted substrate AMP or GMP due to the polyanionic phosphate group. The reaction product, adenosine or guanosine migrated to section B, +40 to +90 mm from the origin.

The chromatographic mobilities of both substrate

TABLE II

ASSAY MEDIUM FOR ESTIMATION OF 5'NASE ACTIVITY

Reagent	Concn. mM	Vol. added µl	Final concn. mM
Tris/HCl buffer pH 7.4	220	25	55
$MgCl_2$	50	10	5
AMP-[8-^{14}C]	2.4	25	0.6
GMP-[8-^{14}C]			
Water	-	15	-
Enzyme	-	25	-

and product were checked by developing mixtures of unlabelled nucleotides and nucleosides which were located by their absorption of ultraviolet light.

For each group of assays, three control chromatograms were developed to correct for any fast migrating nucleotides. 25 µl of water was added to the assay medium in place of the enzyme sample and 25 µl of 10% v/v TCA. 30 µl samples were applied to the control chromatograms and developed at the wall edges and centre of the solvent tray. From these control chromatograms the mean of the counts from section B was subtracted from the counts due to the product from the assay chromatograms to correct for fast migrating nucleotides.

The enzyme activity was calculated as follows:-

Counts from product adenosine or guanosine (section B) = Cp

Counts from substrate AMP or GMP (section A) = Cs

Counts from fast migrating substrate (control section B) = Cc

The per cent transformation of substrate is given by:-

$$\%T = \frac{Cp - Cc}{Cp + Cs} \times \frac{100}{1}$$

Enzyme activity was expressed as nmole of substrate transformed per hour and related to total protein. Where possible, enzyme activities were determined in duplicate.

Example of calculation

Cp = 8,500 Incubation time = 15 min.

Cs = 91,500 Enzyme sample 7.5 mg/ml protein

Cc = 500

$$\%T = \frac{8,500 - 500}{8,500 + 91,500} \times \frac{100}{1} = 8.0\%$$

nmole of substrate in assay medium = 63

8.0% of 62 = 4.95 nmole substrate transformed

= 19.8 nmole/h

Protein in enzyme sample = 7.5 µg/µl

Volume of sample = 25 µl

∴ total protein 188 µg

nmole substrate transformed /h/mg protein

$$= 19.8 \times \frac{1000}{188} = 105$$

Result 105 nmole/h/mg protein

2.7a Electrophoresis of Purine Phosphoribosyltransferases

Electrophoresis of human cellulose acetate was carried out as described by Kohn (1969). Cellulose acetate strips (Millipore) 170 x 25 mm were used in a Shandon

horizontal electrophoresis tank using a barbitone buffer pH 8.6 of the following composition: sodium diethyl barbiturate 10.3 g and diethyl barbituric acid 1.84 g in 1 litre of water.

The strips were floated on the surface of the buffer to ensure uniform moistening and, after soaking for 30 min., were blotted dry. They were then connected to the buffer compartment by paper wicks soaked in buffer, and a potential difference of 5 volts/cm applied at a constant current of 2 mA.

After 30 min. to allow for any electroendosmotic flow effect to reach a steady state, the sample of 5 - 10 µl was applied, using a microcapillary pipette, as a line, 15 mm from the end of the strip nearest the cathode.

After 3 - 4 h electrophoresis, the haemoglobin band, if present, migrated 40 mm towards the anode. Strips were then removed from the tank using forceps and placed on a glass thin-layer chromatography plate. The bands of PRTase activity were located as described in the following section.

Electrophoresis of the PRTases was also carried out in starch gel. The gel was prepared and poured as described by Bodman (1969) using the gel buffer of Smithies (1955). The starch gel in the perspex gel former

(Shandon) 50 x 185 mm was laid across the supports in a Shandon horizontal electrophoresis tank and connected to the buffer tanks by wicks of Whatman No. 1 paper soaked in reservoir buffer (Smithies, 1955).

A potential difference of 5.4 volts/cm was applied at a constant current of 2.0 mA and the gel allowed to equilibrate with respect to buffer flow for 1 h.

The most satisfactory technique of sample application to attain high resolution was achieved as follows: a 30 µl sample was absorbed on to a piece of Whatman No. 1 paper 20 x 4 mm and inserted using forceps into a slit of similar dimensions cut into the gel 15 mm from the cathodic end. The slit was sealed with a silicon greased microscope cover slip, and the entire gel covered with a thin sheet of polythene to prevent the gel drying as a result of evaporation.

After electrophoresis for 20 h, the haemoglobin band, if present, migrates 5 - 6 cm towards the anode.

Following electrophoresis, the gel was removed from the former and sliced into two layers using a Shandon gel slicer. The lower layer was transferred to a glass thin layer chromatography plate and the bands of PRTase activity located as described in the following section.

2.7b Location of Purine Phosphoribosyltransferase Activity in the Electrophoresis Support Medium

The bands of PRTase activity in the electrophoresis support medium were located at its surface by placing a marked strip of DEAE ion exchange paper saturated with PRTase reaction medium in contact with it. Zones of PRTase activity in the electrophoresis support convert the radiochemical substrate base on the DEAE paper into the charged product nucleotides.

The strip of DEAE paper 150 x 15 mm marked off in 1 cm units was moistened with 500 µl of a medium of composition shown in Table III.

The detection medium is essentially identical to the assay medium with water added in place of enzyme sample. This addition adjusts the molarities to those in the assay system, while the enzyme is immobilised in the solid phase on the support medium. The moist DEAE paper was laid over the electrophoresis support medium with the first centimetre on the paper opposed to the first centimetre of the origin on the support medium. This unit was then sandwiched between two glass thin-layer plates which were lightly clamped together with spring clips. The assembly was incubated for 3 h in the moist atmosphere provided by a lidded water bath. The temperature of this atmosphere was 37°C. At the end

TABLE III

DETECTION MEDIUM FOR ZONES OF PRTase ACTIVITY

Reagent	Concn. mM	Vol. added µl	Final concn. mM
Tris/HCl buffer pH 7.4	220	125	55
$MgCl_2$	50	50	5
PRPP - di Mg.	10	50	1
Adenine-[8-^{14}C]	2.4	125	0.6
Hypoxanthine-[8-^{14}C]			
Water	-	150	-

of this period, the DEAE paper was carefully lifted off the surface of the support medium, and washed with slow magnetic stirring for 4 h in 4 l of 8 mM tris buffer pH 9.5. During the wash, the unreacted substrate was removed from the DEAE paper while the charged nucleotide products of PRTase activity were tightly bound by the anionic exchange groups. The paper was oven dried for 30 min., cut into 1 cm sections and counted. The peaks of radioactivity due to the products of PRTase activity, AMP or IMP, indicated the position of the bands of PRTase activity in the electrophoresis support medium.

Although this method has not yet provided the high resolution reported by Bakay and Nyhan (1971) who used 1.5 mm slices of polyacrylamide gel, it is considerably easier to perform and represents an improvement on previously published methods (Kaloustian et al., 1969). Since this work has been completed a number of authors have described similar methods.

2.7c Confirmation of the Identity of Regions of Radioactivity, Corresponding to PRTase Activity in the Electrophoresis Support Medium

As four distinct peaks of radioactivity were detected, indicating four bands of PRTase activity which conflicted with previous reports of one band (Atkinson

and Murray, 1965; Kelley et al., 1969; Kaloustian et al., 1969), it was necessary to establish that the activity was due to the products of PRTase activity. This was achieved by using DEAE paper moistened with a modified assay medium. This medium contained either EDTA to complex the Mg^{+2} ion essential for the PRTase reaction, or 6-mercaptopurine, a specific inhibitor of the reaction ($Ki = 0.0039 \times 10^{3}$)(Krenitsky, Papaioannou and Elion, 1969).

The assay medium contained the components shown in Table IV.

In the presence of 40 mM EDTA no peaks of activity were detected whereas four were detected in a control.

When 6-mercaptopurine was present in the assay medium, peaks of radioactivity were reduced by 90% compared to a control.

To determine if the peaks of radioactivity were due to 5'AMP or 5'IMP, a DEAE strip was halved longitudinally subsequent to assay. One half was treated for 2 h with 500 µl of 5'-nucleotidase in 220 mM tris/HCl buffer pH 7.4 (1 mg protein/ml) above a water bath with a moist air temperature of $37^{o}C$. Both halves of the DEAE were then washed and counted as described in section 2.4.

No peaks of radioactivity were detected in the half

TABLE IV

ASSAY MEDIUM FOR CONFIRMATION OF IDENTITY OF ZONES OF RADIOACTIVITY

Reagent	Concn. mM	Vol. added µl	Final concn. mM
Tris/HCl buffer pH 7.4	220	125	55
$MgCl_2$	50	50	5
PRPP di Mg.	10	50	1
Adenine-[8-^{14}C]	2.4	125	0.6
Hypoxanthine-[8-^{14}C]			
EDTA or	200	100	40
6-Mercaptopurine	3.5 µM	100	0.7 µM

treated with 5'-nucleotidase. As 5'-nucleotidase is specific to 5'-mononucleotides (Bodansky and Schwartz, 1968) it was concluded that the peaks of radioactivity were due to 5'-nucleotides.

2.8 DETERMINATION OF SPECIFIC REACTION RATE CONSTANTS FOR THERMAL DENATURATION OF PURINE PHOSPHORIBOSYL-TRANSFERASES

Dialysed samples of red cell lysates or tissue homogenates were diluted to a protein concentration of 5 mg/ml by the addition of 220 mM tris/HCl buffer pH 7.4. The enzyme sample was incubated, with shaking, in 100 x 10 mm thin walled glass tubes. A temperature of $80^{o}C \pm 1^{o}$ was used for the denaturation of HGPRTase, and $56^{o}C \pm 1^{o}C$ was employed for the denaturation of APRTase (Kelley et al., 1969).

Aliquots of 25 µl were removed prior to incubation to determine initial PRTase activity. Further 25 µl samples were removed after 2, 4, 6 and 8 min. incubation and the residual PRTase activity determined. Results of the assays were expressed as a percentage of the initial PRTase activity.

Plotting the logarithm of the percentage residual PRTase activity against time of incubation consistently yielded straight lines. The rate of denaturation of the PRTases under the defined conditions was thus found

to approximate to first order unimolecular reaction kinetics. Fitting the experimental data to second or third order rate equations showed that these treatments were inapplicable (Glasstone and Lewis, 1960).

The first order rate equation is

$$k = \frac{1}{t} \ln \frac{Co}{C} \qquad \underline{\text{Eqn. 1}}$$

where k is the specific reaction rate constant; Co is the initial concentration of the reactant (PRTase); and C is the concentration of catalytically competent PRTase at subsequent time t. A more useful form of equation 1 can be obtained by assuming that "a" moles of the reactant (100%) are initially present and that "x" is the number of moles that will have reacted after time t, leaving a - x unreacted. Thus, Co is proportional to "a" and C to a - x so that equation 1 becomes

$$k = \frac{1}{t} \ln \frac{a}{a - x} \qquad \underline{\text{Eqn. 2}}$$

<u>or</u>

$$k = \frac{2.303}{t} \log_{10} \frac{a}{a - x} \qquad \underline{\text{Eqn. 3}}$$

and a - x as defined is equal to the percentage residual activity and is a pure number without units.

By rearrangement, equation 3 can be written

$$t = \frac{2.303}{k} \log_{10} a - \frac{2.303}{k} \log_{10} (a - x) \qquad \text{Eqn. 4}$$

so that plots of t against $\log_{10} (a - x)$ will be linear for first order reactions, the slope of the line being equal to $\frac{-2.303}{k}$. This enables the specific reaction rate constant k to be evaluated. The constant k has the dimensions of reciprocal time.

2.9 DETERMINATION OF APPARENT MICHAELIS CONSTANTS

Apparent Michaelis constants (Km) and maximal velocities (Vmax) for adenine and hypoxanthine in their respective systems were obtained by determining initial velocities from a series of substrate concentrations. Excess of the second substrate PRPP, and optimal Mg^{+2} concentrations were present in the assay medium.

Purine substrates were prepared initially at approximately 1 mmolar with a specific activity of 20 μCi/μmole. The precise molarity was determined spectrophotometrically using a Pye S.P. 500 ultraviolet spectrophotometer and the following data:-

Adenine: absorption maximum at 262.5 nm pH 1-3, with a molar absorptivity of 13.5×10^3.

Hypoxanthine: absorption maximum 248 nm at pH 1.0, with a molar absorptivity of 10.8×10^3.

Standards thus prepared were diluted with distilled water to give the range of substrate concentrations required for kinetic studies. For APRTase the concentrations of adenine used were 0.5, 1.0, 2.0, 5 and 10 μM, and for HGPRTase the concentrations of hypoxanthine used were 2, 4, 8, 16 and 32 μM. Red cell lysates or tissue homogenates prepared as described in section 2.2 were diluted twenty times with distilled water, and dialysed for 12 h against 10 litres of water to remove any endogenous substrates and possible inhibitors. The dialysis residue was used directly for kinetic studies.

Prior to determining initial velocities at varying substrate concentrations, the linearity of both time of incubation and volume of enzyme sample against mass of substrate transformed were checked. The method of assay was similar to that described in section 2.3 except that a control chromatogram was developed for each substrate concentration used, and the counts from this control subtracted from the counts in the assay chromatogram. A major alteration was a reduction of the incubation time from 15 min. to 90 sec.

Km and Vmax values were obtained by entering the experimental data into an Olivetti Programma 101 desk top computer. This was programmed to calculate Km and Vmax from a regression line of the experimental points for Lineweaver-Burk and Hofstee-Eadie plots.

The programmes were written by Dr J.A. Nimmo, Department of Biochemistry, University of Edinburgh.

2.10 HISTOLOGICAL LOCALISATION OF HYPOXANTHINE GUANINE PHOSPHORIBOSYLTRANSFERASE USING AUTORADIOGRAPHY

Human tissues were obtained at necropsy within twelve hours of death. Portions of testis, cerebral cortex, and thyroid gland were divided into cubes of approximately 2 mm face. The fragments were placed on tantalum wire grids (Trowel, 1959) and placed in Trowel's T8 organ culture medium with added [^{3}H]-hypoxanthine (specific activity 1.0 Ci/mmole). Fragments were also placed free in the medium. The tissue was incubated for 2 h at 37^{o}C, and after this time the fragments were washed three times with Hank's basic salt solution before being fixed in 10% neutral formalin. The material was then paraffin processed, sections cut at seven microns and stained with haematoxylin.

Autoradiographs were prepared using Kodak AR 10 stripping film and an exposure period of twenty-eight

days. The film was developed with Kodak D19, fixed in 12.5% Kodafix and mounted in Depex.

This work was carried out in collaboration with Dr J.M. Anderson, Department of Paediatric Pathology, The Royal Hospital for Sick Children, Edinburgh.

2.11 DETERMINATION OF THE SUBCELLULAR DISTRIBUTION OF HYPOXANTHINE GUANINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY

All procedures were carried out in a cold room at 4°C.

Approximately 1 g of tissue was homogenised in 8 ml of 220 mM tris/HCl buffer pH 7.4. Six passes of a Teflon homogeniser driven at 2000 r.p.m. were used. An aliquot of the homogenate was twice frozen and thawed and used to determine total HGPRTase activity. The remainder of the homogenate was filtered through glass wool and further separated by centrifugation procedures. All volumes were noted throughout the following stages. The nuclei and cell debris were removed by centrifuging at 3,300 g for 10 min. at 4°C.

The mitochondrial fraction was separated by centrifuging at 17,000 g for 1 h at 4°C, and the microsomal fraction by centrifuging at 105,000 g for 5 h at 4°C.

Determinations of HGPRTase activity were carried out on all the supernatant fractions and the total activity of the fractions calculated.

2.12 PURINE TRANSPORT

2.12a Labelling of Rabbit Erythrocytes

To demonstrate that erythrocytes have the capacity to transport purines between tissues *in vivo* it was necessary to load the rabbit red cell with labelled hypoxanthine. This purine was used as it has been shown to be an intermediate in purine metabolism and not an end product (Bradford *et al.*, 1968) and is the major purine released by erythrocytes (Jorgensen and Poulsen, 1955).

Hypoxanthine passes into erythrocytes *in vitro* by a facilitated diffusion mechanism; it flows into the cell down a concentration gradient, and is converted intracellularly into IMP by HGPRTase; this enzyme has been shown to be essential for the uptake of purines by erythrocytes (Sperling *et al.*, 1971) and may also be essential for the uptake of purines into other tissues.

The charged phosphate group on IMP prevents the efflux of IMP from the cell, and the lowered intracellular hypoxanthine levels maintain the flow of extracellular hypoxanthine into the erythrocyte until

equilibrium is attained.

Four male New Zealand White rabbits were used. Allopurinol was administered, at 2, 4 and 10 mg/kg in experiments 2, 3 and 4 respectively. No allopurinol was administered in experiment 1. The drug was given as a suspension by a stomach tube, French Gauge 10.

Allopurinol (4-hydroxypyrazolo(3,4-d)pyrimidine) is a competitive inhibitor of xanthine oxidase EC 1.2.3.2 and its administration delays the catabolism of hypoxanthine via xanthine to allantoin. Allopurinol has been shown to augment the incorporation of hypoxanthine into nucleic acids (Pomales *et al.*, 1963).

10 ml samples of whole blood were obtained by cardiac puncture and withdrawn via a 17 gauge needle into a syringe containing about 1000 units of free acid heparin. The blood was mixed and dispensed into a 50 ml Quickfit conical flask with a ground glass stopper. The purine, hypoxanthine-[8-^{14}C] 50 μCi-59 μCi/μmole, was added to the 10 ml of blood in 1 ml of a medium of the following composition, described by Mager *et al.* (1967): 100 μmoles of glucose; 100 μmole potassium phosphate pH 7.4; 10,000 units of benzylpenicillin; 1 mg Streptomycin sulphate. The antibiotics were added to prevent bacterial growth

during the incubation procedure. The blood containing the hypoxanthine-[8-^{14}C] and antibiotics was incubated for 2 h at 37^{o}C with gentle shaking, then centrifuged at 5000 g for 10 min. The supernatant was removed and stored at -4^{o}C. The packed cells were washed twice with five volumes of isotonic saline buffered with 0.01M potassium phosphate to pH 7.4. Washings were retained and stored at -4^{o}C.

The washed, packed, cells were resuspended in 2.5 volumes of isotonic saline and reinjected into the marginal ear vein of the donor rabbit. The hypoxanthine-[8-^{14}C] added represented a 100% increase in the erythrocyte purine nucleotide concentrations, however, Mager et al. (1967) reported that similar increases did not affect the rate of efflux of hypoxanthine from the erythrocyte.

After 4 h in experiment 1 and 45 h in experiments 2, 3 and 4, the animal was killed by injecting 20 ml of air into the marginal ear vein.

Tissues were removed as soon as possible, generally within 20 min. of death, and the nucleotide-containing fractions (acid soluble, RNA and DNA) were obtained by the procedures described in the following sections.

The procedures directly involving these rabbits were performed by Dr R. Dow, M.R.C. Brain Metabolism Unit, University of Edinburgh and Dr R.A. Harkness, Department of Paediatric Biochemistry, The Royal Hospital for Sick Children, Edinburgh.

2.12b Extraction of Acid Soluble Nucleotides

Acid soluble nucleotides were extracted from the excised tissues by homogenising 0.5 g of tissue in 10 ml of 0.2 M perchloric acid for 1 min. using an Ultraturrax homogeniser.

The homogenate was centrifuged at 10,000 g for 10 min. and the supernatant removed.

Samples of the supernatant were counted as described in section 2.13 to determine the radioactivity in the acid soluble fraction.

2.12c Preparation of Ribonucleic Acid

RNA was extracted according to the procedure of Birnstiel, Sells and Purdom (1972). This procedure involves the treatment of homogenised tissue with phenol to remove the protein and DNA, and yields a DNA-free RNA preparation.

The homogenising medium was of the following composition: 0.15M sodium chloride, 0.05M tris, and 0.1% w/v sodium dodecyl sulphate (S.D.S).

Immediately before use sufficient polyvinyl sulphate was added to the homogenising medium and sodium acetate to achieve a concentration of 2 µg/ml. Polyvinyl sulphate is reported to be an inhibitor of ribonuclease (Scherrer and Darnell, 1962). Phenol was prepared as a water saturated solution. Dilute saline citrate was prepared 0.015 M with respect to sodium chloride and 0.0015 M with respect to trisodium citrate.

The following procedures were carried out in a cold room at 4°C.

Approximately 1 - 2 g of tissue were minced and washed with cold homogenising medium before blotting dry. The minced tissue was then homogenised in 20 ml of homogenising medium for about 1 min. using an Ultraturrax homogeniser at 20,000 r.p.m. to give a homogenate of smooth consistency. An equal volume of water saturated phenol was added and the mixture shaken for 30 min. in a bench shaker. The phenol emulsion was then broken by centrifuging at 10,000 g for 10 min. at 4°C, and the supernatant containing the RNA removed by pipette.

An equal volume of water saturated phenol was added to the supernatant and the mixture shaken as before. The phenol emulsion was broken by centrifuging at 10,000 g for 10 min. at 4°C, the supernatant removed by pipette and sufficient sodium acetate added to achieve a concentration of 2% w/v at

pH 5.0. The RNA was precipitated by the addition of 2 volumes of ice-cold ethanol and by storage overnight at -20°C. The RNA was separated by centrifuging and the resulting pellet washed twice with absolute ethanol, and dissolved in dilute saline citrate solution.

