Developmental Changes in Purine Phosphoribosyltransferases in Human and Rat Tissues

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1. The hypoxanthine/guanine and adenine phosphoribosyltransferase activities in a wide variety of human tissues were studied during their growth and development from foetal life onward. A wide range of activities develop after birth, with especially high values in the central nervous system and testes. 2. Postnatal development of hypoxanthine/guanine phosphoribosyltransferase was also defined in the rat. Although there were increases in the central nervous system and testes, there was also a rise in activity in the liver, which was less marked in man. 3. A sensitive radiochemical assay method, using dTTP to inhibit 5'-nucleotidase activity, suitable for tissue extracts, was developed. 4. No definite evidence of the existence of tissue-specific isoenzymes of hypoxanthine/guanine or adenine phosphoribosyltransferase was found. Hypoxanthine/guanine phosphoribosyltransferase in testes, however, had a significantly different thermal-denaturation rate constant. 5. The findings are discussed in an attempt to relate activity of hypoxanthine/guanine phosphoribosyltransferase to biological function. Growth as well as some developmental changes appear to be related to increases in the activity of this enzyme.

Hypoxanthine/guanine phosphoribosyltransferase (IMP-pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) has a central role in the regulation of purine nucleotide biosynthesis. This enzyme catalyses the condensation of hypoxanthine or guanine with 5-phosphoribosyl 1-pyrophosphate to form IMP or GMP, and thus salvages the free purine bases that would otherwise be oxidized and excreted. This avoids four ATP-requiring reactions involved in the synthesis of purines *de novo*.

This activity was first reported in 1953–55 (Williams & Buchanan, 1953; Remy *et al.*, 1955; Korn *et al.*, 1955) and was then thought to be an unimportant part of purine nucleotide metabolism. The importance of this enzyme was recognized in 1967, when a group of retarded boys with cerebral palsy, choreo-athetosis and gout were found to have an almost complete deficiency of hypoxanthine/guanine phosphoribosyltransferase (Seegmiller *et al.*, 1967). This group of children had a 20-fold increase in glycine conversion into urate, and a large increase in purine biosynthesis *de novo* (Nyhan, 1968). Thus hypoxanthine/guanine phosphoribosyltransferase was shown to have a complementary role in purine nucleotide biosynthesis. Subsequent studies have indicated that some patients with hypoxanthine/guanine phosphoribosyltransferase deficiency produce a protein which

reacts with antibodies to the enzyme but is catalytically incompetent, suggesting a point mutation in a structural gene on the X chromosome (Felix & de Mars, 1969; Rubin *et al.*, 1971; Arnold *et al.*, 1972). Later immunochemical work has generally failed to detect cross-reacting material (Upchurch *et al.*, 1975; Ghangas & Milman, 1975). However, such evidence does not exclude defects of a structural gene.

The clinical features of the Lesch–Nyhan syndrome have been summarized by Nyhan (1968), who demonstrated the slow development of neurological and behavioural abnormalities in the first 2 years of life, with more rapid deterioration thereafter. The development and severity of the neurological disorders in children with hypoxanthine/guanine phosphoribosyltransferase deficiency directed the present studies to defining the distribution of this enzyme in the brain, and the developmental changes that occur. The results show that there is a significant increase in hypoxanthine/guanine phosphoribosyltransferase in the cerebral cortex and medulla after the age of 2 years.

A balanced supply of purine nucleotides is needed in rapidly growing tissues and in tissues with high nucleic acid turnover rates. The distribution and development of hypoxanthine/guanine phosphoribosyltransferase in a variety of such tissues was therefore studied. Adult testes had the highest activity of any tissue studied. The distribution of an apparently related enzyme, adenine phosphoribosyltransferase (AMP-pyrophosphate phosphoribosyltransferase,

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EC 2.4.2.7) was also studied; however, no known enzymic mechanism exists in man for the formation of its substrate, adenine (Murray, 1971).

The properties of hypoxanthine/guanine phosphoribosyltransferase from different tissues at various stages of development were also studied, by using electrophoresis and thermal denaturation, in an attempt to detect tissue-specific isoenzymes. Neither the differences nor the developmental pattern of activity could be accounted for by tissue-specific isoenzymes.

Experimental

Materials

All general laboratory chemicals were AnalaR grade from British Drug Houses, Poole, Dorset, U.K. [8-¹⁴C]Adenine and [8-¹⁴C]hypoxanthine (sp. radioactivity >50 μ Ci/ μ mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Adenine, hypoxanthine and 6-mercaptapurine were purchased from Sigma (London) Chemical Co., Kingston, Surrey, U.K. All compounds were more than 98% pure, except 5-phosphoribosyl 1-pyrophosphate, which was stated to contain 5–10% 5-phosphoribose 1:2-cyclic phosphate. 5'-Nucleotide phosphohydrolase (5'-nucleotidase, EC 3.1.3.5), grade II (Sigma), was from *Crotalus adamanteus* venom. DEAE-cellulose chromatography paper (DE-81) was manufactured by Whatman Ltd., Maidstone, Kent, U.K.

Preparation of human and rat tissues for enzyme assay

The erythrocytes from heparinized venous blood were separated from the leucocytes and plasma after centrifugation at 3000g for 5 min. The erythrocytes were washed with 2 $\times$ 5 vol. of 0.9% NaCl. A sample (100 μ l) of packed erythrocytes was lysed in 900 μ l of water for 5 min at 4°C. The stroma was then removed by centrifugation at 3000g for 5 min. The lysate was dialysed for 1 h against 1 litre of water and a sample of the dialysis residue assayed for enzyme activity.

Tissue homogenates were prepared in a cold-room at 4°C. Approx. 1 g of tissue was washed in 20 ml of 220 mM-Tris/HCl buffer, pH 7.4, cut into small pieces and homogenized in 5 ml of the above buffer, by using five passes of a Teflon homogenizer (Jencons, Hemel Hempstead, Herts., U.K.) at 2000 rev./min. in about 30 s. The resulting homogenate was centrifuged at 26000g for 1 h at 4°C. The supernatant was removed and then dialysed as described above.

