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Metabolism of hypoxanthine in isolated rat hepatocytes

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1. The hepatic metabolism of hypoxanthine was investigated by studying both the fate of labelled hypoxanthine, added at micromolar concentrations to isolated rat hepatocyte suspensions, and the kinetic properties of purified hypoxanthine/guanine phosphoribosyltransferase from rat liver. 2. More than 80% of hypoxanthine was oxidized towards allantoin; less than 5% of the label was incorporated into the purine mononucleotides, and a similar proportion appeared transiently in inosine. The maximal velocity of oxidation (approx. 750 nmol/min per g of cells) was in close agreement with the known activity of xanthine oxidase in liver extracts. In contrast, the maximal velocity of the incorporation of labelled hypoxanthine into mononucleotides reached only 30 nmol/min per g of cells, compared with an activity of hypoxanthine/guanine phosphoribosyltransferase, measured at substrate concentrations analogous to those prevailing intracellularly, of 500 nmol/min per g of cells. 3. Hypoxanthine incorporation into the mononucleotides was decreased by allopurinol, anoxia and ethanol, despite inhibition of its oxidation under these conditions; it was increased by incubation of the cells in supraphysiological concentrations of P_i. Allopurinol and anoxia decreased the concentration of phosphoribosyl pyrophosphate inside the cells by respectively 40 and 60%, ethanol had no effect on the concentration of this metabolite and P_i increased its concentration up to 10-fold. 4. The kinetic study of purified hypoxanthine/guanine phosphoribosyltransferase showed that a mixture of ATP, IMP, GMP and GTP, at the concentrations prevailing in the liver cell, decreased the $V_{max.}$ of the enzyme 6-fold, increased its K_m for hypoxanthine from 1 to 4 μM and its K_m for phosphoribosyl pyrophosphate from 2.5 to 25 μM. In the presence of 5 μM-hypoxanthine and 2.5 μM-phosphoribosyl pyrophosphate, the mixture of nucleotides inhibited the activity of purified hypoxanthine/guanine phosphoribosyltransferase by 95%. 5. It is concluded that this inhibition results in a limited participation of hypoxanthine/guanine phosphoribosyltransferase in the control of the production of allantoin by the liver.

Investigations of the uptake of supraphysiological concentrations of hypoxanthine (100–200 μM) by isolated rat hepatocytes have shown that only small amounts were incorporated into the cellular nucleotide pool (Smith *et al.*, 1977; Lalanne & Lafleur, 1980). The reasons for this are not immediately apparent, since the K_m for hypoxanthine as well as the $V_{max.}$ of HGPRT, the enzyme catalysing the first step of this incorporation, are in the same range (Olsen & Milman, 1974; Wohlhueter, 1975) as those of the two other enzymes that can compete for the utilization of hypoxanthine in the liver, namely nucleoside phosphorylase (EC 2.4.2.1) and xanthine oxidase (EC 1.2.3.2) (Stirpe & Della Corte, 1969; Zimmerman *et al.*, 1971; Shenoy & Clifford, 1975). Moreover, the influence of the concentration of *PP*Rib*P*, the other substrate of HGPRT, on the utilization of hypoxanthine by isolated hepatocytes, has not been evaluated. An interrelationship is likely, since the hepatic concentration of

Abbreviations used: APRT, adenine phosphoribosyltransferase (EC 2.4.2.7); HGPRT, hypoxanthine/guanine phosphoribosyltransferase (EC 2.4.2.8); *PP*Rib*P*, 5-phosphoribosyl 1-pyrophosphate.

*PP*Rib*P* (Clifford *et al.*, 1972; Lalanne & Henderson, 1974, 1975) and the K_m of liver HGPRT for this metabolite (Olsen & Milman, 1974; Wohlhueter, 1975) are both around 5 μM. In the present work, the reasons for the restricted incorporation of hypoxanthine in the hepatic purine mononucleotides have been investigated. This has been done by studying, under various experimental conditions, the fate of the purine base, added at micromolar concentrations, to isolated rat hepatocyte suspensions, and by assessing the kinetic properties of purified HGPRT from rat liver at concentrations of substrates and effectors analogous to those prevailing inside the cells.