A standard graph was prepared of RNA in dilute saline citrate solution, at 10, 20, 30, 40 and 50 µg RNA per ml, and the optical density of these standards determined at 260 nm.

2.12d Preparation of Deoxyribonucleic Acid

DNA was extracted by a modified method of Marmur (1961). The method can be outlined as follows: the cells are first disrupted mechanically and by the action of the anionic detergent sodium dodecyl sulphate (S.D.S.); the cell debris, protein and fat removed by denaturation and centrifugation, RNA is removed by ribonuclease; this is followed by the selective precipitation of DNA with isopropanol.

The homogenisation medium of 0.15 M sodium chloride, 0.1 M EDTA pH 8.0 inhibits DNAase activity due to high pH or EDTA or both. Saline/citrate solution, 0.15 M sodium chloride, 0.015 M trisodium citrate maintains the ionic strength of the dissolved DNA and chelates divalent ions. The high concentration of sodium

perchlorate helps to dissociate the protein from the nucleic acid while the chloroform/isoamyl alcohol denatures protein and removes fat. The protein and fat move into the phenol layer and this is separated by centrifuging.

Tissue homogenisation was carried out in a cold room at 4°C; subsequent stages at room temperature.

Approximately 2 g of tissue was minced, washed in saline/EDTA and blotted dry. The minced tissue was then homogenised in 20 ml of saline/EDTA for about 30 sec. using an Ultraturrax homogeniser. A 5 ml aliquot of the homogenate was made 2% w/v with S.D.S. and incubated in a water bath at 60°C for 10 min. If this preparation had the consistency of syrup, then the remainder was made 2% w/v with S.D.S. and incubated at 60°C. If the 5 ml trial aliquot became lumpy on treatment with S.D.S. then the bulk of the homogenate was diluted by the addition of 10 ml of saline/EDTA before being made 2% w/v with S.D.S. and incubated as described. The incubated, S.D.S.-treated, homogenate was cooled to room temperature, made 1 M with respect to sodium perchlorate, and $^{1}/_{10}$ of the total volume (V1) of saturated Tris pH 8.0 added.

This was followed by half a volume ($V_{\frac{1}{2}}$) of chloroform/

isoamyl alcohol 24 : 1 v/v, and a half volume ($V_{\frac{1}{2}}$) of water saturated phenol. This preparation was mixed for 30 min. using a bench shaker, then centrifuged at 10,000 g for 10 min. The viscous supernatant containing the crude DNA was removed with a wide bore pipette, and DNA precipitated by the addition of two volumes of ice-cold ethanol. The DNA was then spooled onto a twisted glass rod, and the DNA fibres washed in turn with 90%, 95% and 99.8% ethanol. The DNA was dissolved in 10 ml dilute saline/citrate solution (0.015 M sodium chloride 0.0015 M trisodium citrate) and gently shaken to help dissolve the DNA. This solution was made to 0.15 M sodium chloride, 0.015 M trisodium citrate by the addition of 1 ml of 1.5 M sodium chloride - 0.15 M trisodium citrate solution.

Impurities in the DNA preparation were removed by treatment with ribonuclease (RNase). 20 mg of RNase were dissolved in 1 ml of 2% w/v sodium acetate pH 5.0, and heated to 100°C for 5 min. to denature any deoxyribonuclease (DNase) present. This DNase free RNase solution was added to the crude DNA preparation to give a RNase concentration of 50 µg/ml protein, and the mixture incubated at 37°C for 2 h.

Protein was removed by treatment with protease

prepared from Streptomyces griseus at 20 mg/ml protein in saline/citrate solution. This was added to the DNA preparation to give a final concentration of 400 µg/ml protein, and incubation was at 37°C for 3 h.

After deproteinising, half of the total volume (V2) of chloroform/isoamyl alcohol and a half volume ($\frac{V2}{2}$) of water saturated phenol were added, the mixture shaken and centrifuged as before, and the supernatant removed.

The purified DNA was spooled onto a twisted glass rod after the addition of 2 volumes of propan-2-ol and during the spooling a $^{1}/_{10}$th volume of sodium acetate/EDTA was added. The spool was washed in 90%, 95% and 99.8% ethanol and dissolved in 5 ml of 0.015 M sodium chloride, 0.0015 M trisodium citrate.

A standard graph was prepared of DNA in saline/citrate solution at 10, 20, 30, 40 and 50 µg DNA/ml and the optical density of these standards determined at 260 nm.

2.12e Determination of Purity of Prepared Deoxyribonucleic Acid and Ribonucleic Acid

The possibility of DNA contamination of RNA was checked by the attempted digestion of the RNA samples

by DNase using the Sigma modification of the method of Kunitz (1950).

The RNA contamination of DNA was checked by the attempted digestion of DNA samples with RNase using the method of Kunitz (1950).

Both DNA and RNA were hydrolysed by DNase and RNase respectively using the above methods.

2.13 LIQUID SCINTILLATION COUNTING OF AQUEOUS SAMPLES

Aqueous samples were counted by the addition of 100 µl of the sample to 10 ml of Bray's solution (Bray, 1960).

The samples were counted in a Packard Tricarb liquid scintillation counter; settings were as before (section 2.4).

The counting efficiency for ^{14}C determined by internal standardisation was between fifty and sixty per cent.

2.14 THIN LAYER CHROMATOGRAPHY OF PURINES AND PURINE DERIVATIVES

Purines and derivatives were separated chromatographically using commercially prepared cellulose plate 20 x 20 cm (Kodak, Ltd, Liverpool).

Using a solvent of 1 M ammonium acetate pH 7.0 (Hochstadt-Ozer and Stadtman, 1971) a running time of 1 • 5 h gave satisfactory separations. The spots were identified under u/v light.

TABLE V

Rf VALUES FOR PURINES AND DERIVATIVES ON A SUPPORT OF CELLULOSE AND SOLVENT OF 1M AMMONIUM ACETATE pH 7.0

Compound	Rf Value
Adenine	0.3
Hypoxanthine	0.44
Xanthine	0.32
Urate	0.21
AMP	0.6
GMP	0.098
IMP	0.34

SECTION III

RESULTS AND DISCUSSION

3.1 ERYTHROCYTE PURINE PHOSPHORIBOSYLTRANSFERASE

3.1a Introduction

Erythrocytes provide a readily available source of the purine phosphoribosyltransferases for both research and diagnostic purposes.

These enzymes can be studied in erythrocytes by several methods, for example that of Fujimoto et al. (1968) or of Chow et al. (1970); the assay method developed in this work was based on that used for erythrocyte purine phosphoribosyltransferases by Cartier and Hamet (1968).

Initially, activities of both HG and APRTase from erythrocytes were determined to ensure that the assay system would yield results very similar to those obtained by Cartier and Hamet (1968).

In an attempt to obtain maximal PRTase activity, the effects of the reducing agent sodium borohydride were investigated, since HGPRTase is known to be inhibited by the sulphydryl binder p-chloromercuri-benzoate (Krenitsky and Papaioannou, 1969).

When there are -SH groups at the active site, the addition of a reducing agent to the enzyme assay medium often results in considerable increases in the measured activity (Adams, 1969).

Modifications of the PRTase assay method The products of the PRTase reactions IMP, GMP and AMP, are

hydrolysed to nucleosides by 5'-nucleotidase (EC 3.1.3.5) (Adams et al., 1971), an enzyme found in nearly all tissues except erythrocyte lysates. When the assay of the PRTases depends on the determination of the product nucleotide, a consequence of tissue 5'-nucleotidase activity will be the reduction of nucleotide levels and hence underestimation of PRTase activity. In order to minimise this artefact, some potential inhibitors of 5'-nucleotidase were studied. In agreement with the finding of Ahmed and Reis (1958), nickel ions were found to be powerful inhibitors of 5'-nucleotidase; unfortunately nickel ions were found to also inhibit HGPRTase. The effects of thymidine triphosphate (TTP) as a selective inhibitor of 5'-nucleotidase (Murray and Friedrichs, 1969) were studied to determine its effects on HGPRTase, and TTP was shown to be an effective selective inhibitor of 5'-nucleotidase.

Relationship of HGPRTase activity to known alterations in urate excretion Cyclic changes in manic depressive illness are mirrored by changes in daily urinary uric acid excretion (Anumoyne et al., 1968). Urinary urate is increased during the early phase of remission. These changes in urate excretion occur during natural remissions and remissions induced therapeutically by lithium carbonate. A

possible cause for the increase in urinary urate excretion could be an inhibition of HGPRTase by lithium ions. A higher proportion of hypoxanthine might then be converted to xanthine and hence to urate.

Therapy of manic depressive illness with lithium carbonate aims at plasma lithium levels of 2 mM, and the effect of 2 mM lithium on HGPRTase activity was therefore studied.

The effects of testosterone at 5 and 10 μM on HGPRTase activity were studied, as urinary excretion of urate falls and plasma urate levels rise during and after puberty (Harkness and Nicol, 1969). It seemed possible that these changes could be related to increased testosterone levels.

3.1b Results

It was confirmed that the amount of enzyme in the incubation medium was linearly related to the mass of substrate transformed. The increase in nmoles of hypoxanthine transformed per minute with increase in enzyme volume is shown in Fig. 8. This relationship was linear and remained so with increase in enzyme volume up to a 25% transformation of substrate hypoxanthine (1.35 mg/ml protein)

The effects of increasing incubation time on the

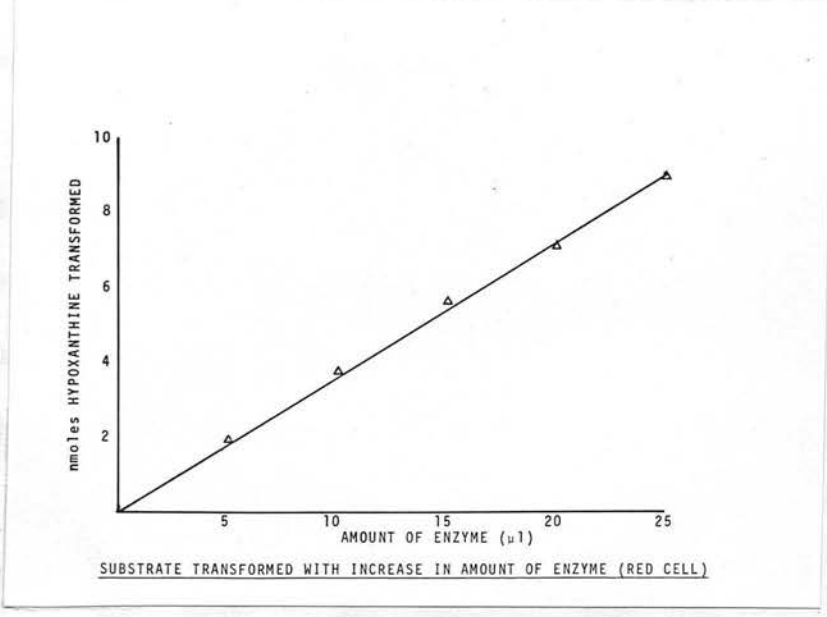

10
8
6
4
2
nmoles HYPOXANTHINE TRANSFORMED
5
10
15
20
25
AMOUNT OF ENZYME (µl)
SUBSTRATE TRANSFORMED WITH INCREASE IN AMOUNT OF ENZYME (RED CELL)

Fig. 8

mass of hypoxanthine transformed per minute, for a fixed volume of red cell lysate (25 µl), are shown in Fig. 9. The relationship becomes non-linear after 25 min. incubation, but is linear up to twenty-five per cent transformation of substrate hypoxanthine. Similar results were obtained with erythrocyte APRTase.

Sodium borohydride at 10 mM had no effect on HGPRTase activity; mean ($\pm$ S.D.) values of 583 $\pm$ 75 nmole/min./ml red cells were obtained for a control series and of 583 $\pm$ 78 nmole/min./ml red cells when assayed in the presence of 10 mM sodium borohydride.

Erythrocyte HGPRTase activities from the 0 - 2 year old group were 611 $\pm$ 81.9 nmole/min./ml red cells (n = 8) and 104.3 $\pm$ 4.5 nmole/h/mg protein (n = 3). Mean ($\pm$ S.D.) HGPRTase activities in the 2 - 10 year age range were 587.3 $\pm$ 72.6 nmole/min./ml red cells (n = 11) and 111.0 $\pm$ 7.5 nmole/h/mg protein (n = 4).

Erythrocyte APRTase, from the same group of patients in the 0 -2 year group, were 188.6 $\pm$ 50.6 nmole/min./ml red cells (n = 10) and 37.0 $\pm$ 9.0 nmole/h/mg protein. In the 2 - 10 year age group mean ($\pm$ S.D.) values obtained were 194.6 $\pm$ 51.3 nmole/min./ml red cells (n = 11) and 36.5 $\pm$ 5.3 nmole/h/mg protein

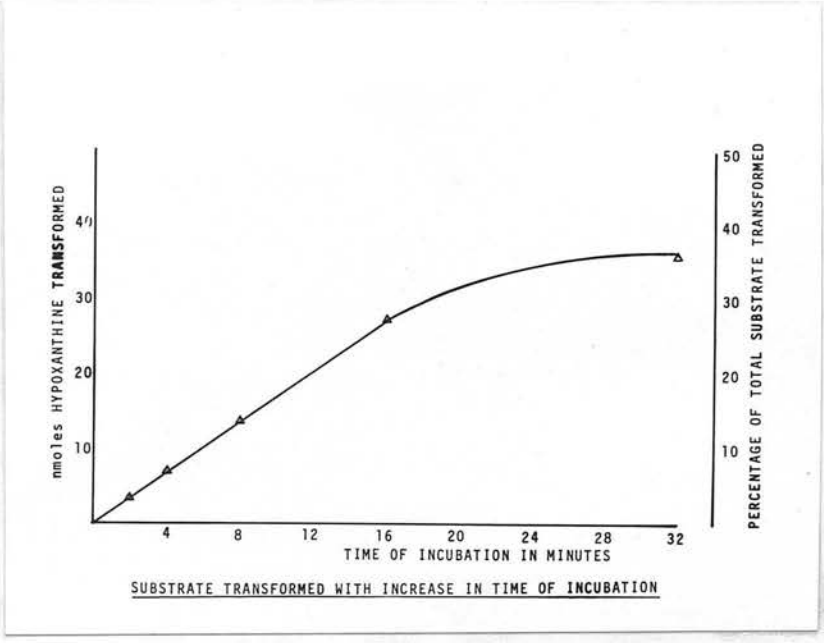

nmoles HYPOXANTHINE TRANSFORMED
40
30
20
10
4
8
12
16
20
24
28
32
TIME OF INCUBATION IN MINUTES
50
40
30
20
10
PERCENTAGE OF TOTAL SUBSTRATE TRANSFORMED
SUBSTRATE TRANSFORMED WITH INCREASE IN TIME OF INCUBATION

Fig. 9

(n = 4).

The presence of 5 μM testosterone had no significant effect on erythrocyte HGPRTase activity. A control series of assays gave mean ($\pm$ S.D.) values of 11.59 $\pm$ 4.19 percentage transformation of substrate compared with 11.17 $\pm$ 5.65 (n = 11) percentage transformation in the presence of 5 μM testosterone. No significant effect was found in the presence of 10 μM testosterone, control mean values of 11.87 $\pm$ 9.25 per cent transformation of substrate compared with 10.79 $\pm$ 9.0 (n = 14) in the presence of 10 μM testosterone.

Nickel at 2.5 mM, an inexpensive and powerful inhibitor of 5'-nucleotidase, was found also to strongly inhibit HGPRTase. In a series of controls a mean ($\pm$ S.D.) value of 9.8 $\pm$ 5.8 per cent transformation of substrate was obtained, and when assayed in the presence of 2.5 mM nickel mean values ($\pm$ S.D.) of 3.4 $\pm$ 1.8 (n = 10) per cent transformation of substrate were obtained.

The effects of adding 1.5 mM thymidine triphosphate to the assay medium were not significant. In a control series a mean ($\pm$ S.D.) value of HGPRTase activity of 10.33 $\pm$ 8.54 per cent transformation of substrate was obtained while in the presence of 1.5 mM

TABLE VI

EFFECTS OF LITHIUM IONS ON HGPRTase ACTIVITY

PERCENTAGE TRANSFORMATION OF SUBSTRATE	
CONTROL	2.0 mM LITHIUM
7.6	7.9
7.8	8.1
8.1	6.8
7.2	7.3
7.7	7.4

TABLE VII

EFFECTS OF 4, 6, 8, AND 10 mM LITHIUM IONS ON HGPRTase ACTIVITY

Percentage Transformation of Substrate					
Control	2 mM Li^+	4 mM Li^+	6 mM Li^+	8 mM Li^+	10 mM Li^+
1.54	1.61	1.45	2.02	1.6	1.41
2.06	1.23	2.02	2.84	2.1	1.74
10.4	10.6	11.3	12.9	10.3	9.7
10.2	10.4	10.4	10.6	9.9	10.3

thymidine triphosphate a mean ($\pm$ S.D.) of 10.45 $\pm$ 8.63 (n = 10) per cent transformation of substrate was obtained.

Assays of 5'-nucleotidase activity on erythrocyte lysates prepared as described in section 2.2a showed activities of less than 0.5 nmole/h/mg protein.

Lithium ions had no effect on HGPRTase activity at 2 mM but slight inhibition may be evident at higher concentrations (Tables VI and VII).

3.1c Discussion

Validity of assay method After initial difficulties in the preparation of purine substrates, mainly due to the low solubility of purine at pH 7.4, the present assay method proved to be a practical method of determining PRTase activity.

The mass of substrate purine transformed under the conditions of assay was linear with time of incubation up to sixteen minutes (Fig. 9) or twenty-eight per cent transformation of available substrate base. When larger percentages of transformation of substrate were found, deviations from linearity were noted, probably due to the contribution from the back reaction.

$$\text{AMP} + \text{PPi} \xleftrightarrow[Mg^{+2}]{} \text{Adenine} + \text{PRPP}$$

Similar results were obtained for increasing enzyme volume and increasing time of incubation for erythrocyte APRTase.

The coefficient of variation of the assay method for HGPRTase was determined from a series of fifty duplicate assays according to the method of Henry and Dryer (1963). A coefficient of variation of 9.6% was obtained for a mean of 95.3 nmole/h/mg protein.

Sodium borohydride at 10 mM had no activating effect on HGPRTase and was not subsequently added to the assay medium.

HGPRTase is inhibited by the sulphydryl binder p-chloromercuribenzoate(Krenitsky and Papaioannou, 1969) and therefore it would appear that an -SH group is involved at the active centre. However, these results in conjunction with *in vivo* stability studies (section 3.4) would indicate that this -SH group is relatively stable to oxidation.

Erythrocyte PRTase activities Erythrocyte HG and APRTase activities from a hospital population were determined in bloods obtained from the Haematology Department, Royal Hospital for Sick Children. The results obtained are similar to those of Cartier and Hamet (1968) and of Kelley *et al*.

(1967a). The latter group of workers used a different assay method.

The values obtained by Cartier and Hamet (1968) and by Kelley et al. (1967) are compared in Table VIII with the values obtained in this study.

Effects of potential modifiers of HGPRTase activity Testosterone at 5 μM and 10 μM had no significant effect on HGPRTase activity $0.4 < P < 0.5$. Recent work by Oliver (1972) has, however, demonstrated that steroid hormones do effect nucleotide biosynthesis. Oestradiol-17-β was shown to activate ribose-5-phosphate pyrophosphokinase (EC 2.7.6.1) leading to an increase in PRPP concentrations and hence an increase in both de novo and salvage nucleotide synthesis. Oestradiol has also been shown to increase the uptake of purines by intact erythrocytes by Christensen and Jones (1961). Further studies on the effects of steroid hormones on tissue nucleotide synthesis are indicated.

5'-Nucleotidase inhibitors Nickel ions at 2.5 mM were effective inhibitors of 5'-nucleotidase, but were also strong inhibitors of HGPRTase $P > 0.001$ and were not further investigated.

TABLE VIII

A COMPARISON OF ERYTHROCYTE PRTase ACTIVITIES (MEANS ± S.D.) OBTAINED IN THE PRESENT INVESTIGATION AND IN THE TWO PREVIOUS SERIES

Authors' Age Group	HGPRTase		APRTase	
	nmole/min/ml R.B.L.	nmole/h/mg pro.	nmole/min/ml R.B.L.	nmole/h/mg pro.
This work 0-2 yr.	611 ± 81.9 (8)	104.3 ± 4.5 (4)	188.6 ± 50.6 (8)	37.0 ± 9.0 (4)
This work 2-10 yr.	587 ± 72.6 (11)	111 ± 7.5 (4)	194.6 ± 51.3 (11)	36.5 ± 5.3 (4)
Cartier & Hamet 0-2 yr.	600 ± 91	-	244 ± 26.0	-
Cartier & Hamet 2-10 yr.	669 ± 59	-	210 ± 18.0	-
Cartier & Hamet Adults	647 ± 74	111.0 ± 13	203 ± 19.0	34.8 ± 3.3
Kelley et al. Adults	600 ± 122	103 ± 21	182 ± 35.0	31.2 ± 6.0

Thymidine triphosphate has been shown to be a powerful inhibitor of 5'-nucleotidase by Murray and Friedrichs (1969) and no inhibitory or activating effects were detected on HGPRTase $0.7 < P < 0.8$. Thymidine triphosphate was therefore added to the PRTase assay medium to a final concentration of 1.5 mM when tissue PRTase activities were determined. Tissue homogenates, other than erythrocyte lysates, have high 5'-nucleotidase activity, some of which is solubilised during homogenisation with consequent hydrolysis of the PRTase reaction products.