Human and rat tissues

Human tissues were obtained from the Department of Paediatric Pathology, Royal Hospital for Sick Children, Edinburgh, and from the Department of Pathology, Royal Infirmary, Edinburgh. In studies of

human tissues, samples were obtained at necropsy about 12–14 h after death. The effects of storage at 4°C for this period of time on purine phosphoribosyltransferase stability was therefore studied in tissues removed surgically. Portions of liver, ileum and appendix were chilled immediately after surgical removal and the purine phosphoribosyltransferase activities determined. The tissues were stored at 4°C and assayed at 12, 24 and 48 h subsequently. Small variations were observed between tissues, but the half-lives were never less than 60 h for hypoxanthine/guanine phosphoribosyltransferase and 30 h for adenine phosphoribosyltransferase. The almost unavoidable use of post-mortem tissues should not therefore greatly distort the results. The foetal material used was obtained surgically and assays were performed within a few hours.

Rats of the PVG strain developed by Glaxo Research Ltd., Greenford, Middx., U.K., were obtained from the Department of Pharmacology, University of Edinburgh. The rats were fed *ad libitum* on Oxoid breeding diet (Oxoid Ltd., London S.E.1, U.K.). Groups of four littermates were used when possible and were killed by asphyxiation under N₂ to minimize mechanical brain damage.

Cultured human cells were grown in Ham's F10 medium (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.), supplemented by 10% foetal calf serum, and harvested by trypsin treatment with added EDTA, both 0.4 g/l. The washed cells were homogenized with an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen i. Breisgau, Germany), the homogenates centrifuged at 26000g for 1 h at 4°C, and the supernatants assayed, as for other tissues.

Assay of purine phosphoribosyltransferases

Hypoxanthine/guanine and adenine phosphoribosyltransferases were assayed in duplicate by a modification of the method of Cartier & Hamet (1968). This method involves the use of radioactive purines and the separation of the unchanged substrate from the polyanionic reaction product by ion-exchange chromatography on DEAE-cellulose paper.

However, this method and others (Chow *et al.*, 1970; Fujimoto *et al.*, 1968; Krenitsky *et al.*, 1969) are unsuitable for tissues other than erythrocytes, as the product nucleotides are subject to hydrolysis to nucleosides by the widely distributed 5'-nucleotidase (see Bodansky & Schwartz, 1968). In the present method, it was possible to inhibit 5'-nucleotidase with dTTP. Since Murray & Friedrichs (1969) showed that the dTTP concentration for maximum inhibition of hydrolysis of AMP and IMP was about 0.5 mM, a dTTP concentration of 1.5 mM was used.

The effect of dTTP on the phosphoribosyltransferase reactions was studied by using erythrocyte lysates, since these contain no 5'-nucleotidase which could result in the formation of nucleosides. No

marked activation or inhibition of the phosphoribosyltransferase, as determined by nucleotide formation by erythrocyte lysates, was detected with 1.5mM-dTTP. Fujimoto & Seegmiller (1970) also found no marked activation or inhibition of hypoxanthine/guanine phosphoribosyltransferase activity by dTTP, using nucleotide and nucleoside determinations in fibroblasts. A concentration of 1.4mM-dTTP was effectively used by Fujimoto & Seegmiller (1970) in determining low activities in fibroblasts. Concentrations of dTTP greater than 3.3mM are reported to affect hypoxanthine/guanine phosphoribosyltransferase activity (Itiaba *et al.*, 1972).

Assays on all tissues, except erythrocytes, carried out in the presence of dTTP showed between 5 and 400% increase in activity compared with duplicate assays carried out in the absence of dTTP. The increase in measured phosphoribosyltransferase activities by the inhibition of 5'-nucleotidase was most marked in central nervous tissues. As expected, this tissue also contained the highest 5'-nucleotidase activity of the tissues studies.

Hypoxanthine/guanine and adenine phosphoribosyltransferase activities were measured by the following method. Assay medium (75 μ l) containing Tris/HCl buffer, pH 7.4 (55mM), $MgCl_2$ (5mM), 5-phosphoribosyl 1-pyrophosphate (1mM), dTTP (1.5mM) and [8- ^{14}C]adenine or [8- ^{14}C]hypoxanthine (0.6mM; a total of 20000 c.p.m. was used) was pre-incubated at 37°C for 5 min before the reaction was started by the addition of 25 μ l of the enzyme preparation. After 5-30 min incubation, depending on the tissue, the reaction was halted by the addition of 25 μ l of EDTA (100mM) and subsequent cooling to 3-4°C. A 30 μ l sample of the reaction mixture was applied to DEAE-cellulose paper strips (150mm $\times$ 15mm), and developed with aq. 95% (v/v) ethanol and 1M ammonium acetate adjusted to pH 3.8 with acetic acid (2:1, v/v) as described by Paladini & Leloir (1952). The separation of purine bases from nucleotides was verified by the use of unlabelled standards, which were located by their u.v. absorption. The DEAE-cellulose paper chromatograms were oven-dried for 30 min, and the areas of paper corresponding to the location of the nucleotide and the free base were counted for radioactivity by liquid-scintillation spectrometry. The procedure has been described in detail (Adams, 1973).

The efficiency of the extraction of the hypoxanthine/guanine phosphoribosyltransferase from the tissue samples was checked by determining the activity remaining in the residual pellet after centrifugation at 26000g. The tissue homogenate was prepared and centrifuged as described, and the supernatant was removed. The residual pellet was washed with 2 $\times$ 5 ml of water and the washed pellet rehomogenized and centrifuged as above. This supernatant was used to determine the activity 'in' the pellet.