Materials and methods

Chemicals and enzymes

6-Mercapto-9-(tetrahydro-2-furyl)purine was a gift from Dr. Ven L. Narayanan, National Cancer Institute, Silver Spring, MD, U.S.A. Allopurinol [4-hydroxypyrazolo(3,4-*d*)pyrimidine], AMP- and GMP–agarose were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. DEAE-cellulose (DE 52) was from Whatman Biochemicals, Maidstone, Kent, U.K. Sephadex G-25 (fine grade) and Sephacryl S-300 were from Pharmacia Fine Chemicals, Uppsala, Sweden. APRT was purified from liver by affinity chromatography on AMP–agarose (Hershey & Taylor, 1978), to a specific activity of 0.2unit/mg of protein at 37°C. The enzyme was stored in 0.3% bovine serum albumin. Immediately before use, in order to remove *PP*Rib*P*, it was filtered by centrifugation, as described by Penefsky (1977), through a column (0.4cm × 6cm) containing Sephadex G-25 (fine grade) equilibrated with 50mM-Tris/HCl buffer (pH7.4)/10mM-$MgSO_4$/30mM-KCl. GTP and *PP*Rib*P* (tetrasodium salt) were from Boehringer GmbH, Mannheim, Germany. GTP was purified immediately before use by DEAE-cellulose chromatography, with a 0–0.5M linear gradient of NaCl in 20mM-Tris/HCl buffer, pH7.0. It was free of GMP and contained less than 2% of GDP as checked by high-pressure anion-exchange chromatography. *PP*Rib*P* was found to be 25–30% pure by the assay described below. [8-^{14}C]Hypoxanthine (53Ci/mol), [8-^{14}C]inosine (55Ci/mol), [8-^{14}C]IMP (56Ci/mol) and [U-^{14}C]adenine (296Ci/mol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. For the assay of *PP*Rib*P*, it was purified on polyethyleneimine–cellulose thin-layer plates that were developed in butan-1-ol/methanol/water (1:1:8, by vol.) (Reibel & Rovetto, 1978). 1,4-Dithio-DL-threitol was obtained from Fluka, Buchs, Switzerland. The source of all other chemicals has been given (Van den Berghe *et al.*, 1980).

Experiments with isolated hepatocytes

The results shown are representative of at least three experiments. The preparation of hepatocytes from fed adult male Wistar rats and the method used for the extraction of metabolites after incubation of the hepatocytes (at a concentration of approx. 50mg of cells/ml) with labelled purines have been given in detail (Van den Berghe *et al.*, 1980). The radioactivity in hypoxanthine, in inosine, in the sum of xanthine, uric acid and allantoin, and in the purine mononucleotides (the latter remaining at the origin) was determined by chromatography with appropriate carriers (50nmol) on cellulose thin-layer plates that were developed four times in the same dimension in butan-1-ol/methanol/water/conc. NH_3 (sp.gr. 0.910) (60:20:20:1, by vol.) (Crabtree & Henderson, 1971). One-dimensional chromatography of the purine mononucleotides on polyethyleneimine–cellulose thin-layer plates (Crabtree & Henderson, 1971) was hampered by the presence of labelled uric acid, which trailed over the plates. In some experiments, determination of the radioactivity in the individual nucleotides was therefore performed by separating first the nucleosides and bases from the nucleotides on cellulose plates. Thereafter the nucleotide spot (remaining at the origin), was scraped off, the powder was eluted with water, and the concentrated eluate was applied on the polyethyleneimine–cellulose thin-layer plate. The concentration of hypoxanthine, measured spectrophotometrically by the method of Jorgensen (1974), which detected approx. 1nmol/ml of cell suspension, was unmeasurable in the hepatocytes unless allopurinol was present. Except in the latter condition, the concentration of endogenous hypoxanthine was therefore neglected and the amount of metabolites formed from added [^{14}C]hypoxanthine was calculated from its specific radioactivity.