Lithium therapy and HGPRTase The changes in urinary excretion of urate as a consequence of lithium carbonate therapy of manic depressive illness are obtained by maintaining plasma lithium levels of 2 mM. Using erythrocyte HGPRTase as a model for tissue HGPRTase, lithium ions at 2 mM had no effect on HGPRTase activity $0.6 < P < 0.7$.

Slight inhibition was observed at higher lithium concentrations, and the possibility of intracellular concentrations of 8 - 10 mM lithium with inhibition of HGPRTase may be worth further investigation. The evidence available would suggest, however, that intracellular lithium is lower than plasma lithium

levels (Glen, personal communication, 1972).

Role of the PRTases in the erythrocyte

Erythrocytes have relatively high activities of the PRTases whose role in the nucleated precursors of the developing reticulocytes in the bone marrow is probably in the generation of nucleotides for RNA and DNA synthesis. The PRTase activities found in the bone marrow (section 3.4) were somewhat lower than those found in the mature erythrocyte, but this may be due to sampling difficulties with bone marrow.

The half-lives of HG and APRTase in erythrocytes have been determined by Rubin et al. (1969), and a $t_{\frac{1}{2}}$ of 82 days for HGPRTase and $t_{\frac{1}{2}}$ of 34 days for APRTase have been found. APRTase from erythrocytes from Lesch-Nyhan patients had a $t_{\frac{1}{2}}$ of 86 days, the increased stability probably being a result of stabilisation of the APRTase protein by PRPP.

The role of the PRTases in the mature erythrocyte with no RNA or DNA metabolism is discussed more fully in section 3.7.

3.2 CLINICAL STUDIES

3.2a Introduction

During the course of this work some patients admitted to the Royal Hospital for Sick Children were

investigated. Children who had signs and symptoms resembling those found in the Lesch-Nyhan syndrome were investigated by determining their erythrocyte HGPRTase and APRTase.

The patient I.I. was referred by Dr D. Eccleston, Royal Edinburgh Hospital and the patients K.J. and E.J. by Dr D. Patrick, Great Ormond Street Hospital, London.

A short summary of the case notes is presented, along with the PRTase activities and other relevant data.

3.2b Results

Patient A.L. Male aged 2.5 years on admission. This child presented with recurrent haematuria, and after surgery was found to have a large oxalate/urate bladder calculus. At the time of admission serum urate levels were 9.2 mg/100 ml, and urinary urate 191 mg/100 ml. Blood electrolyte and calcium levels, and renal function tests were normal. A.L. then developed a urinary tract infection, and serum urate levels which had fallen to 4.6 mg/100 ml began to rise and reached 8.0 mg/100 ml. Erythrocyte HG and APRTase determined at this time were, HGPRTase 575 nmole/min/ml erythrocytes and APRTase,

150 nmole/min/ml erythrocytes.

Erythrocyte PRTase activity was determined one year later and values of 640 nmole/min/ml erythrocytes for HGPRTase, and 231 nmole/min/ml erythrocytes for APRTase were obtained. These values are normal (section 3.1).

Patient J.S. Male aged 5 years on admission. This patient presented as a markedly atoxic child with choreoarthetoid movements of limbs and a coarse bilateral tremor. J.S. exhibited hyperactive behaviour and self-mutilation by head banging and arm biting. The E.E.G. showed slight generalised abnormalities. Haematological reports showed a marked anaemia of undetermined origin.

Erythrocyte PRTase activity was determined and HGPRTase activity was 621 nmole/min/ml erythrocytes and APRTase 217 nmole/min/ml erythrocytes. These values are normal (section 3.1).

Patient S.B. Male aged 6 years on admission. Patient S.B. was slightly retarded in both motor and intellectual development. He was referred due to his self-mutilating behaviour, biting the flesh from his hands and arms. His general health was good. His teeth were removed to prevent further

damage to himself, and erythrocyte HGPRTase activity determined. HGPRTase activity was 588 nmole/min/ml erythrocytes; this is normal (section 3.1).

Patient I.I. Female aged 65 years

Patient I.I. was admitted to the Royal Edinburgh Hospital with manic depressive illness. She had a serum urate level of 11.3 mg% which was subsequently found to be due to psoriasis. There was family history of anaemia of undetermined type.

Erythrocyte PRTases were determined, and HGPRTase activity of 500 nmole/min/ml erythrocytes and APRTase activity of 164 nmole/min/ml erythrocytes were obtained. These values are at the lower end of the normal range (section 3.1).

Patients E.J. and K.J. Females aged 1 year and 2.5 years on admission. These sisters were admitted to the Great Ormond Street Hospital, London and were mentally retarded. The elder sister, K.J., had mutilated her lower lip by biting and both children exhibited marked choreoarthetoid movements. K.J. is shown in Fig. 10.

Erythrocyte PRTase activities were determined

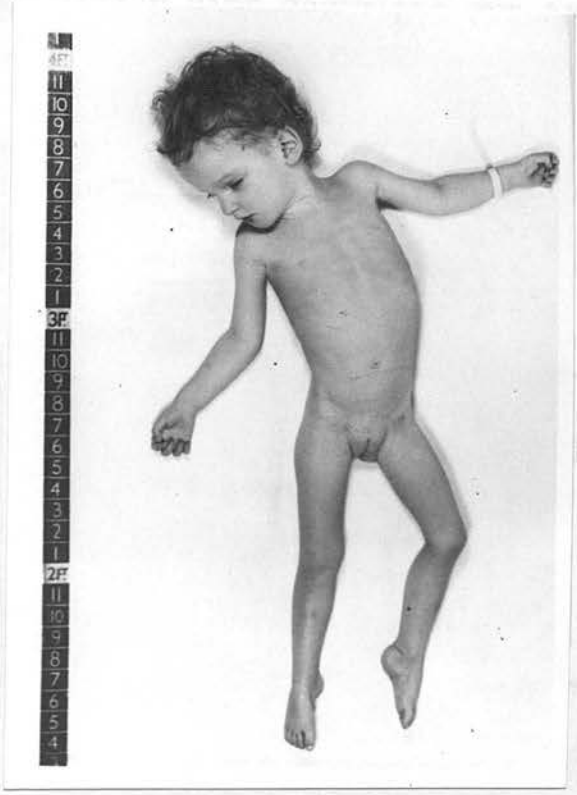


Fig. 10

Unusual posture adopted by
patient K.J.

TABLE IX

THERMAL DENATURATION RATE CONSTANTS FOR APRTase AT 56°C FROM CONTROLS AND PATIENTS E.J. AND K.J.

Subjects	No. of Determinations	Denaturation Rate Constant for APRTase at 56°C (MIN^{-1})	
		Erythrocytes	Cultured Fibroblasts
Controls	(4)	0.182 ± 0.009	0.482 ± 0.054
E.J.		0.30	0.42
K.J.		0.41	0.47

and the HGPRTase activity was found to be 580 and 630 nmole/min/ml erythrocytes for K.J. and E.J. respectively. APRTase activity was 198 and 195 nmole/min/ml erythrocytes for K.J. and E.J. respectively. In view of the low APRTase activity this enzyme was further investigated.

The thermal denaturation rate constants for APRTase from erythrocytes and cultured fibroblasts at 56^{o}C were determined as described in section 2.8, and are presented in Table IX.

The erythrocyte APRTase from these patients was more heat labile than control APRTase.

In a further attempt to determine if an abnormal APRTase protein was present, the electrophoretic and kinetic properties of the erythrocyte APRTase were investigated. The electrophoretic properties and apparent Km for adenine for both E.J. and K.J. were similar to those obtained for controls determined at the same time.

3.2c Discussion

Most of the patients studied had neurological symptoms resembling those found in Lesch-Nyhan children. No abnormal HGPRTase activity was detected; this provides further evidence that a

large number of brain disorders can result in similar signs and symptoms.

The studies on APRTase from the children K.J. and E.J. would suggest a normal APRTase in spite of the apparently lower denaturation rate constant detected for the enzyme in erythrocytes. This destabilisation could be the result of high intracellular oxypurine or reduced PRPP concentrations (Rubin and Earl Balis, 1972).

The low erythrocyte APRTase activity recorded for patient A.L. when his serum urate was 9.2mg% is unlikely to have been a result of an error in the laboratory as controls run at the same time gave normal results. Normal erythrocyte APRTase activities were recorded for this child when his serum urate was normal one year later. The possible significance of this finding is being investigated. However, the results may be due to the greater lability of APRTase compared to HGPRTase (section 3.4).

3.3 TISSUE 5'-NUCLEOTIDASE ACTIVITY

3.3a. Introduction

The results of enzyme assays carried out using tissue homogenates as an enzyme source can often be invalidated by the presence of any one of

several factors. These include the presence of inhibitors or activators of the enzyme studied, which may act by mass action or by allosteric mechanisms.

A tissue homogenate containing high levels of pyrophosphate would be inhibitory in the PRTase reactions, due to product inhibition.

$$\text{Base} + \text{PRPP} \longrightarrow \text{Nucleotide} + \text{PPi}$$

When a reaction product is being measured to determine enzyme activity this compound may be further metabolised by other enzymes present in the tissue homogenate. This situation occurs with the products of the PRTase reactions; AMP, IMP, and GMP are substrates for 5'-nucleotidase EC 3.1.3.5 (5'Nase) which hydrolyses these nucleotides to the corresponding nucleosides. Assays of PRTase activity in tissue homogenates which depend on the measurement of the nucleotide product will therefore underestimate PRTase activity.

Assays of PRTase activity by determining the radioactivity in both the nucleotide and nucleoside corresponding to the substrate base used are a justifiable method of overcoming the

effects of 5'-nucleotidase. However, such methods will tend to overestimate PRTase activity. This is due to the formation of nucleosides from the free base in the presence of ribose-1-phosphate by the enzyme nucleoside: orthophosphate ribosyltransferase activity EC 2.4.2.a (Sweetman et al., 1972).

Hypoxanthine or Guanine + Ribose-1-P $\longrightarrow$ Nucleoside + Pi

This limitation does not apply in mammalian tissues in the assay of APRTase, as no enzyme has been detected capable of the direct formation of adenosine from adenine (Huennekens et al., 1956).

The results presented in this section show the 5'Nase activities in some human tissues, and the efficiency of 1.5 mM TTP as an inhibitor of 5'Nase.

The effects of 1.5 mM TTP on the APRTase and HGPRTase activities measured as described in section 2.3 are presented.

3.3b. Results

5'-Nucleotidase activity was detected in the supernatants of tissue homogenates from all the

tissues studied. Results are presented in Table X of 5'-nucleotidase activities in nmole/h/mg protein with GMP as a substrate assayed as described in section 2.6.

In foetal liver and cerebral cortex, 5'-nucleotidase levels are low compared to the adult tissue, much higher activity was detected with AMP as a substrate.

Highest activities were found in central nervous system and in liver. Very much lower activities were detected in adult kidney and bone marrow.

No 5'-nucleotidase activity was detected in erythrocyte lysates.

The inhibition of 5'-nucleotidase by 1.5 mM thymidine triphosphate for various tissues is shown in Table Xl. The percentage inhibition ranges between 19.2% for foetal liver and 100% inhibition in adult cerebral cortex. Higher percentage inhibition was obtained when larger amounts of substrate were transformed (Tables XII and XIII).

The effects of 1.5 mM thymidine triphosphate in the PRTase assay system are shown in Table XII for HGPRTase and Table XIII for APRTase.

TABLE X

DISTRIBUTION OF 5'-NUCLEOTIDASE ACTIVITY IN HUMAN TISSUES USING GMP AS SUBSTRATE

Tissue	Age	5'Nase Activity nmole/h/mg Protein
Liver	35 week foetus	49.5 [112]
Cerebral cortex Grey matter		81.5 [107]
Basal nuclei	0.5 years	380
Cerebral cortex Grey matter		940
Cerebral cortex White matter		96
Liver	2.0 years	292
Cerebral cortex Grey matter		354
Cerebral cortex White matter		139
Thalamus		260
Liver	Adult	40
Basal nuclei		287
Testis		149
Kidney		33
Bone marrow		16.4
Medulla		358
Cerebral cortex Grey matter		252
		144
Cerebral cortex White matter		192

The figures in parenthesis represent the 5'nucleotidase activity with AMP as a substrate

TABLE XI

INHIBITION OF HUMAN TISSUE 5'-NUCLEOTIDASE BY 1.5 mM THYMIDINE TRIPHOSPHATE

Tissue	Age	Percentage Inhibition
Liver	35 week foetus	19.2 [75]
Cerebral cortex Grey matter		47 [49]
Basal nuclei	0.5 years	90.4
Cerebral cortex Grey matter		81.5
Cerebral cortex White matter		100
Liver	Adult	61
Thalamus		85
Cerebral cortex Grey matter		100
Cerebral cortex White matter		100

The figures in parenthesis represent percentage inhibition 5'-nucleotidase with AMP as a substrate

TABLE XII

PERCENTAGE INCREASE IN MEASURED HGPRTase ACTIVITY ASSAYED IN THE PRESENCE OF 1.5 mM THYMIDINE TRIPHOSPHATE

Tissue	Age	Percentage Increase
Thalamus	0.002 year	89
Cerebral cortex Grey matter		39
Adrenals	0.45 year	25
Cerebral cortex Grey matter		38
Cerebral cortex White matter		430
Kidney		23
Basal nuclei	0.5 year	180
Cerebral cortex Grey matter		31
Cerebral cortex White matter		0
Cerebral cortex Grey matter	1.5 year	440
Basal nuclei		380
Liver		29
Liver	6.0 year	150
Thalamus		335
Liver	Adult	190
Cerebral cortex Grey matter		2
Thalamus		68

TABLE XIII

PERCENTAGE INCREASE IN MEASURED APRTase ACTIVITY ASSAYED IN THE PRESENCE OF 1.5 mM THYMIDINE TRIPHOSPHATE

Tissue	Age	Percentage Increase
Thalamus	0.02 year	Not detected in absence of TTP
Cerebral cortex Grey matter		310
Liver	6.0 year	510
Thalamus		585
Liver	Adult	15.5
Cerebral cortex Grey matter		Not detected in absence of TTP
Thalamus		85

Significant increases in HGPRTase activity were observed after inhibiting 5'-nucleotidase with 1.5 mM thymidine triphosphate. These increases in detected HGPRTase activity were particularly marked in the tissues of the central nervous system and in liver, increases of up to 440% being noted. Percentage increases in activity in tissues other than central nervous tissue and liver were generally much lower, 23% increase in kidney HGPRTase and 25% with adrenal HGPRTase. The small increases in activity of 0 and 2% may have been due to more prolonged storage of these tissues.

The percentage increase in measured APRTase activity (Table XIII) was also high in nervous tissue and liver although a value of only 15.5% was obtained for adult liver. As the levels of APRTase are low in the central nervous system (see section 3.4), theoretically infinite increases in activity were obtained in the thalamus (adult) and cerebral cortex grey matter (0.02 yr.) when no APRTase activity was detected in the absence of thymidine triphosphate. Similar results were obtained with rat tissues.

Nickel ions at 5 mM totally inhibited

5'-nucleotidase, obtained from all tissues examined. Nickel ions were also powerful inhibitors of HGPRTase (section 3.1) and were therefore not further investigated.

3.3c. Discussion

5'-Nucleotidase, first described by Reis (1934), hydrolyses the phosphate group of the fifth carbon atom of ribose nucleotides.

A wide variety of assay methods has been reported (see Bodansky and Schwartz, 1968), and difficulties in interpretation of results include the contribution to hydrolysis from non-specific acid and alkaline phosphatases EC 3.1.3.1 and EC 3.1.3.2. 5'-Nucleotidase was reported to be present in all human tissues except the intestinal mucosa (Reis, 1951; Planteydt and Willighagen, 1960) while Pearse and Reis (1952) reported that the tissue distribution of 5'-nucleotidase was different from that of the non-specific phosphatases.

Comparisons of tissue 5'-nucleotidase activities obtained in this study with other reported values are difficult due to the wide variety of methods of assay used and to the report by Bjork (1964) that the nature of the buffer anion affects activity.

The available evidence would suggest that in this work true 5'-nucleotidase activity is being measured because magnesium ions activate 5'-nucleotidase at pH 7.4 (Belfield and Goldberg, 1969) and at the pH used in the assay the non-specific phosphomonoesterases show only 5% of their maximal activity at optimal pH (King, 1965)

5'-nucleotidase activity has therefore been measured under conditions almost identical to those of the PRTase activities. This enables an evaluation of the contribution of 5'-nucleotidase breakdown of the PRTase reaction products in tissue homogenates to be made.

In this work, high 5'-nucleotidase activities were found in the central nervous tissues (Table X), higher values being found in the white matter than in the grey matter. Foetal 5'-nucleotidase showed a higher catalytic activity with AMP as substrate than with GMP (Table X). This may indicate the presence of separate enzymes in the foetus for the hydrolysis of AMP and GMP - as is found in chick brains (Manzoli, Ipata and Wegelin, 1971), but further studies on this would be required to arrive at a positive conclusion.

Little is known about the physiological role

of 5'-nucleotidase, but as AMP is important in the regulation of at least three important enzymes in the Embden Meyerhoff pathway and the tricarboxylic acid cycle (Atkinson, 1966) it is possible that amongst other functions, 5'-nucleotidase, by lowering AMP levels, influences the activities of these enzymes. The role of 5'-nucleotidase in growth has been studied by Panda et al., 1962 who found significant increases in 5'-nucleotidase after injections of growth hormone. This work in conjunction with the finding of elevated serum 5'-nucleotidase in a wide variety of neoplastic diseases by Kowlessar et al., 1961 strongly suggests that 5'-nucleotidase is important in the turnover of nucleotides during periods of rapid growth.

The inhibition of 5'-nucleotidase by thymidine triphosphate was reported by Murray and Friedrichs (1969), and this study shows effective inhibition of 5'-nucleotidase particularly in tissues with a high specific activity.

Assays of HG and APRTase carried out in the absence and presence of 1.5 mM thymidine triphosphate showed significant increases in the PRTase activity detected. This was shown by initial studies on red

cell lysates not to be due to any stimulation of the PRTase by TTP, or via any increase in PRPP levels. These lysates, with no detectable 5'-nucleotidase activity, showed no increase in PRTase activity (section 3.1).

As thymidine triphosphate was shown to inhibit 5'-nucleotidase and to increase the values of PRTase activity it was subsequently added to the assay system for the PRTases at a final concentration of 1.5 mM. As 5'-nucleotidase inhibition is unlikely to be total at all times, the PRTase activities reported in section 3.4 may still represent slight underestimations of true tissue PRTase activity.

3.4 HUMAN TISSUE PURINE PHOSPHORIBOSYLTRANSFERASE ACTIVITIES

3.4a. Introduction

Previous estimates of tissue PRTase activities are probably low due to the breakdown of the product nucleotide AMP, IMP or GMP by the action of 5'-nucleotidase.

5'-Nucleotidase, EC 3.1.3.5, can be inhibited by thymidine triphosphate, and the PRTase activities determined in this work were made using a new assay method incorporating 1.5 mM thymidine triphosphate

to inhibit the destruction of the assay product.

Purine phosphoribosyltransferase activities have been determined in adult human tissues by Rosenbloom et al. (1967), and in tissues from the Rhesus monkey by Krenitsky (1969). Murray (1966) who determined PRTase activities in mouse and rat tissues, and in Erlich ascites tumour cells, observed changes in the activity of the PRTases in rat liver extracts following partial hepatectomy.

Boyle et al. (1970) determined the activity and stability of PRTases in tissues from a human foetus, and Epstein (1970) studied the early developmental increases in PRTase activity in pre and post-implantation mouse embryos.

There is an absence of detailed work on the distribution of the PRTases in human tissues, and no information on the changes in the activities of these enzymes which occur between foetal and adult life.

The present work was carried out in order to provide this information.

Knowledge of changes in PRTase activity with growth and development, and the relative importance of HGPRTase and APRTase between tissues, may reveal some role for these enzymes in growth and development and may help to explain the delayed onset of the

neurological symptoms in children with the Lesch-Nyhan syndrome (Nyhan, 1968). The distribution of activities between different tissues may help to clarify the role of these salvage pathways in the purine metabolism of the whole animal.

The human tissues were obtained at necropsy, generally within twenty-four hours of death, by Dr J.M. Anderson, Department of Paediatric Pathology, the Royal Hospital for Sick Children.

As tissues were generally obtained for study of the PRTase activity between twelve and twenty-four hours after death, the *in vivo* stability of HG and APRTase was determined in tissues removed surgically and stored at $4^{o}C$.

As the PRTases are synthesising nucleotides for RNA and DNA biosynthesis which occurs in the nuclear compartment of the cell, a study was carried out on the cellular localisation of HGPRTase.

In an attempt to determine the specific cell type with high HGPRTase activity in the testis, autoradiographic investigations were carried out.