Three representative tissues were studied, cerebral cortex and liver from four children, and testes from four adults. The mean hypoxanthine/guanine phosphoribosyltransferase activities in the residual pellet, expressed as a percentage of the mean tissue activities, were 28, 15 and 22% for cerebral cortex, liver and testes respectively. The mean activities ($\pm$ S.E.M.; $n = 4$) in the residual pellet from cerebral cortex, liver and testes were 36 ± 9 , 6.3 ± 1.7 and 93 ± 26.5 nmol/h per mg of protein respectively. The percentage of activity in the extract is thus similar to that found in the soluble and microsomal fractions (see below), as might be expected from the centrifugation used.

It was confirmed that the mass of substrate transformed was a linear function of both the volume of tissue extract added to the incubation medium and the time of incubation. Deviations from linearity occurred at about 25% transformation of substrate. The specific activity of the phosphoribosyltransferase is expressed as nmol of substrate converted into AMP or IMP/h, and related to the total protein in the enzyme sample as determined by the Miller (1959) modification of the method of Lowry *et al.* (1951). The coefficient of variation of 9% for an individual assay was estimated from the difference between duplicate assays (Snedecor, 1952). However, all results are presented as the means of duplicate assays.

Electrophoresis of the purine phosphoribosyltransferases

Electrophoresis of the purine phosphoribosyltransferases on cellulose acetate was carried out as described by Kohn (1969) by using a barbitone buffer, pH 8.6 at room temperature (20-25°C,) as described by Smithies (1955). A potential difference of 5V/cm was applied at a constant current of 2mA for 3-4h.

Electrophoresis was also carried out in starch gel. The gel was prepared and poured as described by Bodman (1969) in the gel buffer of Smithies (1955). A potential difference of 5.4V/cm was applied at a constant current of 2mA. The 30 μ l sample was inserted into a slit in the gel at the cathodic end and the entire gel covered with a polythene sheet. After electrophoresis for 20h at room temperature, the gel was sliced into two layers.

Location of purine phosphoribosyltransferase activity after electrophoresis

The support medium was covered with a strip (150mm $\times$ 15mm) of Whatman DEAE-cellulose paper saturated with 500 μ l of purine phosphoribosyltransferase assay medium with the composition and concentrations described previously, but omitting the 1.5mM-dTTP. Both were then clamped between two glass plates. This assembly was then incubated for 3h in the moist atmosphere at 37°C provided by a

covered water bath. At the end of this period the DEAE-cellulose paper was carefully removed and washed with slow magnetic stirring for 4 h in 4 litres of 8 mM-Tris/HCl, pH 9.5. During the wash, the reaction products were retained in the DEAE-cellulose paper. The DEAE-cellulose paper was cut into 1 cm sections and counted for radioactivity.

Specificity of the detection method used after electrophoresis

The specificity of the detection method was confirmed by incorporating either 40 mM-EDTA or 0.7 mM-6-mercaptopurine into the enzyme-assay medium used for location of the activity as above. Untreated controls showed a normal pattern in each case. In the assay containing EDTA, a non-specific inhibitor of the purine phosphoribosyltransferase, no zones of activity were detected in the DEAE-cellulose paper, whereas with the assay medium containing 6-mercaptopurine, a specific inhibitor of hypoxanthine/guanine phosphoribosyltransferase (Krenitsky *et al.*, 1969), much smaller peaks of radioactivity were detected. In similar duplicate experiments, subsequent to electrophoresis and treatment with the assay medium, one strip of DEAE-cellulose paper was treated for 2 h at 37°C with 500 units of 5'-nucleotidase in 220 mM-Tris/HCl buffer, pH 7.4 (1 mg of protein/ml). No zones of radioactivity were detected in the 5'-nucleotidase-treated DEAE-cellulose paper, but zones of radioactivity were detected in the control. These experiments demonstrate that the bands of radioactivity on the DEAE-cellulose paper are the products of the purine phosphoribosyltransferase reaction and are 5'-AMP or 5'-IMP.

Determination of the specific reaction rate constants for thermal denaturation of hypoxanthine/guanine phosphoribosyltransferase

Dialysed samples were diluted to give a protein concentration of 5 mg/ml by the addition of 220 mM-Tris/HCl buffer, pH 7.4. The sample was then incubated at $80 \pm 1^\circ\text{C}$ with shaking in glass tubes (100 mm $\times$ 10 mm); 25 μl samples were removed before incubation and after 2, 4, 6 and 8 min at 80°C and the hypoxanthine/guanine phosphoribosyltransferase activities determined.

Plotting the logarithm of the percentage residual enzyme activity against time of incubation consistently yielded straight lines. The rate of denaturation of hypoxanthine/guanine phosphoribosyltransferase under the defined conditions was thus found to approximate to first-order unimolecular reaction kinetics. Where A_0 is the initial concentration of the reactant (hypoxanthine/guanine phosphoribosyltransferase) and $a-x$ is the percentage concentration of catalytically competent enzyme at a subsequent

time, the rate constant K is obtained from the rearranged first-order rate equation:

$$t = \frac{2.303}{K} \log A_0 - \frac{2.303}{K} \log(a-x) \quad (1)$$

$\log(a-x)$ is plotted against t and the slope of the line is then equal to $-2.303/K$. The thermal-denaturation rate constant K has the dimensions of reciprocal time (Table 5).

Determination of apparent Michaelis constants and maximal velocities

Apparent Michaelis constants (K_m) and maximal velocities ($V_{\max}$) 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, 5-phosphoribosyl 1-pyrophosphate (1 mM), and optimal Mg^{2+} concentration (5 mM) were used.

Samples were diluted about 20-fold with water and dialysed for 12 h against 10 litres of water to remove any endogenous substrate or possible inhibitors. The dialysis residue was used directly for kinetic studies. It was confirmed that the reaction was linear with time for 1.5 min at the lowest substrate concentration used. The incubation time was therefore 1.5 min; otherwise the assay was that previously described.

K_m and $V_{\max}$ values were calculated by fitting regression lines to the experimental points by using the Lineweaver-Burk and Hofstee-Eadie methods.