For the measurement of *PP*Rib*P*, 0.25ml of the hepatocyte suspension was mixed with 0.1ml of cold 10% (w/v) trichloroacetic acid and promptly centrifuged at 500*g* for 1min. The supernatant was immediately neutralized with 1M-Tris/HCl buffer, pH9.0. *PP*Rib*P* was measured by adding a portion of the extract to 0.6nmol of [^{14}C]adenine and 0.2munits of purified APRT in 50mM-Tris/HCl buffer (pH7.4)/5mM-$MgCl_2$, in a total volume of 0.1ml. Incubations were run in duplicate at 37°C for 30–40min. The reactions were stopped by spotting 10 μl portions of the mixture on a polyethyleneimine–cellulose plate on which 10 μl of 5mM carrier solutions of adenine and AMP had been applied. After development in butan-1-ol/methanol/water (1:1:8, by vol.) the amount of *PP*Rib*P* was calculated from the quantity of radioactive AMP formed. Recovery of *PP*Rib*P* added

to the trichloroacetic acid just before mixing with the cell suspension was 90–100%.

Measurement of enzyme activities

The activity of HGPRT was measured at 37°C by the rate of incorporation of [^{14}C]hypoxanthine into IMP. The incubation medium contained 50mM-Tris/HCl buffer, pH7.4, 5mM-$MgCl_2$, various concentrations of *PP*Rib*P* and of [8-^{14}C]-hypoxanthine and other additions as indicated, in a total volume of 0.1ml. The reaction was initiated by the addition of the enzyme and terminated at various time intervals by spotting 10μl of the incubation on polyethyleneimine–cellulose plates, on which carrier solutions (50nmol) of hypoxanthine and IMP had been applied. These were developed as described for the measurement of *PP*Rib*P*. The activity of APRT was measured as described for HGPRT, except that [^{14}C]hypoxanthine was replaced by labelled adenine and that this base and AMP were utilized as carriers. The activity of nucleoside phosphorylase was determined with 0.1mM-[^{14}C]inosine in 50mM-potassium phosphate buffer (pH7.4)/5mM-$MgCl_2$. The reaction was terminated by spotting 10μl of the incubation on polyethyleneimine–cellulose plates that had been preloaded with inosine and hypoxanthine and were developed as described for the measurement of *PP*Rib*P*. The activities of 5'-nucleotidase and xanthine oxidase were measured with labelled IMP and hypoxanthine respectively (each 0.1mM) in 50mM-Tris/HCl buffer (pH7.4)/5mM-$MgCl_2$. The assays were also terminated by spotting portions of the incubation on polyethyleneimine–cellulose plates that had been preloaded with appropriate carriers and were developed in butan-1-ol/methanol/water/conc. NH_3 (sp.gr. 0.910) (60:20:20:1, by vol.).

Purification of hypoxanthine/guanine phosphoribosyltransferase

All steps were carried out at 0–4°C. Rat liver (8–10g) was homogenized in 2vol. of 50mM-Tris/HCl buffer, pH7.4, containing 50mM-KCl, 6mM-$MgCl_2$, 0.1mM-EDTA and 2mM-dithiothreitol (buffer A). After centrifugation for 60min at 30000*g*, the supernatant was heated for 3min at 70°C. After cooling, it was centrifuged for 10min at 14000*g* and the resulting supernatant was applied on a Sephacryl S-300 column (2.6cm × 35cm) equilibrated with buffer A. The column was eluted with the same buffer. Fractions (2ml) containing maximal enzyme activity (accounting together for approx. 5% of total proteins) were applied on a GMP–agarose column (0.7cm × 6cm) equilibrated with 20mM-Tris/HCl buffer, pH7.4, containing 20mM-KCl, 6mM-$MgCl_2$, 0.1mM-EDTA and 2mM-dithiothreitol (buffer B). After washing with 10ml of buffer B, elution of HGPRT was performed by adding 0.25mM-*PP*Rib*P* in the same buffer. The enzyme was purified 800–1300-fold (to a specific activity of 12–19units/mg of protein) and was free of adenine phosphoribosyltransferase, xanthine oxidase, nucleoside phosphorylase and 5'-nucleotidase activity. It was stored at 0°C in 0.2% bovine serum albumin. Immediately before use, the enzyme was filtered by centrifugation on a column (0.4cm × 6cm) containing Sephadex G-25 (fine grade) in buffer A, as described by Penefsky (1977), to remove *PP*Rib*P*.