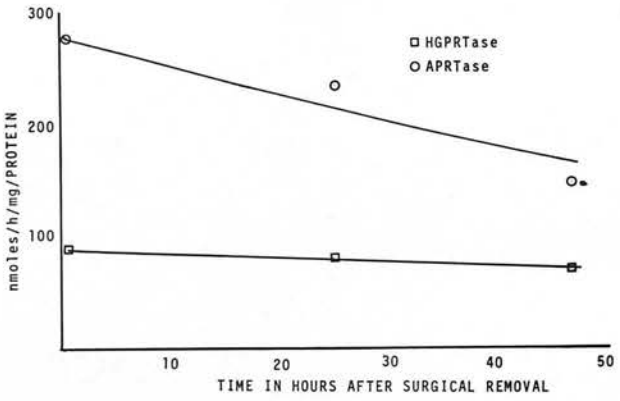
In view of the importance of cells from tissue culture in clinical diagnosis and in experimental work, the PRTase activities from a number of human

cell lines were determined.

Results are presented of HGPRTase activities in a series of human tissue culture cell lines obtained from Dr P. Gormley, M.R.C. Clinical and Population Cytogenetics Research Unit, and from Dr A.D. Bain, Department of Paediatric Pathology, the Royal Hospital for Sick Children.

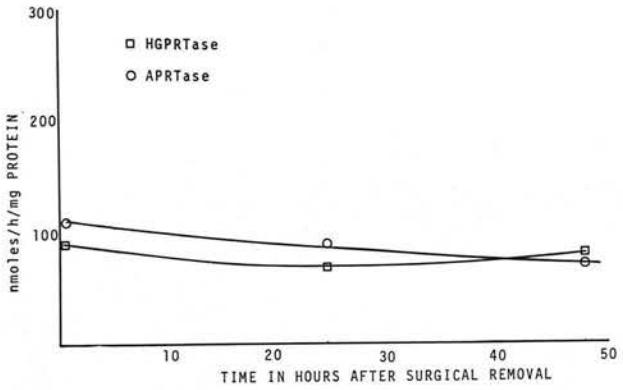
3.4b. Results

Stability of the PRTases The stabilities of the PRTases in surgically removed tissue obtained from Mr F.H. Robarts and Mr W.H. Bisset, the Royal Hospital for Sick Children, are presented in Fig. 11 (liver), Fig. 12 (ileum) and Fig. 13 (appendix). The tissues were stored at $4^{o}C$ and HG and APRTase activities determined immediately after surgery, at twenty-four and at forty-eight hours subsequently. HGPRTase is slightly more stable than APRTase in the tissues studied. A half-life of about fifty hours was calculated for HGPRTase and of thirty for APRTase when stored at $4^{o}C$. Slight variation in half-lives was observed between the tissues studied. The enzymes were less stable in liver than in ileum. From the results obtained there would be a loss of about 15% activity on storage for 24 hours as in many of the necropsy specimens.



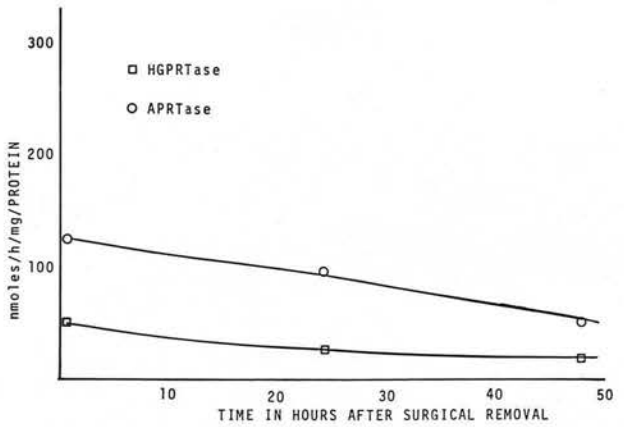
LIVER: STABILITY OF HG and APRTase IN TISSUES STORED AT 4°C

Fig. 11



ILEUM: STABILITY OF HG and APRTase in TISSUES STORED AT 4°C

Fig. 12



APPENDIX: STABILITY OF HG and APRTase in TISSUES STORED AT 40°C

Fig. 13

Foetal tissue PRTase activities Foetal tissue PRTase activities were determined on foetuses made available by Dr F. Cockburn, Simpson Memorial Maternity Pavilion, Edinburgh. Foetal HGPRTase activities are presented in Table XIV and APRTase activities in Table XV. Means and standard errors of the mean are presented where appropriate.

HG and APRTase activities are presented throughout this section in units of nmole of hypoxanthine or adenine transformed per hour per milligram of protein.

Foetal HGPRTase activity is lower than adult tissue levels, however, the relationship of activities between the tissues was generally similar to that found in adults. One exception to this was found in ovarian tissue where activity was not detected in the adult.

Foetal HGPRTase activity was low in liver, 35.3 $\pm$ S.E.M. 6.84, cerebral cortex, 47.6 $\pm$ S.E.M. 16.6, and in basal nucleii, 53 $\pm$ S.E.M. 28.6, when compared to adult levels. Higher than adult HGPRTase activities were found in the cerebellum, 150 and high activity was detected in the foetal ovary. Foetal testis had a high HGPRTase activity,

TABLE XIV

FOETAL TISSUE HGPRTase ACTIVITY

Tissue	Gestation Age Weeks	HGPRTase Activity nmole/h/mg Protein
Liver	8	44
	12	21
	12	9.3
	14	40
	21	54.5
	22	43
Kidney	12	19
Spleen	12	63
Lower trunk	12	39
Upper trunk	12	28.2
Whole brain	8	143
	12	27.7
	14	73
Cerebral cortex	12	30
	21	81
	22	32
Cerebellum	21	150
Basal nuclei	12	0
	21	98
	22	63
Ovary	22	130
Testis	21	203
Placenta	12	23

TABLE XV

FOETAL TISSUE APRTase ACTIVITY

Tissue	Gestation Age Weeks	APRTase Activity nmole/h/mg Protein
Liver	8	30
	12	37
	14	32
	22	31
Lower trunk	12	72
Upper trunk	12	41
Whole brain	8	52
	12	64
	14	61
Cerebral cortex	22	42
Basal nuclei	22	27
Ovary	22	0

203, but considerably lower than adult values.

Placental tissue had low activity, 23, although this organ is metabolically very active in transport of nutrients to the foetus.

Foetal APRTase activity in liver, 32.5 $\pm$ S.E.M. 1.1 was lower than that found in adult liver, but foetal central nervous system had considerably higher APRTase activities, 59 $\pm$ S.E.M. 3.6 than that found in the adult. No APRTase activity was found in foetal ovary.

PRTase activities in tissues from children aged 0 - 2 years The HG and APRTase activities in tissues from children aged 0 - 2 years are presented in Table XVI and Table XVII.

Highest HGPRTase activities were found in the testis and the central nervous system. Considerably higher HGPRTase activities were detected in the cortical grey matter than in the cortical white matter. The basal nuclei had the highest activity found. Lower levels of activity were detected in the medulla, while the cerebellum had very low levels of activity. This contrasts with the high HGPRTase activity in the foetal cerebellum (Table XIV).

Low levels of HGPRTase activity were detected

TABLE XVI

HYPOXANTHINE GUANINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY IN TISSUES FROM CHILDREN AGED 0 - 2 YEARS

Tissue	HGPRTase Activity Mean ± S.E.M. nmole/h/mg Protein	No. of Determinations
Liver	39.8 ± 7.0	11
Kidney	47.2 ±15.9	7
Adrenals	21.0 ±7.2	4
Thymus	53.2 ±13.9	5
Pancreas	19.0 ±9.4	4
Skeletal muscle	12.8 ±3.2	5
Cardiac muscle	37.8 ±9.3	5
Bone marrow	42.3 ±11.9	3
Testis	126.2 ±47.4	4
Cerebral cortex Grey matter	128.4 ±42.1	9
Cerebral cortex White matter	68.2 ±15.9	8
Basal nuclei	157.3 ±22.0	3
Thalamus	81.8 ±19.0	8
Internal capsule	52.7 ±18.8	3
Medulla	75.7 ±18.0	6
Cerebellum	29.3 ±4.3	3

TABLE XVII

ADENINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY IN TISSUES FROM CHILDREN AGED 0 - 2 YEARS

Tissue	APRTase Activity Mean ± S.E.M. nmole/h/mg Protein	No. of Determinations
Liver	88.7 ±38.6	6
Kidney	42.0 ±7.0	3
Thymus	95.6 ±38.4	4
Skeletal muscle	69.2 ±50.7	2
Cardiac muscle	33.2 ±16.7	2
Testis	52.5 ±24.4	4
Cerebral cortex Grey matter	12.7 ±5.5	4
Cerebral cortex White matter	21.0 ±7.7	4
Basal nuclei	5.7 ±2.2	3
Thalamus	12.3 ±6.8	4
Medulla	19.0 ±2.8	3

in the liver, adrenals, kidney, thymus, bone marrow and muscle. Cardiac muscle had higher HGPRTase activity than did skeletal muscle.

APRTase activities in tissues from the 0 - 2 year age group (Table XVII) were highest in liver and thymus.

Skeletal muscle had higher APRTase activity than cardiac muscle. Kidney and testis had low APRTase activity. Central nervous tissues had low APRTase activity, often not detectable in the absence of thymidine triphosphate.

PRTase activities in tissues from children aged 4 - 8 years The HG and APRTase activities in tissues from children aged 4 - 8 years are shown in Table XVIII and Table XIX. Many of the children in this study had been on cytotoxic drugs prior to death.

Liver HGPRTase activity is higher than in the 0 - 2 age group, 78 $\pm$ S.E.M. 26.8 as is the cortical grey matter, 170.7 $\pm$ S.E.M. 34.6, medulla, 157 and thalamus, 136.

Liver APRTase, 122, is increased greatly compared to the 0 - 2 age group, while central nervous system APRTase shows only slight increases

TABLE XVIII

HYPOXANTHINE GUANINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY IN TISSUES FROM CHILDREN AGED 4 - 8 YEARS

Tissue	Age Years	HGPRTase Activity nmole/h/mg Protein
Liver	6	25
	7	103
	8	108
Appendix	4	50
Ileum	4	89
Ovary	7	0
Testis	8	93
Cerebral cortex	6	196
	7	95
	7	138
Grey matter	8	254
Occipital grey	7	105
Occipital white	7	0
Thalamus	6	122
Thalamus	7	145
Medulla	6	157

TABLE XIX

ADENINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY IN TISSUES FROM CHILDREN AGED 4 - 8 YEARS

Tissue	Age Years	APRTase Activity nmole/h/mg Protein
Liver	6	122
Skeletal muscle	6	53
Appendix	4	121
Ileum	4	107
Cerebral cortex Grey matter	6	28
Cerebral cortex White matter	6	35
Thalamus	6	24
Medulla	6	44

in activity.

PRTase activities in tissues from adults aged 60 - 80 years Adult tissue HG and APRTase activities are presented in Table XX and Table XXI, and reproductive tissues in Table XXII.

Liver HGPRTase activity is similar to that found in the 4 - 8 year age group, while cortical grey matter shows a higher activity than that found in the 4 - 8 year age group. Thalamic and medullary HGPRTase are of similar levels. Testicular HGPRTase is higher than that previously detected by a factor of at least three

PRTase activities in reproductive tissues

Reproductive tissues were studied in view of their rapid growth rate and close involvement with nucleic acid biosynthesis.

HGPRTase activity was detected in the myometrium and amnion membrane; activities were not high relative to other tissues (Table XXII). Antenatal diagnosis of the Lesch-Nyhan syndrome depends on the assay of HGPRTase from amniotic material. Low HGPRTase activity was detected in cells from amniotic fluid obtained by amniocentesis and very low activity was detected in the amniotic fluid. No HGPRTase activity

TABLE XX

HYPOXANTHINE GUANINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY IN TISSUES FROM ADULTS AGED 60 - 80 YEARS

Tissue	HGPRTase Activity Mean ±S.E.M. nmole/h/mg Protein	No. of Determinations
Liver	71.3 ±10.9	6
Kidney	42.3 ±15.9	4
Bone marrow	56.7 ±11.9	3
Testis	431.0 ±63.7	5
Cerebral cortex Grey matter	294.0 ±57.8	4
Cerebral cortex White matter	135.3 ±25.0	4
Thalamus	192.7 ±23.7	3
Medulla	142.0 ±5.0	4

TABLE XXI

ADENINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY IN TISSUES FROM ADULTS AGED 60 - 80 YEARS

Tissue	APRTase Activity Mean ±S.E.M. nmole/h/mg Protein	No. of Determinations
Liver	165.2 ±11.7	4
Kidney	28.5 ±12.0	3
Bone marrow	16.4 ±5.8	4
Testis	59.7 ±10.9	4
Cerebral cortex Grey matter	21.1 ±10.7	4
Cerebral cortex White matter	13.0 ±5.5	4
Thalamus	19.4 ±6.8	4
Medulla	30.7 ±17.2	2

TABLE XXII

HYPOXANTHINE GUANINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY IN MATERNAL REPRODUCTIVE TISSUES AND IN SEMINAL FLUID

Tissue	HGPRTase Activity nmole/h/mg Protein
Endometrium	52
Myometrium	29
Amnion membrane	22
Amniotic cells obtained by amniocentesis	11
Amniotic fluid	0.5
Seminal fluid	0
Sperm	0

was detected in homogenised sperm or in seminal fluid.

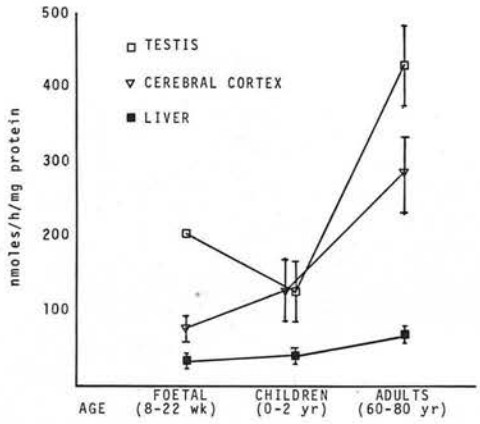
Pattern of developmental changes in tissue PRTase activity The developmental changes in tissue HGPRTase activity are summarised graphically in Fig. 14 and for APRTase in Fig. 16.

The individual points for testis, cerebral cortex, and liver HGPRTase in foetus, children 0 - 2 years of age and adults are shown in scattergrams in Fig. 15

Subcellular distribution of HGPRTase activity The subcellular location of HGPRTase was studied in testis and cerebral cortex where the great majority of activity was located in the soluble cytoplasmic fraction (Table XXIII)

The cellular localisation of HGPRTase in testis Autoradiographic studies carried out on HGPRTase localisation in the testis of a child aged 1 year are shown in Fig. 17.

The HGPRTase activity is localised around the periphery of the tubules and the cell type is therefore the germ cells or the sertoli cells or both (Houssay, 1955).

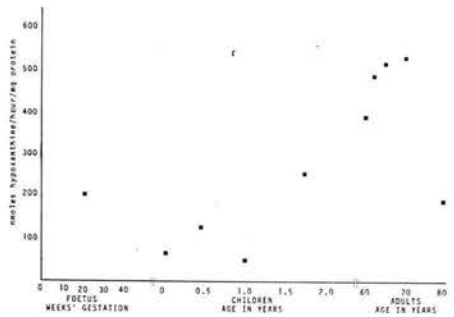


DEVELOPMENTAL CHANGES IN HGPRTase ACTIVITY IN HUMAN TISSUES

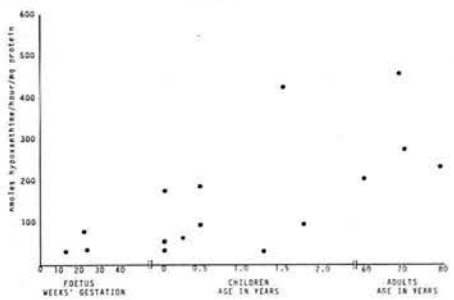
(Points represent Mean ± S.E.M.)

Fig. 14

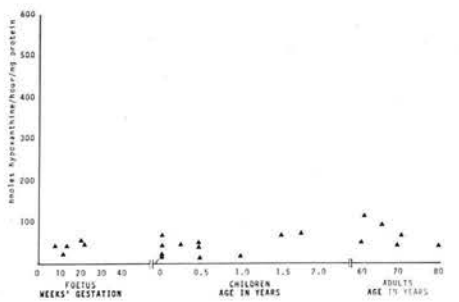
From Tables XIV, XVI and XX



SCATTERGRAM OF INDIVIDUAL RESULTS FOR HGPRTase ACTIVITY FROM HUMAN TESTIS DURING DEVELOPMENT

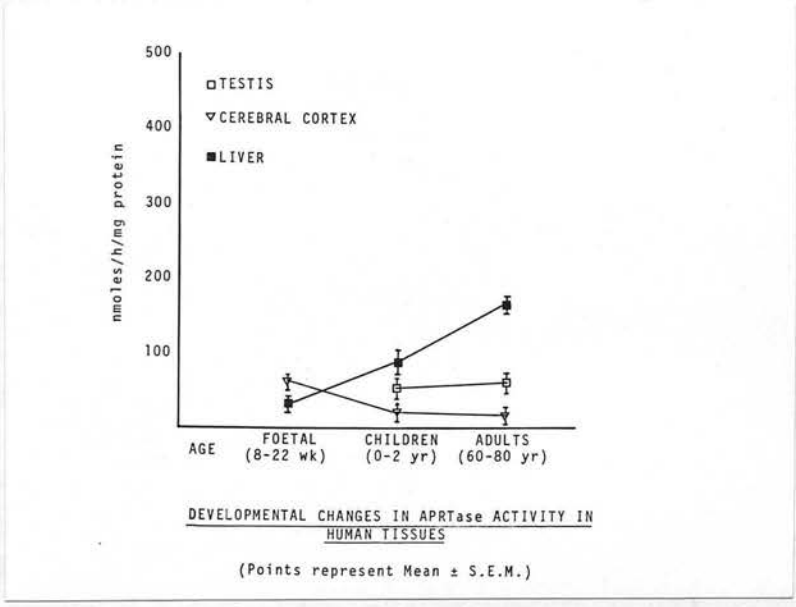


SCATTERGRAM OF INDIVIDUAL RESULTS FOR HGPRTase ACTIVITY FROM HUMAN CEREBRAL CORTEX (GREY MATTER) DURING DEVELOPMENT



SCATTERGRAM OF INDIVIDUAL RESULTS FOR HGPRTase ACTIVITY FROM HUMAN LIVER DURING DEVELOPMENT

Fig. 15



DEVELOPMENTAL CHANGES IN APRTase ACTIVITY IN HUMAN TISSUES

(Points represent Mean ± S.E.M.)

Fig. 16

From Tables XV, XVII and XXI

TABLE XXIII

SUBCELLULAR DISTRIBUTION OF HYPOXANTHINE GUANINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY IN CEREBRAL CORTEX GREY MATTER AND IN TESTIS

Subcell Fraction	HGPRTase Activity nmole/h/mg Protein	
	Testis	Brain
Filtered homogenate	207	107
Frozen and thawed homogenate	197	128
Supernatant S_1 3,300 g	203	113
Supernatant S_2 17,000 g	211	109
High speed supernatant 105,000 g	218	118

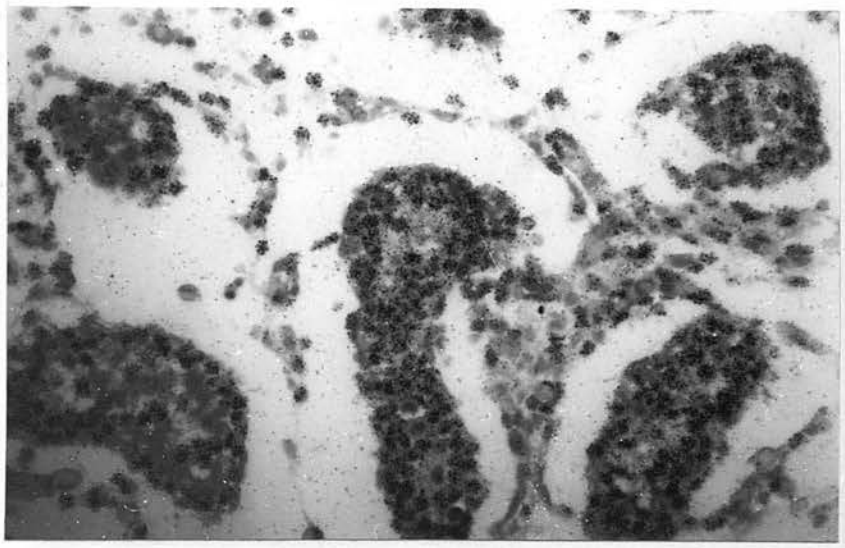


Fig. 17

Autoradiograph of transverse section
of semeniferous tubule

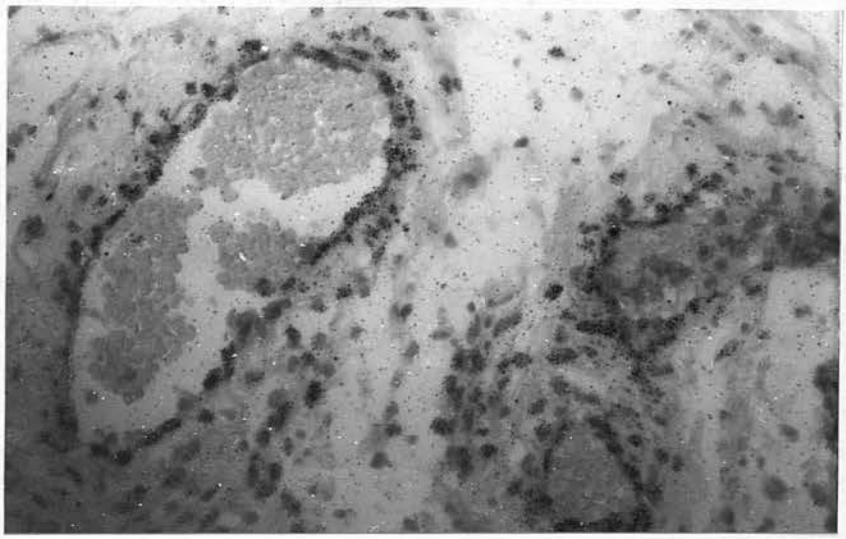


Fig. 18

Autoradiograph of testicular capsule showing a transverse section of a small blood vessel

TABLE XXIV

HYPOXANTHINE GUANINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY IN CULTURED HUMAN CELLS

Cell Line	HGPRTase Activity nmole/h/mg Protein
Human lung fibroblasts H-120-16	19.5
Fibroblasts: genotype XXX	3
Embryonic lung fibroblasts	3
Human amnion cell line transformed with virus SV40 224/168	146
Human amnion cell line derived with subsequent transformations from 224/168 244	170
Human amnion cell line derived from 244. Resistant to AZAGUANINE and thioguanine at 50 µg/ml	2.6

An autoradiograph of the testicular capsule is shown in Fig. 18. This Figure shows HGPRTase activity located around the walls of blood vessels. These findings suggest that the enzyme may be involved in the transport of hypoxanthine from the erythrocyte into tissues; an efficient process in rabbit testis (section 3.7). Blood vessels in the thyroid did not show such activity.