Subcellular distribution of hypoxanthine/guanine phosphoribosyltransferase

Samples of cerebral cortex, liver and testes were obtained at post-mortem of a man aged 59 years who had been dead for approx. 12 h. The minced washed tissue was homogenized in 15 vol. of 220 mM-Tris/HCl buffer, pH 7.4, containing 0.3 M-sucrose, by using a Teflon homogenizer at 1000 rev./min. Total enzyme activities and protein were determined on samples of the homogenate after twice freezing and thawing. Hypoxanthine/guanine phosphoribosyltransferase activity and protein were determined as described above; L-lactate-NAD⁺ oxidoreductase (lactate dehydrogenase, EC 1.1.1.27) activity was measured by the method of Wroblewski & La Due (1955).

The homogenate was filtered through several layers of muslin. This and subsequent procedures were carried out at 4°C . The filtered homogenate was then separated by differential centrifugation into crude nuclear (600g for 5 min), mitochondrial (20000g for 10 min), microsomal (105000g for 60 min) and supernatant fractions. The deposits obtained were resuspended in 220 mM-Tris/HCl buffer, pH 7.4, and enzyme activities and protein determined as above.

Liquid-scintillation counting in a two-phase system

The radioactivity on the sections of DEAE-cellulose paper was counted directly in a two-phase system in 5ml of NE 233 (Nuclear Enterprises, Edinburgh, Scotland, U.K.) in a Packard Tri-Carb model 2002 liquid-scintillation spectrometer. The counting efficiency of 49% was determined with an internal standard (Rogers & Moran, 1966) of [$8\text{-}^{14}\text{C}$]hypoxanthine.

Results*Erythrocyte purine phosphoribosyltransferase activities in man*

The activities of hypoxanthine/guanine phosphoribosyltransferase and adenine phosphoribosyltransferase in human erythrocytes are presented in Table 1. Except for the low hypoxanthine/guanine phosphoribosyltransferase activity in erythrocytes from the newborn (umbilical-cord blood) compared

Table 1. *Purine phosphoribosyltransferase activities in human erythrocytes*

The methods for the preparation of the dialysed lysates of erythrocytes and for the enzyme assay are detailed in the Experimental section. Hypoxanthine was used as a substrate for the measurement of hypoxanthine/guanine phosphoribosyltransferase activity. The heparinized peripheral-venous-blood samples used were generally about 0.5 h old and never more than 4 h old. Results are means $\pm$ S.E.M. with the numbers of individuals studied shown in parentheses. Each estimate, for an individual, was itself the mean of replicate measurements, made in duplicate, that were reproducible within $\pm 9\%$ (coefficient of variation).

Age	Purine nucleotide formed (nmol/h per mg of protein)	
	IMP (hypoxanthine/guanine phosphoribosyltransferase)	AMP (adenine phosphoribosyltransferase)
Newborn	61 $\pm$ 9.8 (15)*	38.7 $\pm$ 2.77 (5)
0-2 years	104 $\pm$ 2.25 (8)	37.0 $\pm$ 2.8 (8)
2-10 years	111 $\pm$ 3.75 (11)	36.5 $\pm$ 2.5 (11)

* Difference between newborn and 0-2 years, $P > 0.01$ (Student's t test).

Table 2. *Purine phosphoribosyltransferase activities in human foetal tissues*

The tissue samples were prepared and the enzyme activities determined by the procedures described in the Experimental section. Hypoxanthine was used as a substrate for the determination of hypoxanthine/guanine phosphoribosyltransferase activity. Results are given as the means of duplicate determinations, which were reproducible within $\pm 9\%$ (coefficient of variation).

Tissue	Gestational age (weeks)	Purine nucleotide formed (nmol/h per mg of protein)	
		IMP (hypoxanthine/guanine phosphoribosyltransferase)	AMP (adenine phosphoribosyltransferase)
Liver	8	44	30
	12	21	37
	12	9	—
	14	40	32
	21	55	—
	22	43	31
Whole brain	8	143	52
	12	28	64
	14	73	61
Cerebral cortex	12	30	—
	21	81	—
Basal nuclei	12	0	—
	21	98	—
	22	63	27
Cerebellum	21	150	—

Table 3. *Purine phosphoribosyltransferase activities in tissues from children aged 0–2 years and from adults aged 60–80 years*

The tissue samples were prepared and the enzyme activities determined by the procedures described in the Experimental section. Hypoxanthine was used as a substrate for the determination of hypoxanthine/guanine phosphoribosyltransferase activity. Results are presented as means ($\pm$ S.E.M.), from the numbers of patients shown in parentheses. Each estimate, for an individual, was itself the mean of replicate measurements, made in duplicate, that were reproducible within $\pm 9\%$ (coefficient of variation). The statistically significant differences (Student's *t* test) between activities in children and in adults are shown by asterisks: * $0.05 > P > 0.025$; ** $0.01 > P > 0.005$; *** $0.1 > P > 0.05$.