HGPRTase activity in cultured human cells The HGPRTase activity in cell lines from cultured human tissues is shown in Table XXIV.

Most tissues examined had low HGPRTase activities, but cells 224/168 transformed by a virus SV40 had high HGPRTase activity, which was increased in line 244 after further viral transformation. A cell line derived from amnion 244 and resistant to the anti-tumour agent 8-azaguanine had very low HGPRTase activity. This illustrates the role of HGPRTase in the drug activation steps converting many anti-tumour drugs into their active form.

3.4c. Discussion

Studies on human tissues obtained at necropsy are likely to give results for enzyme activities somewhat lower than those obtained from fresh tissue.

This is due to the delay between death and post-mortem. During this period, enzyme synthesis is no longer taking place and existing enzyme protein will be liable to destruction by acid hydrolyses released from lysosomes following tissue death (Slater, 1969). The cause of death may also affect the results obtained, for example, liver disease or cerebral oedema may be expected to affect the measured enzyme activities in liver and brain respectively. These limitations are unfortunately almost inevitable when studying human biochemistry, and should be considered when interpreting the results. However, results from a number of necropsies show the same relative distribution of activities between tissues, and often show a close similarity in the individual figures.

Studies on the stability of the PRTases in fresh, surgically removed tissues stored at 4°C (Figs. 11, 12 and 13) would suggest that both HG and APRTase are relatively stable in the tissues studied, with half-lives of approximately fifty hours and thirty hours respectively. This represents a loss of about 15% activity prior to assays being carried out on tissues obtained at necropsy twenty-four hours after death.

These findings on tissues from children are

consistent with the work of Boyle et al. (1970) who determined the stability of the PRTases in foetal tissues.

Both HGPRTase and APRTase activities were detected in all the human tissues studied with the exception of sperm and seminal fluid. The highest HGPRTase activities were detected in the testis and central nervous system at all ages studied. In contrast, APRTase activity was highest in brain in foetuses, highest in the thymus in children aged 0 - 2 years, and was highest in the liver in children aged 4 - 8 years and in adults.

The changes in PRTase activities observed with growth and development were therefore a rise in HGPRTase activity in testis and central nervous system tissues, and a rise in hepatic APRTase activity. A slight fall in central nervous system tissue APRTase activity was observed.

Tissues such as testis, thymus, and bone marrow probably have a high requirement for nucleotides for DNA synthesis as all of these tissues are 'exporting' cells to other parts of the organism.

Central nervous system tissues have high RNA turnover rates (see Glassman, 1969), and will require a constant supply of nucleotides in the appropriate

5'-nucleotide triphosphates form to sustain RNA synthesis.

The nucleotide pools of these tissues can be replenished by de novo synthesis from PRPP and glutamine to generate IMP, or by the salvage pathways via the PRTase reactions to generate AMP, GMP, or IMP.

As both brain and testis have high HGPRTase activities, it would seem likely that this salvage pathway is important in the maintenance of these tissues nucleotide pools. In addition, Howard et al. (1970) has reported that the brain has a low de novo synthetic capacity for purine nucleotides.

In both testis and brain, nucleotides or their degradation products are not totally lost from the organ. In the testis, approximately twenty per cent of the sperm cells are reabsorbed (Amann, 1969) and the sperm DNA is available for degradation to 3' and 5' AMP and GMP by DNases. The high activities of 5'-nucleotidase and non-specific phosphatases in the male genital tract (Mann, 1964) may be involved in the breakdown of these 3' and 5'-nucleotides via adenosine and inosine to hypoxanthine and via guanosine to guanine. These free bases can then be 'salvaged' to reform 5'-nucleotides. Similarly, in the central

nervous system, messenger RNA after hydrolysis by RNase to 3' and 5' AMP and GMP can give rise to moieties which can be recycled intracellularly by HGPRTase to replenish the cell nucleotide pool.

In contrast to brain and testis, the other two tissues which might be expected to have high nucleotide requirements, bone marrow and thymus, do not have high PRTase activity. Activities have not been determined in adult thymus due to sampling difficulties. A possible explanation is that in thymus and in bone marrow the bulk of the nucleotides is synthesised by the de novo pathway.

It is of interest, however, that some children with the Lesch-Nyhan syndrome have megaloblastic anaemia which is improved by adenine therapy but unresponsive to vitamin B_{12} therapy (Ghadimi et al., 1970; Van der Zee et al., 1970). This, and the work of Lajtha and Vane (1955) would suggest that the bone marrow can 'salvage' exogenous adenine; there are no studies on the salvage of hypoxanthine by the bone marrow. Another possible explanation for the low HGPRTase activities in bone marrow and thymus is that DNA synthesised in these tissues is exported from the organ intracellularly, and is therefore unavailable for recycling and salvage as probably occurs in brain and testis.

Moderately high HGPRTase activity, 130, was detected in a foetal ovary of twenty-two weeks gestation, compared to the low activity, 27, found in the ovary of a four-month old child, while no HGPRTase activity was detected in an adult ovary. It may be relevant that meiosis is initiated in the foetal ovary and the period of rapid cell division in this organ is between the fifteenth and twentieth weeks of foetal life (Franchi, Mandl and Zuckerman, 1962). This may be related to the high HGPRTase activity detected at this time.

To summarise the main developmental changes in the PRTases: HGPRTase activity rises with development in the brain and in the testis, while APRTase activity rises with development in the liver but falls in the cerebral cortex and in all central nervous system tissues.

If the rise in HGPRTase activity in the central nervous system tissues is related to functional changes, this would provide an explanation for the delayed onset of the neurological symptoms in children with the Lesch-Nyhan syndrome.

Developmental aspects of enzymology are important in the care of the premature baby as these determine the vulnerability of the child to influences

which can cause irreversible damage. A study of developmental enzymology is a basis for the diagnosis of genetic disease from amniotic cell tissue culture. The enzymes involved in carbohydrate metabolism have been widely investigated during foetal and neonatal life. Some of these enzymes, for example, fructose,1,-6 diphosphatase, rise throughout foetal and neonatal life, while enzymes such as pyruvate carboxylase rise rapidly after birth at a time when it is required for gluconeogenesis (for a review see Walker, 1971).

The enzymes of purine nucleotide metabolism have not been so widely studied, however a study by Manzoli, Ipata and Wegelin (1971) has been reported on these enzymes in chick embryo brain. Manzoli et al. (1971) found that 5'-nucleotidase reached maximal activity at about twelve to fifteen days foetal age and fell rapidly by twenty-one days at hatching. Different activities and a three day difference in maximal activity were found with AMP and GMP as substrates, and two separate enzymes were suggested (section 3.3c).

Ipata, Manzoli and Wegelin (1970) demonstrated the involvement of adenosine deaminase and inosine: orthophosphate ribosyltransferase in the purine salvage

pathway, and that these enzymes increased rapidly after birth. Similarly, Manzoli et al. (1971) reported that guanine aminohydrolase and guanosine: orthophosphate ribosyltransferase rise rapidly after birth.

These studies and the present work would suggest that the enzymes involved in the recycling and salvage of hypoxanthine and guanine in the central nervous system become important functionally some time after birth and its metabolic role increases throughout childhood.

Although a role for HGPRTase has been proposed, the possible role of APRTase remains unclear. Free adenine is found in the urine, but only at very low levels in tissue fluids (Fink and Adams, 1968). However, no enzymic mechanism is known for the formation of free adenine as adenosine is not a substrate for purine nucleoside phosphorylase. (Huennekens, Nurk and Gabrio, 1956). It is possible that free adenine could arise from AMP via APRTase. Although the equilibrium for APRTase greatly favours AMP formation, high concentrations of AMP and pyrophosphate such as those found when the cell is depleted of ATP could result in the formation of adenine, especially when the adenine formed is removed

by the secretion of urine. This would provide a role for APRTase as a shunt for lowering cellular AMP levels in conjunction with AMP aminohydrolase, EC 3.5.4.6.

In this work, HGPRTase activity in tissue homogenates was determined using hypoxanthine as a substrate. Hypoxanthine has two advantages over guanine as a substrate; it is more soluble at pH 7.4, and, more importantly it is not metabolised - as is guanine - by guanine aminohydrolase EC 3.5.4.3, an enzyme present in many tissues and found at high levels in the central nervous system (Rosenbloom et al., 1967). Hypoxanthine is oxidised to xanthine by xanthine oxidase EC 1.2.3.2, but this enzyme is found only in the liver (Watts et al., 1965).

It is perhaps significant that the 'salvage' pathway found in brain and in testis is HGPRTase; with hypoxanthine as a substrate this will result in the formation of IMP which can be converted into AMP and GMP as required, under the cross-over control of the energy requiring reactions of adenylosuccinate synthetase EC 6.3.4.4 and of IMP dehydrogenase EC 1.2.1.14 (see section 1.6).

The role of hypoxanthine as the principal substrate for HGPRTase in vivo is indicated by studies

on the purine metabolism of xanthinuric patients carried out by Bradford et al. (1968) and by Ayvazian and Skaup (1966). Bradford et al. (1968) showed that while only 6% of the 960 mg hypoxanthine pool was excreted daily, the bulk of the xanthine pool was excreted. Ayvazian and Skaup (1966) showed that adenine was metabolised to hypoxanthine, and only 1% of the label was excreted during the first day, whereas guanine which was metabolised to xanthine by guanine aminohydrolase EC 3.5.4.3, was 63% excreted during the first day of the study.

With the exception of the liver, tissues do not contain appreciable xanthine oxidase activity EC 1.2.3.2 (Watts et al., 1965), while guanine aminohydrolase is widely distributed (Stem et al., 1952) and is present at high levels in the central nervous system (Rosenbloom et al., 1967). In view of the excretion rate of purine bases, and the tissue distribution of the purine catabolising enzymes, it is suggested that hypoxanthine is probably the major substrate for HGPRTase in vivo.

Studies on HGPRTase activity in cultured human cells (Table XXIV) are presented. Low levels of HGPRTase were found in 'normal' fibroblasts, but

fast growing cell lines derived from amnion by viral transformation exhibited high levels of activity. Subsequent transformation further increased HGPRTase activity. A fast growing cell line derived from the amnion line but resistant to 50 µg/ml 8-azaguanine showed relatively low HGPRTase activity little different from that of two slow growing fibroblast lines with 'normal' levels of HGPRTase. An alteration of HGPRTase activity in regenerating rat liver has already been noted (Murray, 1966), and some correction of HGPRTase activities for rate and possibly phase of cellular growth may be necessary before attempting to make any comparisons. This is relevant to the problems of antenatal diagnosis from amnion cell tissue culture.

The resistence of HGPRTase deficient cell lines to 8-azaguanine has been suggested by De Mars (1971) to be an effective method for diagnosing heterozygotes for HGPRTase deficiency. The active form of 8-azaguanine is 8-azaguanine nucleotide, and this is formed *in vivo* by HGPRTase; when HGPRTase is absent this drug is not activated and these cells become resistant to the normal growth inhibitory effects.

3.5 DEVELOPMENTAL CHANGES IN RAT TISSUE HYPOXANTHINE GUANINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY

3.5a. Introduction

The data on developmental changes in human tissue HGPRTase activity (section 3.4) are incomplete because few people die in hospital between infancy and old age. As studies on human enzymology are often dependent on tissues obtained at necropsy, the period between two years and sixty-five years of age has not been adequately sampled. It is at some point during this period that the marked rises in testicular and central nervous system HGPRTase activities occur.

The developmental changes in rat tissue HGPRTase activities were therefore studied in an attempt to determine if the testicular development of HGPRTase occurred largely at puberty and the increase in brain HGPRTase at an earlier age as might reasonably be predicted. It was hoped that the rat would provide a model for the changes expected to be seen in man.

The rats used for this study were the P.V.G. strain developed by Glaxo Research Ltd. for nutritional studies; being a relatively slow growing strain they were especially suitable for developmental studies. The animals were obtained from the animal

house in the Department of Pharmacology, University of Edinburgh. The rats were fed ad libitum on oxoid breeding diet supplemented with brown bread and milk. They were weaned from their mothers at three weeks after birth, and reached reproductive age at about fourteen weeks. Groups of four siblings were killed by asphxyiation under nitrogen in order to minimise damage to the central nervous system. The following tissues were excised within twenty minutes of death: liver, testis, kidney, cerebral cortex, basal nuclei, and brain stem. The tissues were homogenised and the PRTase activity determined as described in section 2.3.

The results are presented as the means and standard error of the means of HGPRTase activity in tissues from four rats from the same litter, generally two males and two females. It is possible that there were small sex differences, but a much larger sample would have been required to prove this and would not have been possible with litter mates. The growth curves for P.V.G. rats for total body weight and for combined testes weight is presented from data supplied by Dr H.E.H. Jones of Glaxo Research Ltd., Greenford, Middlesex.

3.5b. Results

The means and standard errors of the means of tissue HGPRTase activity, from four rats in most instances, are presented in Table XXV. These results are presented graphically in Figs. 19 and 20.

The activity of HGPRTase is seen to rise after birth in all tissues studied. The activity in liver rises after birth until four weeks of age and then falls until nine weeks. After nine weeks the activity increases to maximum level at fifteen weeks. Low activities were detected in sixty-five week rats.

Both cerebral cortex and testis activities rise to a first maximum at two to three weeks, fall slightly between three and four weeks, then rise to maximal activity at fifteen weeks. Low activities of HGPRTase were detected in the cerebral cortex and testis at sixty-five weeks.

The kidney HGPRTase activity also increased after birth until two weeks, fell slightly and had not greatly increased when determined at eighteen weeks.

HGPRTase activity in the basal nuclei was constant between the second and fifth week after birth, increased between the fifth and seventh week,

TABLE XXV

DEVELOPMENTAL CHANGES IN RAT TISSUE HGPRTase ACTIVITY
MEAN ± S.E.M. OF 4 RATS

Age	Liver	Cerebral Cortex	Testis	Brain Stem	Basal Nuclei	Kidney
Neonate	57.5 ± 10.4	24.7 ± 4.5	-	55.7 ± 19.2	-	23
1 week	82.3 ± 12.0	60 ± 14.7	37.3 ± 3.8	50.3 ± 2.2	-	
2 weeks	100.5 ± 17.5	85.5 ± 0.5	72 ± 11.0	77.5 ± 6.5	59.5 ± 15.5	56 ± 5.5
3 weeks	135.2 ± 14.8	65 ± 1.08	88 ± 22	55.8 ± 6.3	54 ± 1.0	-
4 weeks	150.7 ± 20	93.7 ± 22.5	67	53.7 ± 7.3	54.5 ± 8.5	32
5 weeks	118.2 ± 10.1	68.3 ± 9.5	100.5 ± 0.5	53.5 ± 6.6	47 ± 15	-
7 weeks	109 ± 7.0	101 ± 11.1	116 ± 7.0	82.5 ± 6.6	102.5 ± 1.5	-
9 weeks	88.2 ± 2.6	86.7 ± 6.0	103 ± 3.0	66.6 ± 10.8	-	-
15 weeks	174 ± 32	182 ± 31	178 ± 2	143 ± 16	-	-
18 weeks	137	84	-	67	47	51
19 weeks	147 ± 27.0	127.3 ± 5.2	156.5 ± 5.7	129.3 ± 11.8	-	-
19.75 weeks	160	120	-	-	-	-
65 weeks	46 ± 1.5	40 ± 5.5	41.5 ± 1.5	-	32 ± 7.0	-

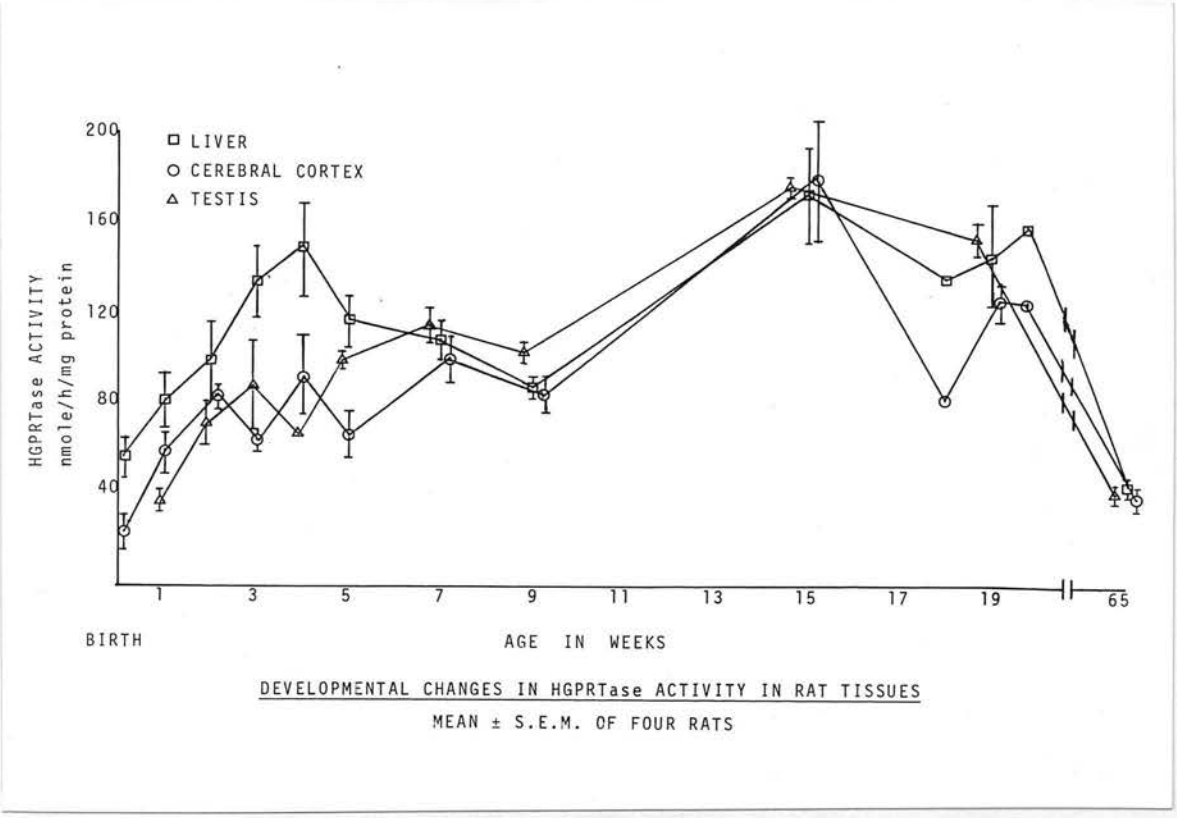
Activities are in nmoles of substrate transformed/h/mg protein

TABLE XXVI

DEVELOPMENTAL CHANGES IN RAT TISSUE APRTase ACTIVITY
MEAN ± S.E.M. OF 4 RATS WHERE APPROPRIATE

Age	Liver	Cerebral Cortex	Testis	Brain Stem	Basal Nuclei	Kidney
15 weeks	21.5 ± 12.3	83.7 ± 6.0	81,64	56	81.5 ± 11.7	179
18 weeks	167	48	-	23	25	200
19.75 weeks	172	38,80	68,54	30	66,49	-

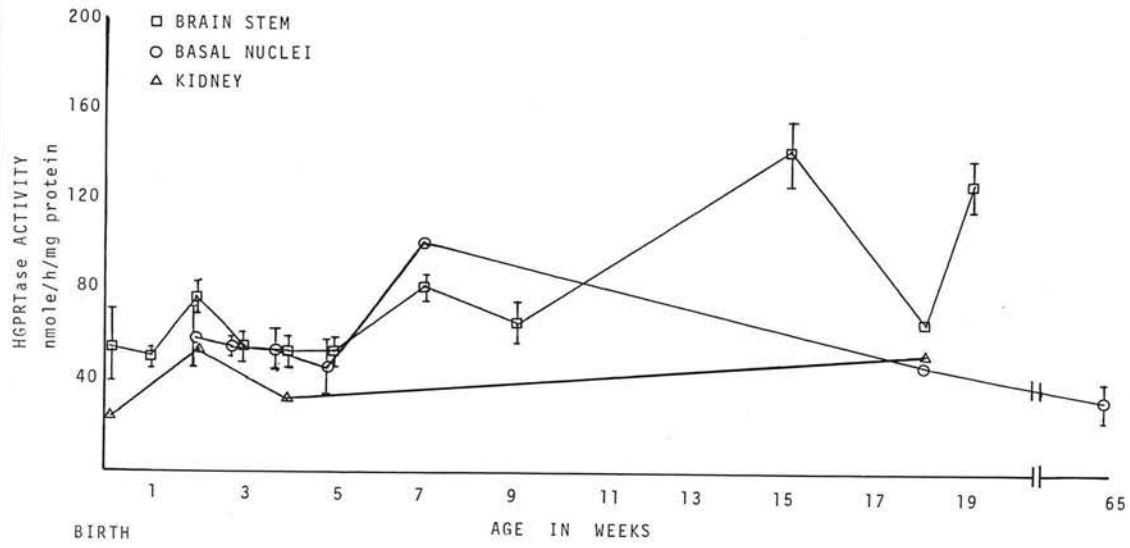
Activities are in nmoles of substrate transformed/h/mg protein



DEVELOPMENTAL CHANGES IN HGPRTase ACTIVITY IN RAT TISSUES

MEAN ± S.E.M. OF FOUR RATS

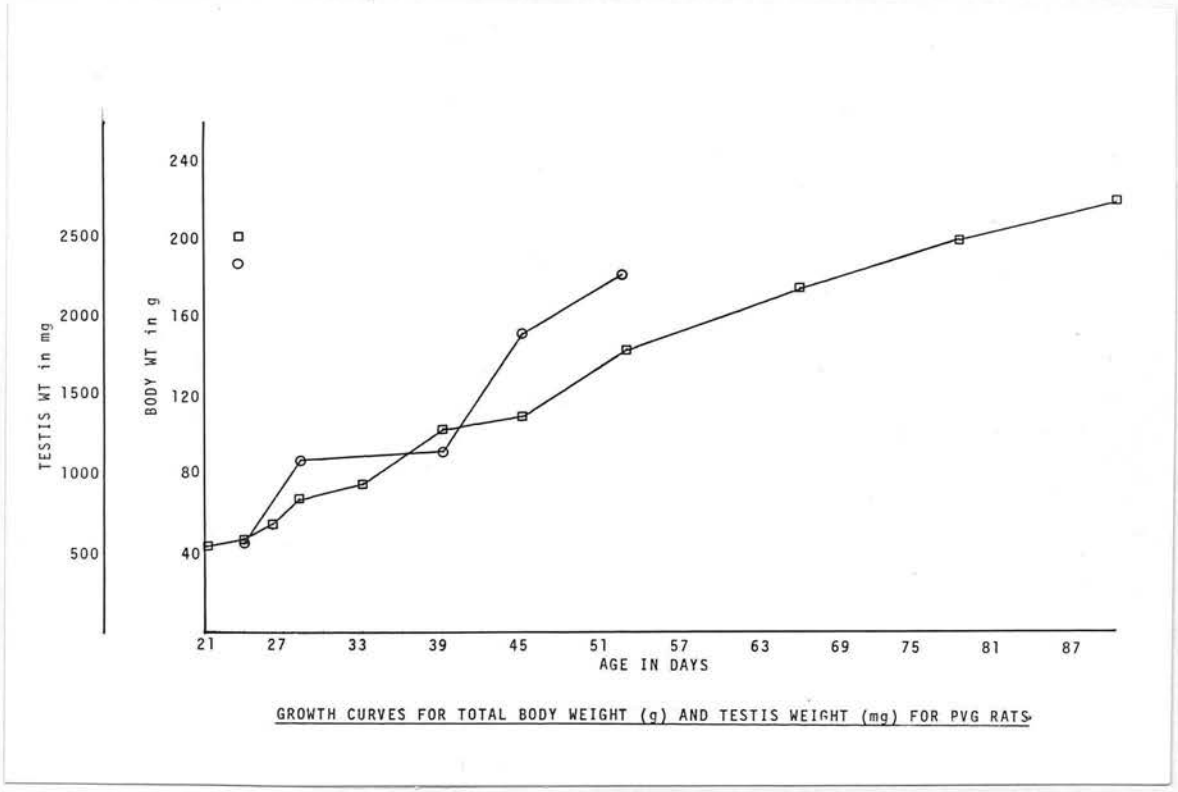
Fig. 19



DEVELOPMENTAL CHANGES IN HGPRTase ACTIVITY IN RAT TISSUES

MEAN ± S.E.M. OF FOUR RATS

Fig. 20



GROWTH CURVES FOR TOTAL BODY WEIGHT (g) AND TESTIS WEIGHT (mg) FOR PVG RATS.

Fig. 21

From data obtained from Dr H.E.H. Jones, Glaxo Research Ltd.

□ - Total body weight

O - Testes weight

and had returned to basal levels by the eighteenth week.

The brain stem differed from all the other tissues studied, in that a slight fall in activity was observed between birth and the first week. This may, however, be an artefact of the small sample size. This tissue showed peaks of activity at the second, seventh and fifteenth weeks.

Some changes in APRTase activities are shown in Table XXVI. Falls in APRTase activity parallel with those in HGPRTase activity, are indicated between the fifteenth and eighteenth weeks, however insufficient figures are available for statistical significance.

Rat erythrocytes had a very low HGPRTase activity of 1.5 nmole/h/mg protein.

Fig. 21 shows the body weight growth curve for P.V.G. rats, along with the growth curve for testis weight, each point being a mean derived from about 10 rats

3.5c. Discussion

The developmental changes in rat tissue HGPRTase activity are complex, and do not follow a simple pattern of increase in activity with age.

In relation to the studies on developmental changes in human tissue HGPRTase activity presented in the previous section, it would appear that the rat may not be a satisfactory model. There are at least two important differences between human and rat tissues with respect to HGPRTase activity. Rat erythrocytes were found to have a very low HGPRTase activity, while Murray (1966) detected no activity. Human erythrocytes have relatively high HGPRTase activity. Also, rat liver contains very high HGPRTase activity with respect to other rat tissues and when compared to human liver.

Further evidence of interspecies differences in the distribution and activities of the PRTases are presented by Krenitsky (1969) who showed that Rhesus monkey skeletal muscle had twice the APRTase activity of monkey liver. Human skeletal muscle has only a small fraction of the APRTase activity of liver (section 3.4; Rosenbloom et al., 1967b). In view of these and other possible differences in the role of the PRTases in different species, the use of animal models may not always be appropriate in the study of human developmental enzymology.

Developmentally, newborn rats are comparable to five to ten day old human neonates (Davison and Dobbing

1966), and the sharp rise in HGPRTase activity observed in several rat tissues during the first week of extrauterine life would correspond to the last weeks of foetal life in human babies.

The tissues studied in this work would appear to be of two groups. The liver, cerebral cortex, and testis, show rapid increases in activity after birth, while the brain stem, basal nuclei, and kidney show smaller increases in activity one week after birth.

A recent study on the development of HGPRTase activity in rat brain by Gutensohn and Guroff (1972) found HGPRTase activities in rat brain comparable to those detected in this study. These authors sampled rats daily during the first three weeks of life and as in this study detected a rapid rise in activity after birth. However, only one determination at seven weeks was subsequently made before the thirteenth week and these authors have concluded that rat brain HGPRTase activity plateaus after three weeks. It would appear that the fluctuations in brain HGPRTase activity have been overlooked due to inadequate sampling, although detailed examination of the data of Gutensohn and Guroff (1972) in fact suggest a slight fall in

activity at twenty and twenty-one days. A final brain HGPRTase activity sample was carried out at twenty-six weeks, at which time the HGPRTase activity was at the 19 - 20 day level. The data presented in the present study show that in old age at sixty-five weeks HGPRTase activity is very low in all the tissues studied.

The tissue distribution of ten week old rat PRTases has been studied by Murray (1966), however, direct comparison with the values obtained in this work is difficult due to this author's choice of 25^{o}C for the assay and also the present finding that the relative tissue HGPRTase activities are time dependent. Murray (1966) found high HGPRTase activity in brain and in bone marrow but detected no activity in the rat erythrocyte. Murray (1966) also noted a rapid rise in PRTase activity in liver occurring one day after partial hepatectomy, and concluded that the physiological significance of the PRTases in rapidly growing tissues was either in the importing of extracellular purines derived from other organs or the conservation of intracellular purines formed by nucleotide degradation.

Murray (1967) also detected increases in PRTase activity in the developing mouse liver and reported

changes in the kinetic constants for PRPP of both HG and APRTase during development.

There are no reported studies on HGPRTase activities on the testis in rats or any other species.

This study shows that testicular HGPRTase activity is related to both morphology and function. The peaks of testicular HGPRTase activity at three and seven weeks (Fig. 19) correspond to infections[1] in the testis growth curve at four and seven weeks (Fig. 21). The maximum testicular HGPRTase activity at fifteen weeks corresponds to the attainment of full reproductive capacity in this strain of rat.

It is not at present possible to relate, in any relatively simple fashion, the changes in HGPRTase activity detected in the central nervous system to functional events.

The fluctuations in HGPRTase catalytic activity may represent increased synthesis of enzyme protein, however, alternative hypotheses are possible..

These include the stabilisation of existing protein by increased substrate levels (see Schimke, 1969) so that catalytic activity increases to meet the increased requirements. Alternatively, the

kinetic constants of HGPRTase could be modified by an allosteric effector, possibly of endocrine origin, thus increasing catalytic rates. Unfortunately, the data available do not allow any conclusion to be made as to which of these mechanisms may be operating

3.6 ATTEMPTS TO DETECT AND CHARACTERISE TISSUE SPECIFIC ISOENZYMES OF HUMAN HYPOXANTHINE:GUANINE PHOSPHO-RIBOSYLTRANSFERASE

3.6a. Introduction

The widely differing tissue activities of HGPRTase, and the developmental changes found to occur with respect to the enzyme's activity would suggest that the role of HGPRTase may vary between tissues. Accordingly, it would be reasonable to expect to find isoenzymes in different tissues with a range of kinetic properties.

The properties of HGPRTase from various tissues at different stages of development were investigated, using the techniques of electrophoresis, thermal denaturation, and enzyme kinetics.

Electrophoresis of HGPRTase and APRTase was carried out using starch gel and cellulose acetate support media as described in section 2.7a. The tissues studied by this method were foetal brain and liver, brain, testis, liver, and erythrocytes from

children, and testis and erythrocytes from adults.

Dialysed homogenates of tissues were treated with sodium dodecyl sulphate and with urea in an attempt to alter the electrophoretic pattern by dissociation of any subunit structure of the HGPRTase protein.

Samples of erythrocyte lysates were heat treated at 80^{o}C for five minutes, and both heat treated and untreated samples were used for electrophoresis.

The specificity of the detection method was confirmed from the identity of both the radiochemical product and some aspects of the enzymic mechanism; the methods used were those described in section 2.7c.

Complementary to the electrophoretic studies, the thermal denaturation rate constants were determined for HGPRTase from various tissues at differing stages of development. The tissues studied were erythrocytes, brain, liver and testis from children and from adults, and adrenals, kidney and spleen from adults only.

Thermal denaturation has been used successfully by Kelley et al. (1969) to demonstrate the existence of both heat labile and heat stable mutants of erythrocyte HGPRTase. The patients studied by Kelley

et al. (1969) had a family history of gout and over-excretion of urinary uric acid. The studies showed that compared with a control population, these families had polymorphic forms of HGPRTase.

The results presented in this section are the specific reaction rate constants for the thermal denaturation of the enzyme protein, measured by the loss of catalytic activity. The treatment used in this work enables the results to be presented numerically and treated statistically. This method is more precise than the visual comparison of the thermal denaturation rate curves.

In spite of the difficulties in interpreting kinetic constants obtained from crude enzyme preparations, apparent Michealis constants (Km) and maximal velocities (Vmax) were determined for hypoxanthine in the HGPRTase system from erythrocytes, liver, cerebral cortex, thalamus, and testis.

Altered kinetic constants have been detected for human erythrocyte HGPRTase of differing heat stabilities (Henderson et al., 1968). Murray (1967) has reported changes in kinetic constants for APRTase during developmental changes in APRTase activity in mouse liver.

The kinetic constants presented were obtained from crude tissue homogenates by determining the initial velocity at a series of substrate concentrations. The values were obtained from an Olivetti Programma 101 programmed to calculate the constants according to the methods of both Lineweaver-Burk and of Hofstee-Eadie (Dixon and Webb, 1967).

3.6b. Results

The electrophoretic pattern obtained for HGPRTase was similar in all tissues studied and at all the ages studied.

The electrophoresis of the PRTases is shown in Fig. 22. The bars of the histograms represent the C.P.M. per centimetre of D.E.A.E. paper used as described in section 2.7b.

Electrophoresis was carried out using both cellulose acetate and starch gel as a support medium, and identical results were obtained from both media.

The electrophoretic separation obtained for HGPRTase and APRTase is presented (Fig. 22). Four bands of activity were detected for the PRTases in every tissue examined.

Initially, only three bands of activity were

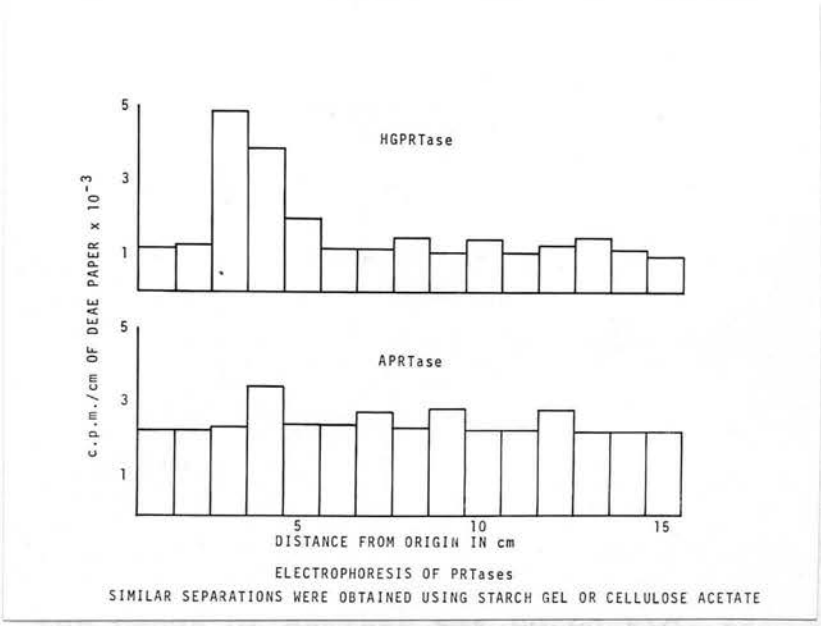

HGPRTase
APRTase
c.p.m./cm OF DEAE PAPER x 10^{-3}
5
3
1
5
10
15
DISTANCE FROM ORIGIN IN cm
ELECTROPHORESIS OF PRTases
SIMILAR SEPARATIONS WERE OBTAINED USING STARCH GEL OR CELLULOSE ACETATE

Fig. 22

located, but by allowing the current to flow for a shorter period of time the fourth band was detected on the thirteenth or fourteenth centimetre from the origin.

In the case of HGPRTase, the peak nearest the origin (cathode) was always the major band and located at the third and fourth centimetre. The second, third and fourth bands, in order of increasing mobility were located at the eighth, tenth and thirteenth centimetres from the origin respectively. Minor variations of one centimetre were observed to occur between the positions of bands when electrophoresis was carried out in triplicate.

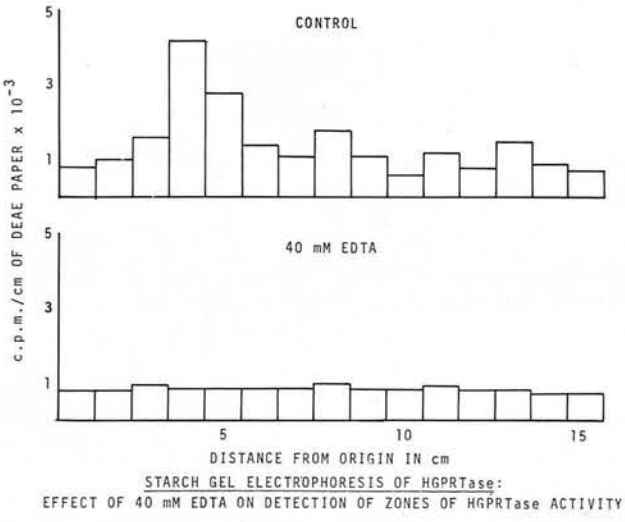
A more regular distribution of activity among the bands of APRTase was noted Fig. 22. The slower migrating band of activity located nearest to the origin represented more catalytic activity than the minor bands, however, the difference between major and minor bands was not so marked as that found with HGPRTase. The bands of APRTase activity were located in the fourth, seventh, ninth and twelfth centimetres from the origin. The distance between the faster migrating minor bands was the same for both HG and APRTase. In order of mobilities, one centimetre separated the second and third bands,

and two centimetres separated the third and fourth bands.

When this work was carried out, only one band of activity had been reported for electrophoresis of both HG and APRTase. It was therefore essential to check that the bands of activity were due to PRTase activity, and that the radiochemical products were in fact 5'-mononucleotides. Following electrophoresis, the bands of activity were located using a medium containing E.D.T.A., a non-specific inhibitor of the PRTases, and also a medium containing 6-mercaptopurine, a specific inhibitor of HGPRTase. When compared to a control, these showed significantly reduced activity (Figs. 23 and 24).

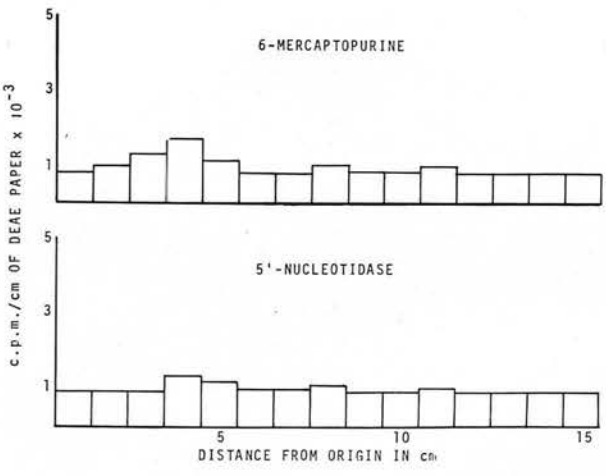
Following localisation of the bands of PRTase activity, the D.E.A.E. strip was halved longitudinally, and one half treated with 5'-nucleotidase EC 3.1.3.5, and enzyme specific for the hydrolysis of the 5' phosphate group from mononucleotides. Compared to the control, this showed (Fig. 24) a significant reduction in activity.

These results would indicate that the enzymes involved are in fact the PRTases, and that the radiochemical products being measured are



STARCH GEL ELECTROPHORESIS OF HGPRTase:
EFFECT OF 40 mM EDTA ON DETECTION OF ZONES OF HGPRTase ACTIVITY

Fig. 23



EFFECTS OF 6-MERCAPTOPURINE AND OF 5'-NUCLEOTIDASE TREATMENT ON DETECTION OF ZONES OF HGPRTase ACTIVITY

Fig. 24

5'-mononucleotides.

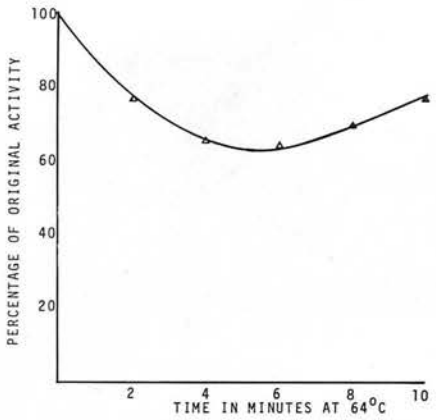
Thermal denaturation studies Thermal denaturation studies on HGPRTase carried out at 55°C and 64°C gave results as shown in Fig. 25.

After initial loss of catalytic activity, a subsequent increase in activity was detected. This phenomenon of thermal reactivation is not fully understood.

The type of curve obtained for thermal denaturation of HGPRTase at 80°C is presented in Fig. 26.

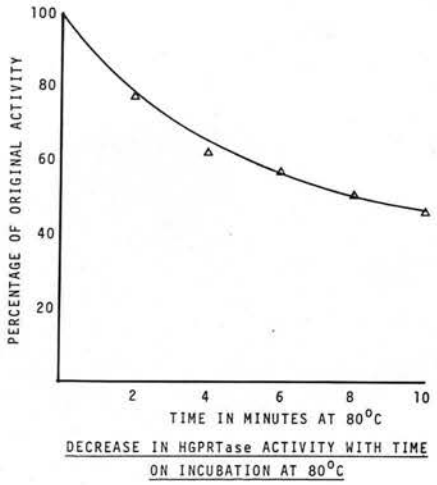
This curve (Fig. 26) can be transformed into a linear plot by the use of logarithims, for the theoretical treatment (section 2.8), and the results obtained are shown in Fig. 27.