Tissue	Purine nucleotide formed (nmol/h per mg of protein)			
	IMP (hypoxanthine/guanine phosphoribosyltransferase)		AMP (adenine phosphoribosyltransferase)	
	Children	Adults	Children	Adults
Liver	39.8 $\pm$ 7.0 (11)	71.3 $\pm$ 10.9 (6)*	88.7 $\pm$ 38.6 (6)	165.2 $\pm$ 11.7 (4)***
Kidney	47.2 $\pm$ 15.9 (7)	42.3 $\pm$ 15.9 (4)	42.0 $\pm$ 7.0 (3)	28.5 $\pm$ 12.0 (3)
Thymus	53.2 $\pm$ 13.9 (4)	—	95.6 $\pm$ 38.4 (4)	—
Skeletal muscle	12.8 $\pm$ 3.2 (4)	—	120.0, 18.5	—
Cardiac muscle	37.8 $\pm$ 9.3 (5)	—	16.5, 50.0	—
Testis	126.0 $\pm$ 47.4 (4)	431.0 $\pm$ 63.7 (5)**	52.5 $\pm$ 24.4 (4)	59.7 $\pm$ 10.9 (4)
Cerebral cortex grey matter	128.4 $\pm$ 42.1 (9)	294.0 $\pm$ 57.8 (4)*	12.7 $\pm$ 5.5 (4)	21.1 $\pm$ 10.7 (4)
Cerebral cortex white matter	68.2 $\pm$ 15.9 (8)	135.3 $\pm$ 25.0 (4)*	21.0 $\pm$ 7.7 (4)	13.0 $\pm$ 5.5 (4)
Basal nuclei	157.3 $\pm$ 22.0 (3)	192.7 $\pm$ 23.7 (3)	5.7 $\pm$ 2.2 (3)	19.4 $\pm$ 6.8 (4)
Medulla	75.7 $\pm$ 18.0 (6)	142.0 $\pm$ 5.0 (4)**	19.0 $\pm$ 2.8 (3)	13.5, 47.5
Cerebellum	29.3 $\pm$ 4.3 (3)	—	—	—
Bone marrow	—	56.7 $\pm$ 11.9 (3)	—	16.4 $\pm$ 5.8 (4)

with that in children aged 0–2 years ($P > 0.01$), there is little variation of erythrocyte purine phosphoribosyltransferase activity with age.

Human tissue hypoxanthine/guanine phosphoribosyltransferase activity

The activities in human tissues from foetuses, children aged 0–2 years and adults of 60–80 years are presented in Tables 2 and 3. These results show that the specific activity of hypoxanthine/guanine phosphoribosyltransferase increases significantly with growth and development in the grey and white matter of the cerebral cortex and in the medulla. At all ages studied, activity was about twice as high in the cerebral grey matter as in the cerebral white matter, suggesting an association with neuronal function. Although the mean activity in the basal nuclei was higher in adults, the difference was not statistically significant. In cerebellum there appears to be a fall in activity between foetal life and 0–2 years; comparison with the more limited results in the foetus (Table 2) suggests that there may be increases in hypoxanthine/guanine phosphoribosyltransferase activity in the central nervous system during the first 2 years of life and/or in the latter half of pregnancy, with the possible exception of the cerebellum.

There was also a significant increase in testes, which in the adult had the highest specific activity of all tissues studied (Table 3). Radioautographic studies on the location of hypoxanthine/guanine phosphoribosyltransferase in the testes of children showed that

there was much activity throughout the sections, with the highest concentrations in the basal cells of the seminiferous tubules in the spermatogonia. Thyroid used as a control tissue showed virtually no activity (Adams, 1973).

One foetal testis and a foetal ovary both showed considerable activity, 203 and 130 nmol/h per mg of protein respectively, but no activity was detectable in the ovary of a child aged 7 years.

In contrast with the developmental increases in hypoxanthine/guanine phosphoribosyltransferase activity in central nervous system and testes, a small but significant increase was detected in liver (Table 3). The relative activity of hypoxanthine/guanine phosphoribosyltransferase in different tissues from individual cadavers when 'ranked' was found to have similar rank in all individuals at all stages of development, although this is not always obvious from the mean values in Tables 2 and 3. The absolute activities in testes and brain, however, increased relatively more than did activities in other tissues.

Adenine phosphoribosyltransferase activity was highest in the foetal central nervous system and appeared to fall during development. No significant developmental changes were detected in testicular specific activity. Hepatic adenine phosphoribosyltransferase activity increased during development.

Hypoxanthine/guanine phosphoribosyltransferase activities were determined in tissues from four children aged between 4 and 8 years. Specific activity in liver was 78 ± 27 nmol/h per mg of protein

(mean $\pm$ S.E.M.), and that in brain ranged from 95 to 254 nmol/h per mg of protein. The values in liver were thus similar to those in adults, and in brain like those in younger children. However, these results must be interpreted with caution, since three of these children were on cytotoxic drugs before death and one was severely ill with cystic fibrosis.

Hypoxanthine/guanine phosphoribosyltransferase activity in cultured human cells from amniotic fluid and skin

A series of eight samples of cultured cells from eight samples of amniotic fluid gave a wide range of activities: 15, 21, 22, 60, 87, 89, 206 and 370 nmol/h per mg of protein.

Samples of three lines of cultured cells from skin known to be deficient in this enzyme had activity less than 1 nmol/h per mg of protein by the present assay method. Hypoxanthine/guanine phosphoribosyltransferase deficiency should therefore be diagnosable from cultured amniotic-fluid cells by using the present assay method. A strain of human amnion cells transformed by the virus SV-40, and in continuous growth, had activities of 146 and 170 nmol/h per mg of protein; a selected strain from this cell line resistant to the growth-inhibitory effects of 8-azaguanine and 6-thioguanine at 50 μ g/ml had an activity of 2.6 nmol/h per mg of protein, confirming that these cells were relatively enzyme deficient.

Rat tissue hypoxanthine/guanine phosphoribosyltransferase activities

The specific activities in various tissues in the rat are presented in Table 4. Activities in liver and

cerebral cortex, but not the brain stem, increased sharply in the first week of life, a time of rapid and general growth. Thereafter the pattern was complex and appeared to differ from that in man. The activity in liver rose after birth until 4 weeks of age and then fell until the 9th week. The activity then increased to a maximum at 15 weeks.

In the central nervous system, a marked increase in activity was found in the cerebral cortex in the first 2 weeks of life. Maximum activities were found at the age of 15 weeks in liver, testis and brain stem. Activity in the basal nuclei remained constant between the second and fifth week after birth and increased between the fifth and seventh week. The brain stem differed from all other tissues studied, in that activities overall were steadier.

The hypoxanthine/guanine phosphoribosyltransferase activity in rat testis is shown in Fig. 1; activity in testis rose parallel to, but lower than, those in liver and cerebral cortex for the first 3 weeks and then increased to a higher specific activity. Activities in these tissues were at similar maximal values in the fifteenth week. All these tissues showed low activities at the sixty-fifth week of life, when the rats were senile.