The thermal denaturation rate constants (k) derived as described in section 2.8 for human tissue HGPRTase, are presented in Table XXVII. The kidney, spleen, adrenals, and cerebral cortex were from adults, while the erythrocyte samples were from three children and two adults, the liver from four children and one adult, and the testis from three children and one adult. When tissues from both children and adults were studied, no marked difference was detected in the values obtained.



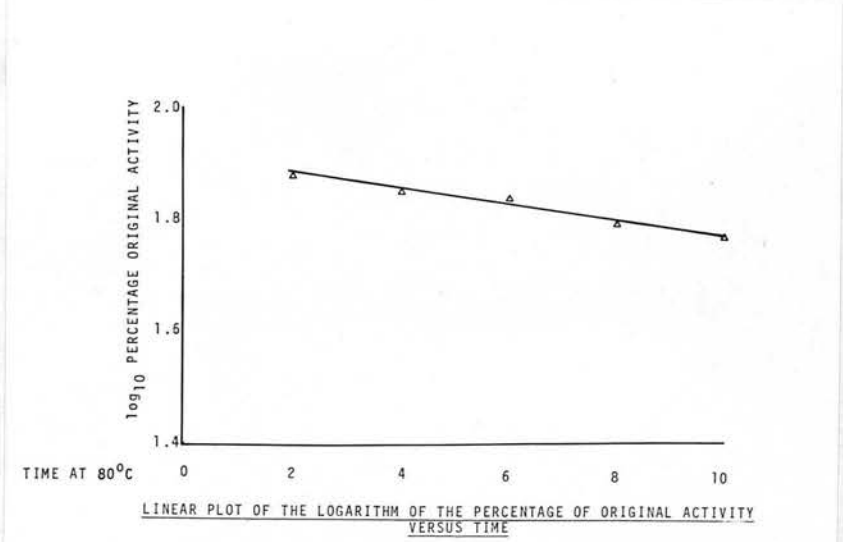
THERMAL DENATURATION AND SUBSEQUENT
THERMAL REACTIVATION OF HGPRTase

Fig. 25



DECREASE IN HGPRTase ACTIVITY WITH TIME ON INCUBATION AT 80°C

Fig. 26



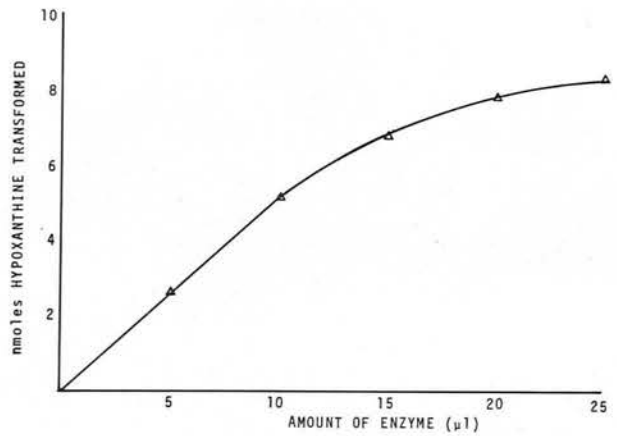
LINEAR PLOT OF THE LOGARITHM OF THE PERCENTAGE OF ORIGINAL ACTIVITY VERSUS TIME

Fig. 27

TABLE XXVII

THERMAL DENATURATION RATE CONSTANTS FOR HYPOXANTHINE GUANINE PHOSPHORIBOSYL-TRANSFERASE FROM HUMAN TISSUES

Tissue	No. of Determinations	Rate Constant (Min^{-1}) Mean ± Standard Deviation
Erythrocytes	5	0.II46 ± 0.006
Liver	5	0.II22 ± 0.035
Testis	4	0.05I5 ± 0.0007
Cerebral cortex	2	0.0855 ± 0.009
Basal nucleus	1	0.064
Kidney	2	0.II0
Spleen	1	0.III
Adrenal	1	0.052



SUBSTRATE TRANSFORMED WITH INCREASE IN AMOUNT OF ENZYME (CEREBRAL CORTEX)

Fig. 28

TABLE XXVIII

APPARENT MICHEALIS CONSTANTS AND MAXIMAL VELOCITIES FOR HYPOXANTHINE GUANINE PHOSPHORIBOSYLTRANSFERASE FROM HUMAN TISSUES WITH RESPECT TO HYPOXANTHINE

Tissue	No. of Determinations	Lineweaver-Burk		Hofstee-Eadie	
		Km ± SD*	Vmax ± SD*	Km ± S.D.*	Vmax ± SD*
Erythrocytes	8	12.2 ± 2.6	29.4 ± 3.1	8.4 ± 2.25	26.3 ± 2.6
Liver	10	8.5 ± 4.9	40.5 ± 30.2	4.9 ± 0.94	30.0 ± 24.8
Cerebral cortex	4	5.3 ± 5.0	20.0 ± 14.1	5.2 ± 4.3	21.0 ± 14.0
Thalamus	4	15.5 ± 13.1	73.5 ± 38.8	8.7 ± 5.5	52.0 ± 5.6
Testis	4	6.7 ± 1.84	6.31 ± 1.77	8.2 ± 2.2	7.21 ± 1.9

* Kms in μM, Vmax in μM/min.

Enzyme kinetics Using tissue homogenates from cerebral cortex, the mass of substrate hypoxanthine transformed was not a linear function of the volume of enzyme used (Fig. 28). Similar results have been obtained by Murray (1971) using brain homogenates. The other tissues studied produced linear plots of substrate transformed versus volume of enzyme added.

The means $\pm$ S.D. of the apparent Kms and Vmax as determined by the methods of Lineweaver-Burk and Hofstee-Eadie are presented for the tissues studied in Table XXVIII. All estimates were prepared in duplicate, thus the number of tissue samples used was half the number of determinations. All the tissues were obtained from children with the exception of one adult testis sample which did not show a markedly different result.

3.6c Discussion

Electrophoretic studies The electrophoretic detection methods used in this study represent a considerable improvement on methods previously used. Prior studies on the electrophoretic properties of HGPRTase using starch gel (Kelley et al., 1969; Kaloustain et al., 1969) detected only one band of HGPRTase activity. In this work, four zones of

HGPRTase activity were detected in all tissues studied. Similarly, four zones of APRTase activity were located.

The identity of the radioactive products were shown to be nucleotide monophosphates by hydrolysis by 5'-nucleotidase, and the enzymes forming this product shown to be HGPRTases by their inhibition by E.D.T.A. and 6-mercaptopurine.

The electrophoretic detection method was found to be semi-quantitative; doubling of the sample loaded onto the electrophoresis support medium produced an approximate doubling of peak height.

Recently, Bakay and Nyhan (1971) using a high resolution polyacrylamide gel slicing technique, have also demonstrated the existence of four bands of activity for normal HGPRTase, although only one band of APRTase activity was reported.

HGPRTase has been purified to homogeneity by Arnold and Kelley (1971), and while only one band of activity was eluted after chromatography on D.E.A.E. cellulose, three bands of activity were detected after isoelectric focusing.

The three bands obtained on isoelectric focusing migrated at different rates in polyacrylamide

disc gel electrophoresis, however, a mixture of the three bands from the isoelectric focusing column migrated in polyacrylamide gel electrophoresis as a single band to a position identical with the isoenzyme band of intermediate mobility. All three of the isoenzyme bands were immunologically identical and all had molecular weights of 34,000 $\pm$ 4,000. No sialic acid residues were detected on any of the three isoenzymes.

In the present study, treatment of the tissue homogenates with 1 M urea or 10% sodium dodecyl sulphate, or heat treatment at 80^{o}C had no detectable effects on the electrophoretic properties of HGPRTase.

It should be noted that although no alteration of the major peak was detected after treatment with 10% S.D.S., this compound was not included in the gel buffer and the unaltered electrophoretic pattern could be due to rapid reassociation.

As a single mutational event results in the complete deficiency of HGPRTase, the existence of multiple forms of HGPRTase from more than one gene is unlikely.

The isoenzymes detected in this and other studies could arise from either post-transcriptional

alterations to the enzyme protein, or alternatively, be due to dissociated subunits of a polymeric protein.

In view of the immunological similarities in the subunits, the identical molecular weights, the reassociations detected (Arnold and Kelley, 1971) and the fact that the slowest migrating band found in this study was always the major band, the evidence available would suggest a polymeric protein, in equilibrium with catalytically competent subunits.

No differences in electrophoretic properties were detected in HGPRTases from different tissues, but the presence of a complex polymeric protein may suggest different associative and regulatory properties in individual tissues.

Thermal denaturation studies Thermal denaturation studies of enzymes can detect small differences in protein structure. London, Wigler and Hudson (1954) studying prostatic acid phosphatase showed that the rate of protein denaturation, as measured by loss of catalytic activity, was a sensitive function of buffer anion, buffer pH, and temperature. Changes of 0.1 pH unit or of 1^{o}C were reflected in changes in the denaturation rate.

Thermal denaturation of HGPRTase carried out at 50 and 64°C showed initial loss of activity and subsequent thermal reactivation. This phenomenon has been reported (Jack, 1971) but is not fully understood.

The rate constants derived and presented in Table XXVII were highly reproducible in erythrocytes and testis, but the results from liver, cerebral cortex and kidney were more variable.

The denaturation rate constant for testicular HGPRTase was significantly different from the mean of all other tissues by the Student t-test $0.02 < P < 0.05$. However, the denaturation rate constant for the adrenal HGPRTase was similar to that for the testis. It is possible that in these two functionally similar tissues with high hexose monophosphate shunt activities and possible high ribose-5-phosphate generating capacity (Mayes, 1967), high PRPP levels may be present stabilising the enzyme (Greene et al., 1970). This does not exclude the possibility that an altered HGPRTase may be present.

APRTase of differing heat stabilities has been reported in sections of rat jejunal villi from the tip to crypt (Earl Balis, Brown and Cappucino, 1971)

and two enzyme forms are suggested, a heat labile and a heat stable form, and that the heat labile form was short-lived *in vivo*.

Kinetic studies Apparent Michealis constants for erythrocyte HGPRTase with hypoxanthine as a substrate have been reported by Henderson (1968). Similar values have been obtained in this study. The apparent Kms for erythrocyte and testis HGPRTase were reproducible, but a wider range of results was obtained when determining these constants for liver, cerebral cortex, and thalamus HGPRTase (Table XXVIII).

Due to the possible presence of enzyme systems other than HGPRTase metabolising either substrate or product, both at very low concentrations, kinetic constants must be interpreted with caution. However, the apparent Km values calculated by the method of Hofstee-Eadie yielded more reproducible results than the method of Lineweaver-Burk, and, under the condition of assay showed a reasonable agreement between the values obtained from the different tissues.

The different denaturation rate constants for the testis would suggest the possibility of a tissue specific form of HGPRTase localised in the testis,

although the possibility of substrate stabilisation has already been indicated.
As the electrophoretic and other evidence suggests that HGPRTase is a polymeric protein, the possibility of tissue specific subunit interactions should be considered, as subunit interactions have been reported by Bakay et al. (1972) to alter the apparent activity of HGPRTase. The phenomenon of protein-protein interactions has been recognised as a major factor in the regulation of enzyme activity in vivo (Frieden, 1971).

3.7 STUDIES ON THE TRANSPORT OF PURINES BY ERYTHROCYTES

3.7a. Introduction

The role of HGPRTase in the purine metabolism of the whole animal and the consequences of its deficiency in the pathology of the Lesch-Nyhan syndrome are less well understood than is its role in the cell.

It has been felt for some time that there might be two types of tissue, one exporting purines, the other importing purines. In 1956 Smellie et al. reported that [^{14}C] formate was incorporated into nucleic acid bases in rabbit bone marrow and

tumour cells, and they suggested that some tissues might be dependent on exogenously supplied purines.

The work of Lajtha and Vane (1958) showed that the bone marrow depended on the liver for its purine supply and many groups of workers have since suggested that the liver may act as a purine exporting tissue.

Liver contains high activities of PRPP amidotransferase - the rate limiting enzyme in de novo purine biosynthesis (Caskey, Ashton and Wyngaarden, 1964) - and has been shown by Howard, Kerson and Appel (1970) to have four to five times the capacity of brain tissue for de novo purine biosynthesis.

Transport of purines between tissues would appear to be a function of the erythrocytes (Henderson and Le Page, 1959) and, as these cells cannot synthesise purines de novo (Lowry and Williams, 1960), any turnover of purine nucleotides in the erythrocyte must result from the entry and release of purines from these cells. Murray (1971) has calculated that for a 70 kg man there is an erythrocyte nucleotide turnover of 6 mg of purine base per kg body weight every twenty-four hours.

Pritchard, Chavez-Peon and Berlin (1971) reported that rat liver prelabelled with adenine-[8-^{14}C] loses label to the non-hepatic tissues, but that erythrocytes maintain labelled hypoxanthine and guanine for six days despite the short turnover time of nine hours for the erythrocyte nucleotide pool.

Hypoxanthine has been shown to be the major purine released from human red cells (Jorgensen and Poulsen, 1955) and Hershko et al. (1967) have reported that hypoxanthine is the major purine released from rabbit red cells irrespective of the purine incorporated.

As the uptake of hypoxanthine by erythrocytes is dependent on a catalytically competent HGPRTase (Sperling et al., 1971), this work was carried out to determine if the uptake of hypoxanthine by tissues from rabbit erythrocytes in vivo was related to the tissue HGPRTase activity.

Results are presented showing the uptake of radioactive hypoxanthine by human erythrocytes, incubated as described in section 2.12a. The PRTase activities found in rabbit tissues are presented along with the uptake of labelled purine from the prelabelled erythrocytes by these tissues.

3.7b. Results

The uptake of hypoxanthine-[8-^{14}C] by human erythrocytes is shown in Fig. 29 and shows a linear relationship with time after thirty minutes. The cause of the lag phase in the first thirty minutes is unknown.

Prior to studying the transportation of purines by rabbit erythrocytes, the tissue PRTase activities from the strain of rabbits was determined. These activities are presented in Table XXIX and the figures represent the means of duplicate determinations on tissues from two adult rabbits.

Highest HGPRTase activity was found in the testis, and high activity was also found in the brain. Lower activity was detected in the liver and erythrocyte, and very little activity was detected in the kidney and muscle.

APRTase activity was high in kidney and in liver, low activities being detected in other tissues.

The tissue uptake of radioactive purines from erythrocytes *in vivo* is shown in Table XXX. The fractions examined for radioactivity were the perchloric acid soluble nucleotide fraction, the

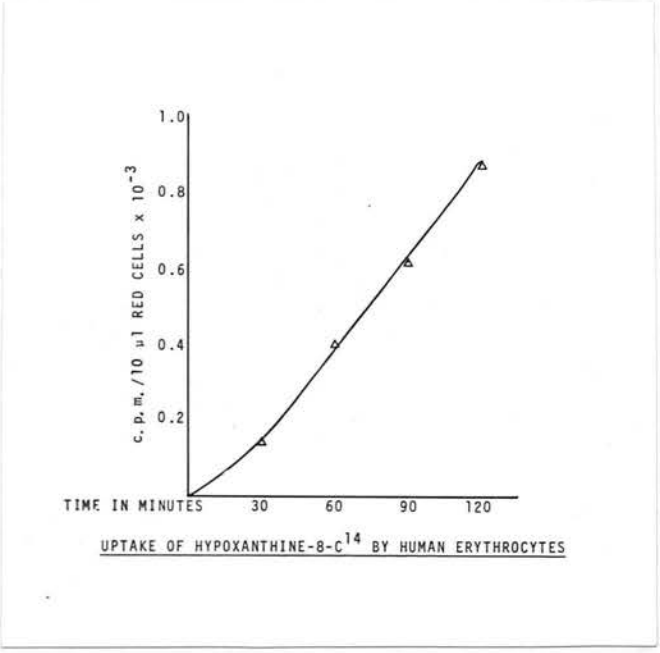

1.0
0.8
0.6
0.4
0.2
c. p. m. / 10 µl RED CELLS x 10^{-3}
TIME IN MINUTES
30
60
90
120
UPTAKE OF HYPOXANTHINE-8-C^{14} BY HUMAN ERYTHROCYTES

Fig. 29

TISSUE	HGPRTase nmole/h/mg/protein	APRTase nmole/h/mg protein
Brain	230	57
Kidney	15	133
Liver	86	92
Muscle	14	23
Testis	290	61
Erythrocyte	125	-

TABLE OF PURINE RIBOSYLTRANSFERASE ACTIVITIES IN RABBIT TISSUES

Table XXIX

TABLE XXX

TISSUE UPTAKE OF RADIOACTIVE PURINES FROM RABBIT ERYTHROCYTES IN VIVO

Fraction	Tissue	Exp. 1		Exp. 2		Exp. 3		Exp. 4	
		1	2	1	2	1	2	1	2
Perchloric acid Soluble	Brain	40	513	72	595	144	910	51	425
	Kidney	71	910	130	1070	160	1010	104	870
	Liver	95	1220	200	1650	164	1040	190	1585
	Muscle	27	346	82	677	136	860	45	375
	Testes	38	487	91	752	176	1110	212	1770
RNA	Brain	1.9	24.4	5.2	43	11.5	73	60.5	505
	Kidney	2.4	30.8	19.5	161	9.7	61.3	29.7	248
	Liver	1.7	21.8	11.5	95	4.9	31	17.1	143
	Muscle	5.7	73.1	6.5	53.7	12.5	79	29.5	246
	Testes	2.5	31.6	4.0	33.1	5.0	31.6	10.6	89
DNA	Brain	1.5	19.2	2.0	16.5	6.1	38.6	2.6	28
	Kidney	3.1	39.7	3.5	28.9	9.5	60	3.5	29.4
	Liver	6.1	78.2	2.0	16.5	7.2	45.5	2.6	22
	Muscle	1.3	16.6	1.5	12.4	2.4	15.2	6.2	52
	Testes	2.8	35.9	1.5	12.4	3.6	22.8	1.8	14.6

Column 1 Disintegration per minute per gram wet weight of tissue $\times 10^2$

Column 2 $\frac{\text{Radioactivity per gram wet weight of tissue}}{\text{Radioactivity administered to whole animal}} \times 10^6$

RNA fraction, and the DNA fraction. These were isolated as described in section 2.12 from brain, kidney, liver, muscle and testis.

The figures represent the disintegrations per minute per gram wet weight of tissue in column one, and this figure as a fraction of the total dose of radioactivity administered x 10^6 in column two.

The figures show that significant uptake of purines by tissues from erythrocytes has taken place. The perchloric acid soluble nucleotide fraction contained the majority of the radioactivity, lower levels being detected in the RNA and DNA fractions.

An approximate measure of the requirements for nucleotides for nucleic acid synthesis in these tissues is given by the ratio of the radioactivity in the perchloric acid soluble fraction to the total radioactivity in the RNA and DNA fractions. This is presented in Table XXXI.

These figures would suggest that brain and muscle have a high requirement for nucleotides for nucleic acid synthesis.

The means $\pm$ S.E.M. for experiments 2, 3, and 4 of uptake of ^{14}C purine are presented in Table XXXII.

TABLE XXXI

THE RATIO OF THE P.C.A. SOLUBLE RADIOACTIVITY TO THE RADIOACTIVITY IN THE RNA AND DNA FRACTIONS FROM EXPERIMENTS 2, 3 and 4

Tissue	P.C.A.-soluble D.P.M./g Tissue	(RNA + DNA) D.P.M./g Tissue	$\frac{\text{P.C.A.}}{\text{(RNA + DNA)}}$
Brain	643	234	2.7
Kidney	983	195	5.0
Liver	1425	116	12.3
Muscle	637	152	4.2
Testis	1201	67	18.0

TISSUE UPTAKE OF RADIOACTIVE PURINES FROM RABBIT ERYTHROCYTES *in vivo*

Erythrocytes were labelled *in vitro*, administered to the donor rabbit, and uptake into tissues measured after 45 hours. Results are expressed as Means ± S.E.M. from 3 experiments and are calculated as

$$\frac{\text{radioactivity per gram wet weight of tissue}}{\text{radioactivity administered to the whole animal}} \times 10^6$$

TISSUE	Uptake of ^{14}C purine (Fraction of dose per gram wet weight of tissue, calculated as above)		
	PCA-soluble	RNA	DNA
Brain	643 ± 142	207 ± 149	27 ± 6
Kidney	983 ± 59	156 ± 54	39 ± 10
Liver	1425 ± 190	89 ± 32	27 ± 8
Skeletal Muscle	637 ± 141	126 ± 60	26 ± 12
Testis	1201 ± 305	51 ± 19	16 ± 3

Table XXXII

TABLE XXXIII

TABLE OF EXPERIMENTAL PROCEDURES AND RESULTS FOR TISSUE UPTAKE OF PURINE FROM RABBIT ERYTHROCYTES IN VIVO

Parameter	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Rabbit total body weight Kg.	4.5	3.5	2.4	3.4
Dose of radioactive hypoxanthine incorporated into erythrocytes μCi	3.5	5.45	7.1	5.4
Duration of *in vivo* incubation in hours	4	45	45	45
Dose of allopurinol mg/kg	0	4	6	10
Percentage of radioactive dose detected in tissues studied	33	57	48	59
Percentage of radioactive dose in urine	22	34	9.59*	17.3
Percentage of radioactive dose remaining in erythrocytes	41	$>$ 0.1	0	0.07
Percentage of radioactive dose unaccounted for	4	9	42*	24

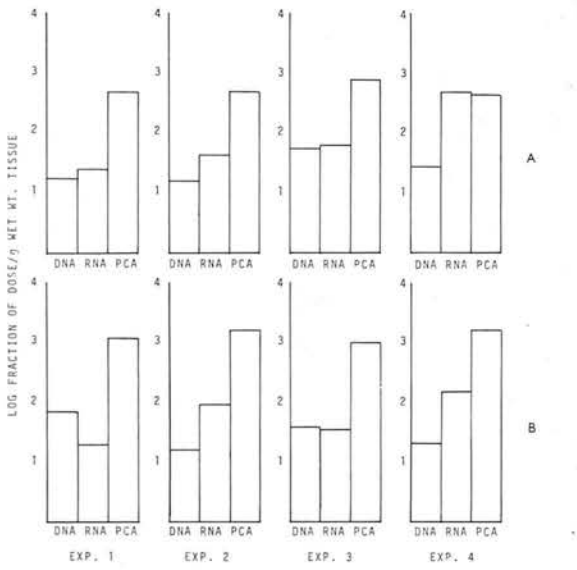
* Due to the rabbit urinating during sacrifice, an incomplete urine collection was obtained

A similar pattern was obtained when the incorporation was related to the RNA and DNA extracted.

The results are also presented graphically in Fig. 30 where the logarithm of the fraction of the radioactive dose is shown for the DNA, RNA, and PCA soluble fractions in brain and in liver. Similar results were obtained in kidney, muscle, and testis.

The data on the animals used and the gross distribution of the radioactivity in the animals for experiments 1 to 4 are presented in Table XXXIII.

The first experiment was carried out with a short *in vivo* incubation, and no allopurinol was given. In experiments 2, 3 and 4, the duration of the *in vivo* incubation was 45 h, and increasing doses of allopurinol were given. About half of the radioactivity administered in the erythrocytes was incorporated into the tissues studied, the bulk of the remainder being in the urine despite the administration of allopurinol. A large proportion of radioactivity was found in the erythrocytes in experiment 1 with the short incubation period, but on increasing the time of *in vivo* incubation the radioactivity remaining in



FRACTION OF DOSE OF HYPOXANTHINE-8-C^{14}/g WET WEIGHT OF TISSUE INCORPORATED INTO DNA, RNA, and PCA SOLUBLE FRACTIONS

A BRAIN

B LIVER

Fig. 30

the erythrocyte fell to nearly zero.

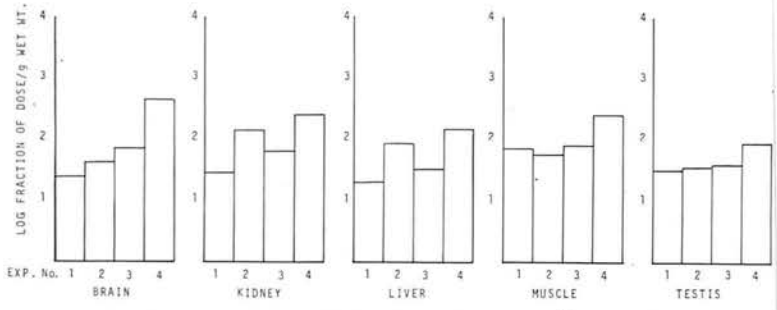
The radioactivity unaccounted for was probably distributed in the tissues not studied.

The urine was examined to determine the source of the radioactivity using a cellulose thin-layer system with a solvent of 1 M ammonium acetate pH 7.0. No radioactivity was detected in spots corresponding to hypoxanthine, xanthine or urate. The urinary radioactive moiety had an Rf value of 0.81 in this system (Table XXXIV) and was probably allantoin or allantoic acid.

The effects of increasing dosage of allopurinol or incorporation of hypoxanthine-[8-^{14}C] into RNA is shown in Fig. 31. The logarithm of the fraction of the radioactive dose incorporated into tissue RNA is shown for experiments 1, 2, 3, and 4.

The histogram shows an increased percentage incorporation as the dose of allopurinol was increased.

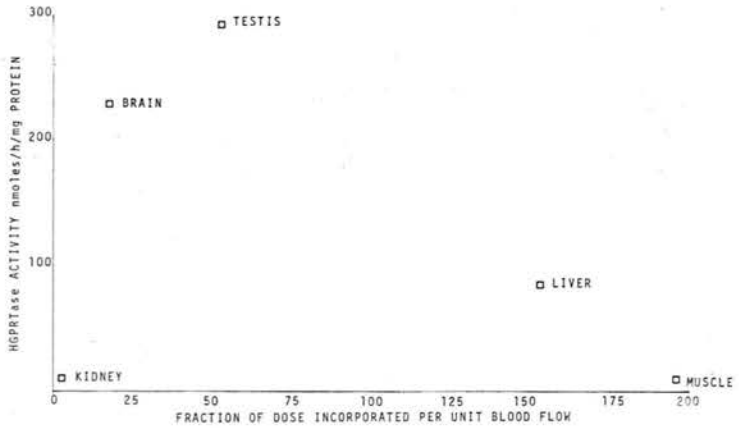
The relationship between tissue HGPRTase activity and fraction of radioactive dose incorporated per unit blood flow is presented in Fig. 32. The fraction of radioactive dose



EFFECT OF ALLOPURINOL ON INCORPORATION OF HYPOXANTHINE-8-C^{14} INTO TISSUE RNA

EXPERIMENT No.	1	2	3	4
DOSE OF ALLOPURINOL mg/Kg	0	?	4	10

Fig. 31



RELATIONSHIP BETWEEN FRACTION OF DOSE OF HYPOXANTHINE-8-C^{14} PER g TISSUE INCORPORATED AND HGPRTase ACTIVITY: CORRECTED FOR BLOOD FLOW RATE

Fig. 32

TABLE XXXIV

Rf VALUES FOR PURINE STANDARDS AND FOR UNKNOWN RADIOACTIVE COMPOUND FROM RABBIT URINE. SUPPORT MEDIUM CELLULOSE WITH A SOLVENT SYSTEM OF 1 M AMMONIUM ACETATE pH 7.0

Compound	Rf
Hypoxanthine	0.44
Xanthine	0.32
Urate	0.21
Urinary radioactivity	0.81

incorporated increases with HGPRTase activity in kidney, brain, and testis, but liver and muscle showed high incorporation of radioactive dose with a low tissue HGPRTase activity.

It was felt justifiable to examine the effect of correcting the incorporation values for blood flow rates, as the mass transfer of compounds by the circulatory system will be dependent on blood perfusion rates. It must be emphasised however that Fig. 32 is speculative.

The blood flow rates were derived from the limited number in the literature (see Hamilton, 1963) but accurate data were available for testis (see Setchell, 1970).

It is possible that in this study the fraction of the radioactive dose incorporated into RNA has been underestimated due to the hydrolysis of RNA by RNase during the period of about 1 h between death and extraction of the RNA fraction.

The rather high incorporation of label into brain DNA could be due to contamination by RNA (see section 2) but active mitotic activity has been reported in the ependyma (Watson, 1965).

3.7c. Discussion

The results of the preliminary experiments

showed that hypoxanthine was rapidly incorporated into the human erythrocyte. The transport of hypoxanthine across the erythrocyte membrane has been shown to be an active process, and linked to urate transport (Larsen and Overgaard-Hansen, 1962; Christensen and Jones, 1961). The reason for the initial lag phase observed is not understood.

About 10% of the label in the incubation medium moved into the rabbit erythrocyte during incubation.

The PRTase activities in adult rabbit tissues were found to be lower than those in man but the ranking of the tissue activities was similar to that found in human tissues with the exception of the high APRTase activity found in rabbit kidney. Whereas human erythrocytes cannot convert IMP into AMP (Lowry, Williams and London, 1962), in the rabbit erythrocyte IMP can be converted into AMP or GMP (Lowry, Williams and London, 1961). This interspecies difference between the rabbit and human erythrocyte could possibly affect the metabolism and release hypoxanthine, but the studies by Herschko et al. (1967) on rabbit erythrocytes suggest that the main purine released by the rabbit

erythrocyte is hypoxanthine irrespective of the purine incorporated.

Radioactive hypoxanthine from rabbit erythrocytes was incorporated into tissue nucleic acids in all the tissues studied, and when corrections were made for blood flow rates, the incorporation into kidney, brain, and testis was related to the tissue HGPRTase activity.

As was expected, a high incorporation into the liver P.C.A. soluble fraction was detected. This was probably a reflection of the role of the liver in the catabolism of the purine ring system to urate and allantoin.

An unexpected finding was the high incorporation of label into skeletal muscle, when corrected for blood flow rates. A large increase in the urinary excretion of hypoxanthine after muscular work has been reported by Nasrallah and Al-Khalidi (1964) and this tissue may depend on exogenous purines to compensate for this loss. No information was found in the literature regarding muscle tissue capacity for de novo purine biosynthesis.

Although the nature of the purine released by the liver is uncertain, some deductions can be

made about its nature. Nucleotides are unable to pass through cell membranes due to the charges in the phosphate group (Davis, 1958), and the inhibition of nucleoside transport across the erythrocyte membrane by p-nitrobenzylthioguanine has no effect on the turnover rates of the erythrocyte nucleotide pool (Brown and Paterson, 1971). It would appear likely, therefore, that the moiety released by the liver is a free purine base. As the erythrocytes do not contain xanthine oxidase EC 1.2.3.2 (Al-Khadimi and Chaglassian, 1965) but possess guanine aminohydrolase EC 3.5.4.3 activity (Jorgensen, 1956) the most likely base would appear to be hypoxanthine. The recycling of hypoxanthine has been reported by Bradford et al. (1968) who studied xanthinuric patients, and found that the bulk of the daily pool of 276 mg of xanthine was excreted, while only 6% of the 960 mg hypoxanthine pool was excreted.

This and other data (see section 3.4) would suggest that hypoxanthine is the principal substrate for HGPRTase, and that this base is extensively salvaged and recycled, both intracellularly and between tissues.

SECTION IV

GENERAL DISCUSSION

A balanced supply of purine and pyrimidine nucleotides for co-enzymes and nucleic acid synthesis is a prerequisite for growth. There is considerable evidence to suggest that the purine phosphoribosyltransferases are important pathways for the synthesis of purine ribonucleotides in rapidly growing tissues. High PRTase activities have been detected in regenerating liver after partial hepatectomy (Murray, 1966) in germinating grain (Price and Murray, 1969) and in tumour cells (Smith et al., 1971). Human spermatozoa do not contain HGPRTase activity (section 3.4), but mouse ova contain low levels of both HG and APRTase activities which, after fertilisation, increase by a factor of about twenty at the blastocyst stage (Epstein, 1970). Low activities of the PRTases were detected in human foetuses at eight weeks (section 3.4).

The biosynthesis of purine ribonucleotides by tissues can proceed via the de novo pathway or by the 'salvage' pathways. Although little information is available on the capacity of human tissues for de novo biosynthesis, it is suggested that a balance between the two pathways exists, whereby tissues whose nucleotide requirements are

not met by de novo synthesis use HGPRTase to maintain these needs. Testis and central nervous tissues are probably in this category, and possibly also bone marrow as children with the Lesch-Nyhan syndrome often present a megaloblastic anaemia. The intracellular salvage of purines is a result of the degradation products of nucleic acid breakdown being partially in the form of 3'-nucleotides, which require to be degraded to the free base level before being reformed into 5'-nucleotides (section 3.4). Another probable role of HGPRTase is in the salvage of extracellular purines, principally hypoxanthine, transported by the peripheral blood. That this process can occur has been demonstrated (section 3.7; Pritchard et al., 1970). The liver is the most probable source of purines for this transport process. The finding of HGPRTase localised in walls of blood vessels (section 3.4) may be of significance in this process. A similar role for APRTase has been demonstrated in bacteria, where adenine uptake was shown to be correlated with APRTase activity, and a large proportion of this enzyme was associated with the cell membrane (Hochstadt-Ozer and Stadtman, 1971).

The role of human erythrocytes in purine transport has been emphasised by calculations made by Murray (1971). As human erythrocytes cannot synthesise purines de novo (Lowry and Williams, 1960) turnover of purines in these cells must be a result of the entry and release of purines. Murray (1971) calculated that for a 70 kg man, a total daily turnover of 450 mg of purine base occurred.

Children with the Lesch-Nyhan syndrome, deficient in HGPRTase, synthesise purine nucleotides de novo greatly in excess of normal. It is suggested that this increase in de novo synthesis is a compensatory result of the deficiency of intracellular purine nucleotides. Free hypoxanthine derived from extracellular sources or from adenosine breakdown will be recycled to form nucleotides; in the absence of HGPRTase these will be oxidised to urate and excreted. This will pull the equilibrium of this system towards purine catabolism, and de novo purine biosynthesis will increase in an attempt to meet the requirements of the tissue. This conclusion is reinforced by the failure to grow of cultured skin fibroblasts from Lesch-Nyhan children without

the addition of supplementary adenine to the culture medium (Felix and De Mars, 1969).

The relationship between behaviour, drive, aggression and intelligence with plasma uric acid levels (Kasl, Brooks and Rodgers, 1970) is of great interest when it is considered that Lesch-Nyhan children exhibit pathologically aggressive and compulsive drive behaviour. There may be a functional relationship between serum urate and the end products of cerebral purine metabolism.

Pyrimidine nucleotide biosynthesis in tissues will be related to purine nucleotide biosynthesis, and recent work by Tatibana and Shigesada (1972) has shown that PRPP regulates the activity of carbamyl phosphate synthetase. PRPP is a very important rate limiting substrate in purine biosynthesis (see section 1.7) and this would suggest a mechanism for the control of both purine and pyrimidine biosynthesis pathways. In addition, XMP has been shown to regulate the activity of orotidine monophosphate decarboxylase (Kelley and Beardmore, 1971), an enzyme in the pyrimidine biosynthetic pathway.

It is of interest that at present a controversy

exists in the literature regarding the capacity of the brain for de novo pyrimidine nucleotide biosynthesis. Some workers, for example, Hogans, Guroff and Udenfriend (1971) and Santos et al. (1968) suggest that the brain relies on preformed pyrimidines and forms pyrimidine nucleotides by the salvage reactions of uridine and cytidine kinase, whereas Bourget and Tremblay (1972) claim that brain is not dependent on the pyrimidine salvage pathways and can meet its needs by de novo synthesis.

The pathology of the Lesch-Nyhan syndrome would, however, strongly suggest that at least in man the central nervous tissues are dependent on preformed purines for nucleotide supplies. Although no testicular pathology has been reported in Lesch-Nyhan children, the present work suggests HGPRTase is important in spermatogenesis.

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The Development of Hypoxanthine/Guanine Phosphoribosyltransferase Activity in Man

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Hypoxanthine/guanine phosphoribosyltransferase (EC 2.4.2.8) is important to man. Its absence is associated with the childhood syndrome of mental deficiency, self-mutilating behaviour and hyperuricaemia (Nyhan, 1968). The enzyme catalyses the conversion of 5-phosphoribosyl 1-pyrophosphate and hypoxanthine or guanine into the corresponding 5′-mononucleotides.

The measurement of hypoxanthine/guanine phosphoribosyltransferase activity in tissues other than erythrocytes is complicated by destruction of the product by 5′-nucleotidase (EC 3.1.3.5). However, 5′-nucleotidase can be inhibited by TTP (Murray & Friedrichs, 1969). Assays based on the method of Cartier & Hamet (1968) were performed on the 25000*g* supernatant from homogenates. When the hydrolysis of GMP by brain homogenates was decreased to 15% of control values by 1.5mM-TTP there was a fourfold increase in measured hypoxanthine/guanine phosphoribosyltransferase activity. Other tissues showed less marked changes, and activity in erythrocytes was unaffected. A survey in foetal, childhood and adult tissues showed that activity was present in all the tissues examined; in most, including liver and kidney, little change occurred with growth and development. In contrast, activity in regions of brain rose during the postnatal period to reach values that, in the adult were markedly higher than those in other tissues. Activity in the testis was also somewhat higher than those in other organs.

Hypoxanthine/guanine phosphoribosyltransferase activity can be separated into at least two components by electrophoresis on cellulose acetate or starch gel, there being no major alteration in the pattern from brain during development. Reaction products from both components were destroyed by 5′-nucleotidase, and the reaction was inhibited partially by 0.7mM-6-mercaptopurine and totally by 40mM-EDTA. No marked alteration in electrophoretic pattern was observed after treatment with 5mM-sodium dodecyl sulphate. Electrophoretic separation of hypoxanthine/guanine phosphoribosyltransferase components on polyacrylamide gel has also been reported by Bakay & Nyhan (1971).

No differences in thermal stability were detected in different tissues.

The distribution and activity of hypoxanthine/guanine phosphoribosyltransferase seem consistent with its postulated 'salvage' role and possibly with its involvement in the use of exogenous purines for cellular nucleotide biosynthesis (see Raivio & Seegmiller, 1970).

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more natural development of the cultivated cells, the use of brain extract may be of importance in establishing preparations of brain-cortex cells that are suitable for metabolic studies.

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Purine Uptake from Erythrocytes by Rabbit Tissues *in vivo*

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Liver can synthesize purines *de novo* about four times as rapidly as can brain (Howard *et al.*, 1970) and has been shown to supply preformed purines for nucleic acid synthesis in bone marrow (Lajtha & Vane, 1958). In tissues other than liver, nucleotide biosynthesis from preformed purines catalysed by the purine phosphoribosyltransferases (the salvage pathways) is important (see Murray *et al.*, 1970).

Transport of purines between tissues appears to be a function of the erythrocytes (see, e.g., Henderson & Lepage, 1959). The uptake of purines by erythrocytes is dependent on hypoxanthine/guanine phosphoribosyltransferase activity (EC 2.4.2.8) (Sperling *et al.*, 1971). Since the major purine released from the erythrocyte is hypoxanthine (see, e.g., Hershko *et al.*, 1967), tissue uptake might be related to its hypoxanthine/guanine phosphoribosyltransferase activity. Therefore the uptake of radioactive purines from rabbit erythrocytes *in vivo* by tissues with widely differing activities of this enzyme was investigated.

The hypoxanthine/guanine phosphoribosyltransferase activities in rabbit testis, brain, liver, kidney, muscle and erythrocytes were 290, 230, 86, 15, 14 and 125 nmol/h per mg of protein respectively. Adenine phosphoribosyltransferase (EC 2.4.2.7) activities for the same tissues, with the exception of erythrocytes, were 61, 57, 92, 133 and 23 nmol/h per mg of protein respectively. Similar results have been obtained in the rat and in man (Adams *et al.*, 1971).

Radioactive hypoxanthine was incorporated into rabbit erythrocytes by the following method. About 10ml of heparinized rabbit blood was incubated with 50 μCi of [8-^{14}C]hypoxanthine (specific radioactivity 59 μCi/μmol) by the method of Mager *et al.* (1967); it was confirmed that 6–15% of this radioactivity was incorporated into the washed erythrocytes. These were injected into the donor rabbit, which was killed 45h later. The 'nucleotide' fraction was extracted into 0.2M-$HClO_4$; RNA and DNA fractions were obtained by the methods of Birnstiel *et al.* (1972) and of Marmur (1961) respectively from testis, brain, liver, kidney and leg muscle. ^{14}C in these extracts was measured by liquid-scintillation counting, and the amounts of RNA and DNA by u.v. spectrophotometry. The incorporation of the administered dose into the tissues,

Table 1. *Tissue uptake of radioactive purines from rabbit erythrocytes in vivo*

Erythrocytes were labelled *in vitro* and administered to the donor rabbit, and uptake into tissues was measured after 45h. Results are expressed as means±S.E.M. for three experiments and are calculated as:

$$\frac{\text{Radioactivity/g wet wt. of tissue}}{\text{Radioactivity administered to the whole animal}} \times 10^6$$

	Uptake of [^{14}C]purine (fraction of dose/g wet wt. of tissue)		
Tissue	$HClO_4$-soluble	RNA	DNA
Brain	643 ± 142	207 ± 149	27 ± 6
Kidney	983 ± 59	156 ± 54	39 ± 10
Liver	1425 ± 190	89 ± 32	27 ± 8
Skeletal muscle	637 ± 141	126 ± 60	26 ± 12
Testis	1201 ± 305	51 ± 19	16 ± 3

expressed as a fraction of the dose per g wet wt., is shown in Table 1: 57, 48 and 56% of the administered radioactivity was incorporated into the tissues studied. Most of the remainder appeared in the urine despite the administration of allopurinol. An initial study of incorporation over only 4h showed 33% uptake; therefore the uptake from erythrocytes was rapid.

The results show that there is no simple relationship between hypoxanthine/guanine phosphoribosyltransferase activity and the tissue uptake, although the testis does show a high uptake into the acid-soluble or 'nucleotide' fraction. This is especially marked when some allowance is made for its relatively low blood flow per unit weight (see Setchell, 1970) when compared with liver and kidney. A relatively high uptake into brain RNA was also noted. The large amounts removed by muscle are difficult to explain except, possibly, by some exchange of muscle purines during the operation of the purine nucleotide cycle (see Lowenstein, 1972). Significant amounts of the dose appeared in the DNA fraction (Table 1), incorporation into RNA being greater and more variable than that into DNA, particularly in brain.

The above evidence shows that tissue uptake of purines from circulating erythrocytes can take place; failure of this transport process may be important in the pathogenesis of the Lesch–Nyhan syndrome (see Kelley, 1972). It appears justifiable from the above results to suggest that hypoxanthine/guanine phosphoribosyltransferase may also be important in the testis for the uptake of purines from erythrocytes.

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