Micro-dissection of rat testes

The distribution of hypoxanthine/guanine phosphoribosyltransferase activity in rat testes was studied after microdissection of seminiferous tubules from interstitial tissue by the method of Christensen & Mason (1965). The activities found were for seminiferous tubules and interstitial tissue respectively 260 and 280 nmol/h per mg of protein, suggesting that the high activity throughout the section after radio-

Table 4. Developmental changes in rat tissue hypoxanthine/guanine phosphoribosyltransferase activity

The tissue samples were prepared and the enzyme activities determined by the procedures described in the Experimental section. Hypoxanthine was used as a substrate for the determination of hypoxanthine/guanine phosphoribosyltransferase activity. Results are presented as means ($\pm$ S.E.M.). Each estimate, for an individual, was itself the mean of replicate measurements, made in duplicate, that were reproducible within $\pm 9\%$ (coefficient of variation). S.E.M. values are given when four, or rarely three, rats were available. No. S.E.M. is given when only two littermates were available.

Age (weeks)	Tissue hypoxanthine/guanine phosphoribosyltransferase activity (nmol/h per mg of protein)				
	Liver	Cerebral cortex	Testis	Brain stem	Basal nuclei
Newborn	57.5 $\pm$ 10.4	24.7 $\pm$ 4.5	—	55.7 $\pm$ 19.2	—
1	82.3 $\pm$ 12.0	60.0 $\pm$ 14.7	37.3 $\pm$ 3.8	50.3 $\pm$ 2.2	—
2	100.5 $\pm$ 17.5	85.5 $\pm$ 0.5	72.0 $\pm$ 11.0	77.5 $\pm$ 6.5	59.5 $\pm$ 15.5
3	135.2 $\pm$ 14.8	65.0 $\pm$ 1.08	88.0 $\pm$ 22.0	55.8 $\pm$ 6.3	54.0 $\pm$ 1.0
4	150.7 $\pm$ 20.0	93.7 $\pm$ 22.5	58, 76	53.7 $\pm$ 7.3	54.5 $\pm$ 8.5
5	118.2 $\pm$ 10.1	68.3 $\pm$ 9.5	100.5 $\pm$ 0.5	53.5 $\pm$ 6.6	47.0 $\pm$ 15.0
7	109.0 $\pm$ 7.0	101.0 $\pm$ 11.1	116.0 $\pm$ 7.0	82.5 $\pm$ 6.6	102.5 $\pm$ 1.5
9	88.2 $\pm$ 2.6	86.7 $\pm$ 6.0	103.0 $\pm$ 3.0	66.6 $\pm$ 10.8	—
15	174.0 $\pm$ 32.0	182.0 $\pm$ 31.0	178.0 $\pm$ 2.0	143.0 $\pm$ 16.0	—
19	147.0 $\pm$ 27.0	127.3 $\pm$ 5.2	156.5 $\pm$ 5.7	129.3 $\pm$ 11.8	—
19.75	138, 182	115, 125	—	—	—
65	46.0 $\pm$ 1.5	40.0 $\pm$ 5.5	41.5 $\pm$ 1.5	—	32.0 $\pm$ 7.0

Fig. 1. Changes in growth and development of rat testes

(a) Total body weight ($n = 20-140$); (b) inflexion in total weight of testes ($n = 20$) from PVG-strain rats (Glaxo Research Ltd.); (c) hypoxanthine/guanine phosphoribosyltransferase specific activity in testes (mean $\pm$ S.E.M. generally on four littermates). Each estimate, for an individual, was itself the mean of replicate measurements, made in duplicate, that were reproducible within $\pm 9\%$ (coefficient of variation). The approximate times of appearance of testicular tubular cell types are indicated by arrows.

autography of human material could be interpreted as indicating that all testicular tissues had high activities, with some concentration in the basal layer of cells, the spermatogonia, in the seminiferous tubules.

Electrophoresis of purine phosphoribosyltransferases from human tissues

The tissues studied electrophoretically were foetal brain and liver, children's brain, testis, liver and erythrocytes, and adult's testis, brain and erythrocytes. Four distinct bands of enzyme activity were detected in all the tissues studied. The method was semi-quantitative; doubling of the volume of the sample applied resulted in the approximate doubling of the measured activity. For hypoxanthine/guanine phosphoribosyltransferase, the band of activity located at 3 and 4 cm from the origin at the cathodic end of the strip was always the major band of activity; the bands at 8, 10 and 13 cm were of similar activities,

all being of lower activity than the major band. The minor bands were consistently detectable.

Tissue adenine phosphoribosyltransferase was located at 4, 7, 9 and 13 cm from the origin at the cathodic end of the strip. The band of activity at 4 cm represented more activity than the bands of higher mobility; however, the difference in activity was less marked with adenine phosphoribosyltransferase than with hypoxanthine/guanine phosphoribosyltransferase. These results, shown in Fig. 2, were obtained by using either cellulose acetate or starch gel as a support medium. Similar results have been obtained by Bakay & Nyhan (1971) using polyacrylamide gel, although they reported only one band for adenine phosphoribosyltransferase.

Treatment of the tissue homogenate with 10% (w/v) sodium dodecyl sulphate had no effect on the subsequent electrophoretic pattern. Sodium dodecyl sulphate was not removed from the sample but was not included in the electrophoretic buffer in these separations. Similarly, tissue homogenates which were heat-treated at 80°C for 2 min resulted in identical separations on electrophoresis. It was therefore unlikely that the isoenzymes had different thermal stabilities.

Subcellular distribution of hypoxanthine/guanine phosphoribosyltransferase in human tissues

The subcellular distribution of activity was determined in human cerebral cortex, liver and testes. The

Fig. 2. Electrophoretic separation of hypoxanthine/guanine (a) and adenine (b) phosphoribosyltransferase activities in human tissues

Activities were located and measured by performing the reaction on the surface of the support medium in a DEAE-cellulose paper saturated with the phosphoribosyltransferase assay solution, as detailed above. Results obtained by using starch gel and cellulose acetate were similar. The origin was at the cathodic end of the strip in every case.

results were similar for the three tissues and showed that the majority, 64–66%, of the activity was in the soluble fraction. The distribution of hypoxanthine/guanine phosphoribosyltransferase activity was similar to that for lactate dehydrogenase used as a control enzyme. The subcellular distribution of lactate dehydrogenase activity in human cerebral cortex was similar to that in rat cerebral cortex (Pull & McIlwain, 1974) and that in human testes was similar to that in mouse testes (Blanco *et al.*, 1976).

Thermal-denaturation rate constants

Thermal-denaturation rate constants determined as described in the Experimental section are presented in Table 5. The kidney, spleen, adrenals and cerebral cortex were taken from adults, whereas the erythrocyte samples were taken from three children and two adults, the liver from four children and one adult, and the testes from three children and one adult. Where tissue from both children and adults was studied, no marked difference was detected in the values obtained. The rate constants obtained from erythrocytes, testis and cerebral cortex were highly reproducible, but those for liver were more varied. The denaturation rate constant for testicular hypoxanthine/guanine phosphoribosyltransferase was significantly different from the mean of the other tissues, excluding the adrenal, by Student's *t* test ($0.02 < P < 0.05$). The single value obtained for the functionally similar adrenal was also markedly different.

Apparent kinetic constants for human tissue hypoxanthine/guanine phosphoribosyltransferase

The apparent Michaelis constants (K_m) and maximal velocities (V_{max}) for hypoxanthine were determined by the Lineweaver–Burk and Hofstee–

Eadie (Hofstee, 1959) methods. The values obtained for the erythrocyte enzyme were highly reproducible and similar to those reported by Henderson (1968). The mean K_m values ($\pm$ S.E.M., $n = 8$) obtained for the erythrocyte enzyme were, by the Lineweaver–Burk method, $12.2 \pm 0.92 \mu M$, and by the Hofstee–Eadie method, $8.4 \pm 0.80 \mu M$. The mean ($\pm$ S.E.M.) values obtained for the K_m for the enzyme from liver, cerebral cortex, thalamus and testes ranged between $4.9 \pm 0.3 \mu M$ for liver (10) and $8.7 \pm 2.75 \mu M$ (4) for thalamus (Hofstee–Eadie plots). The apparent K_m values obtained for tissues were not as reproducible as those for erythrocytes. These K_m values for tissue hypoxanthine/guanine phosphoribosyltransferase probably reflect the presence of enzyme systems other than this enzyme metabolizing the substrate or product, both at very low concentration. These results do not themselves suggest that different forms of this enzyme exist in different tissues.

Discussion

The measurement of purine phosphoribosyltransferase activities in tissues other than erythrocytes is complicated by the destruction of the product by 5'-nucleotidase. The use of methods measuring both IMP and the nucleoside, inosine (Boyle *et al.*, 1970) still suffers from the presence of enzymes metabolizing the nucleosides, e.g. guanosine phosphorylase (EC 2.4.2.15), inosine phosphorylase (EC 2.4.2.1) and adenosine deaminase (EC 3.5.4.4), although such methods have yielded useful results. The present method (Adams *et al.*, 1971) and several subsequent methods have avoided these difficulties by inhibiting 5'-nucleotidase almost completely with 1.5 mM-dTTP (Murray & Friedrichs, 1969; Fujimoto & Seegmiller, 1970).

An alternative and useful method for compensating for product destruction by 5'-nucleotidases has been to measure hypoxanthine/guanine phosphoribosyltransferase-to-adenine phosphoribosyltransferase ratios. However, 5'-nucleotidase from liver and cerebrum was found to hydrolyse GMP and AMP at different rates, hence alterations in this ratio might be due to changes in 5'-nucleotidase activity rather than in hypoxanthine/guanine phosphoribosyltransferase. Development changes in 5'-nucleotidase sometimes occur about the same time as changes in hypoxanthine/guanine phosphoribosyltransferase (see below) and might thus distort the pattern.

The substrate selected for measuring hypoxanthine/guanine phosphoribosyltransferase activity was hypoxanthine, which has advantages over guanine in that it is more soluble at pH 7.4, and that it is not metabolized as is guanine by guanine aminohydrolase (EC 3.5.4.3). Guanine aminohydrolase is present in many tissues and is found in high concentrations in the central nervous system (Rosenbloom *et al.*,

Table 5. *Thermal-denaturation rate constants for hypoxanthine/guanine phosphoribosyltransferase from human tissues*

Thermal-denaturation rates were determined and the rate constants calculated as described in the Experimental section. The results are presented as the means $\pm$ S.E.M. of the numbers of individuals shown in parentheses.

Tissue	Rate constant (min^{-1})
Erythrocytes	0.1146 ± 0.0027 (5)
Liver	0.1122 ± 0.024 (5)
Testis	0.0515 ± 0.00035 (4)*
Cerebral cortex	0.0855 (2)
Basal nucleus	0.064 (1)
Kidney	0.110 (2)
Spleen	0.111 (1)
Adrenal	0.052 (1)

* Significant difference between the mean value for testis and that for all tissues except adrenal ($0.02 < P < 0.05$) by Student's *t* test.

1967). Hypoxanthine is oxidized by xanthine oxidase (EC 1.2.3.2), but this enzyme is found in large quantities only in liver (Watts *et al.*, 1965), with small amounts in intestine and also in milk. Hypoxanthine is probably the principal substrate for hypoxanthine/guanine phosphoribosyltransferase *in vivo*, since hypoxanthine is extensively recycled in man, whereas guanine is largely oxidized and excreted as urate (Ayvazian & Skaupp, 1965; Bradford *et al.*, 1968).

Hypoxanthine/guanine and adenine phosphoribosyltransferase activities in erythrocytes were relatively constant; our findings agree with those of Kelley *et al.* (1967) and Cartier & Hamet (1968). The lower activity of hypoxanthine/guanine phosphoribosyltransferase in umbilical-cord blood is difficult to explain, but has also been reported by Durward & Boyle (1973). Increased adenine phosphoribosyltransferase activity in neonatal blood has been reported by Borden *et al.* (1974), a finding that we have been unable to confirm. Many enzyme activities are altered in erythrocytes from cord blood (Jones & McCance, 1949) and may reflect the widespread physiological and metabolic changes at about the time of birth.

Marked increases in hypoxanthine/guanine phosphoribosyltransferase specific activity were found in several tissues during growth and development. Growth appears to be associated with high activity of this enzyme; increases have been shown in regenerating rat liver, and high activities are found in tumour cells (Murray, 1966). Increases in hypoxanthine/guanine and adenine phosphoribosyltransferase activities are also correlated with morphogenesis in early embryonic life (Epstein, 1970). It is possible to suggest some general differences in function between tissues with high hypoxanthine/guanine phosphoribosyltransferase activity, such as brain and testis, and those with lower activities. In brain and testis extensive recycling of nucleic acid-degradation products takes place within these organs (Glassman, 1969; Amann, 1967). In other tissues with a high DNA or RNA turnover, such as bone marrow or thymus, nucleated viable cells are exported in large numbers. Hypoxanthine/guanine phosphoribosyltransferase is probably still important in bone marrow, supplementing the purine-synthetic pathway *de novo*, as cases of megaloblastic anaemia have been reported in patients with the Lesch-Nyhan syndrome (Ghadimi *et al.*, 1970; van der Zee *et al.*, 1970).

Unfortunately it has not yet been possible to determine the capacity of different human tissues to synthesize purine nucleotides *de novo*, as some of the enzymes involved are very labile. However, Howard *et al.* (1970) have shown low purine biosynthesis *de novo* in rat brain relative to rat liver.

There are some other studies of the developmental changes in the activities of enzymes involved in purine

nucleotide metabolism. Gutensohn & Guroff (1972) have shown somewhat similar changes in hypoxanthine/guanine phosphoribosyltransferase in rat brain over the earlier period of postnatal development. The relative activities of this enzyme in cerebral cortex, basal ganglia and brain stem obtained by Gutensohn & Guroff (1972) were similar to the present results in the rat. In chick brain also, some of the enzymes involved in recycling hypoxanthine become functionally important after 'birth' (Manzoli *et al.*, 1971), although 5'-nucleotidase shows maximal activity before hatching. However, for one woman, Rosenbloom *et al.* (1967) have reported higher activities in basal ganglia than the present results and activities that they had found in frontal lobe. The other results from this woman were somewhat similar to those in the present study, despite the absence of a 5'-nucleotidase inhibitor. No simple explanation for this difference is apparent. The result in the other case presented was similar to the present activities in basal ganglia.

In the liver there were marked species differences in the pattern of developmental changes of hypoxanthine/guanine phosphoribosyltransferase activity. Activity in rat liver increased markedly with age, whereas there was little change in human liver. The enzymes of purine catabolism also alter rapidly in rat liver; Silverstein *et al.* (1969) have shown that xanthine oxidase activity rises sharply between 25 and 40 days of age, and guanine aminohydrolase increases from birth to a maximum activity at about 40 days.

In the testes, marked increases in hypoxanthine/guanine phosphoribosyltransferase occurred in man and the rat. Despite the high activity in adult human testes, no activity could be detected in spermatozoa or seminal fluid. Correlation of the developmental changes in rat testes with known patterns of hormonal, enzymic and cellular change show a number of associations. Some of the patterns are shown in Fig. 1. The sharp increase in hypoxanthine/guanine phosphoribosyltransferase over 5–20 days of life is associated with active synthesis of DNA, RNA and protein; specifically, there is an increase in 5'-nucleotidase (Xuma & Turkington, 1972). In spermatogenesis, active DNA synthesis is associated with spermatogonia, with RNA synthesis continuing up to the spermatid stage (Fox & Fox, 1967). The pattern of hypoxanthine/guanine phosphoribosyltransferase activity is similar to the pattern of testosterone in plasma (Miyachi *et al.*, 1973). Full reproductive capacity is found at about 100 days in the PVG strain studied. At this time (15 weeks) testes as well as central-nervous-system and liver tissues show their highest hypoxanthine/guanine phosphoribosyltransferase activities (Table 4).

The ease with which human erythrocytes can be obtained has allowed many studies of hypoxanthine/guanine phosphoribosyltransferase in these cells.

Since we have found no unequivocal evidence for tissue-specific isoenzymes in man, the results of such work may be applicable to this enzyme obtained from other tissues, at least in man. The erythrocyte enzyme purified to homogeneity (Arnold & Kelley, 1971) still shows three fractions with similar immunological properties. They reassociated after separation by isoelectric focusing, which may account for our failure to alter the electrophoretic pattern by prior incubation with sodium dodecyl sulphate. The available evidence suggests that hypoxanthine/guanine phosphoribosyltransferase is a polymeric protein in equilibrium with catalytically competent subunits (Bakay *et al.*, 1972).

The available evidence does not exclude the possibility of an altered hypoxanthine/guanine phosphoribosyltransferase in testes. Alternatively, the increased stability of this enzyme in testes (Table 5) may be due to substrate stabilization by 5-phosphoribosyl 1-pyrophosphate, possibly produced in relatively large amounts (Mayes, 1967; Greene *et al.*, 1970) and modulated, like testosterone secretion, by trophic hormones (McKerns, 1973). However, dialysis should have lowered the concentration of endogenous substrates in our studies. If substrate stabilization does exist in testes, it may be a factor in the control of hypoxanthine/guanine phosphoribosyltransferase activity in testes.

The results presented show that in man hypoxanthine/guanine phosphoribosyltransferase plays an important role in nucleotide metabolism in the central nervous system and, probably, in the testes.

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