Results

Rates of metabolism of hypoxanthine in isolated hepatocytes

Addition of labelled hypoxanthine, at micromolar concentrations, resulted in its rapid uptake and metabolism. As shown in Fig. 1, 10μM-hypoxanthine was nearly completely metabolized within 60s, 80% being oxidized towards allantoin and 4% incorporated into the mononucleotides; further analysis of this fraction showed that 1.3% of the label was incorporated in IMP, 0.1% in AMP, 0.8% in ADP and 1.8% in ATP. Approx. 4% of the label appeared in inosine at 10s and disappeared progressively thereafter. The curves (Fig. 1) allow a graphic estimate of the initial velocities of the metabolism of hypoxanthine into its two main directions. The rates calculated from Fig. 1 and from data obtained simultaneously with hypo-

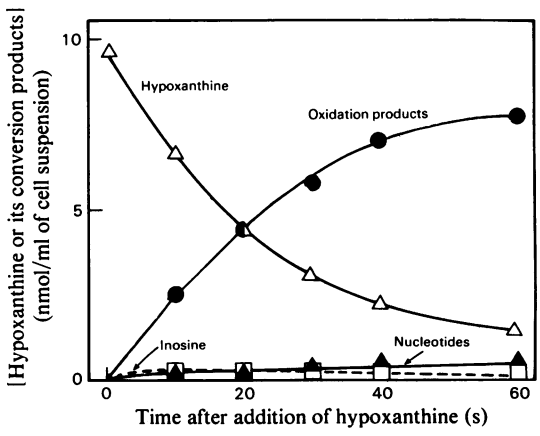


Fig. 1. *Time-course of the conversion of hypoxanthine into nucleotides, inosine and oxidation products in hepatocytes*
[^{14}C]Hypoxanthine was added at a final concentration of 10μM. (For further methodological details, see Van den Berghe *et al.*, 1980).

xanthine concentrations of 2.5–75 μM are depicted in Fig. 2. The maximal rate of oxidation of hypoxanthine into allantoin reached 750 nmol/min per g of cells, the half-maximal velocity being observed at approx. 20 μM. Incorporation into the purine mononucleotides reached a maximum velocity of 30 nmol/min per g of cells at 25 μM-hypoxanthine. Half of this velocity was obtained at approx. 2 μM-hypoxanthine, but hypoxanthine concentrations above 25 μM became inhibitory.

Effects of the inhibition of xanthine oxidase

In view of the very rapid oxidation of hypoxanthine, experiments were designed to inhibit this process. Allopurinol, a potent inhibitor of xanthine oxidase, renders hypoxanthine instead of allantoin the final product of adenine nucleotide catabolism. Accordingly, 100 μM-allopurinol caused a nearly complete block of the oxidation of hypoxanthine into allantoin (Fig. 3*b*). Contrary to expectations, the initial rate of incorporation of hypoxanthine into the purine nucleotides was diminished from 14 to 8 nmol/min per g of cells (Fig. 3*d*). The utilization of hypoxanthine was profoundly decreased (Fig. 3*a*), and a progressive accumulation of the label in inosine became evident (Fig. 3*c*). The rate of incorporation of hypoxanthine decreased rapidly after 10 s. In the control hepatocytes, as shown in Fig. 1, it stopped when the purine base was completely utilized. In the presence of allopurinol it continued over the whole 60 min duration of the experiment, at a rate of 1.5 nmol/min per g of cells (Fig. 3*d*, inset), owing to the persistence of the purine base in the cell suspension.

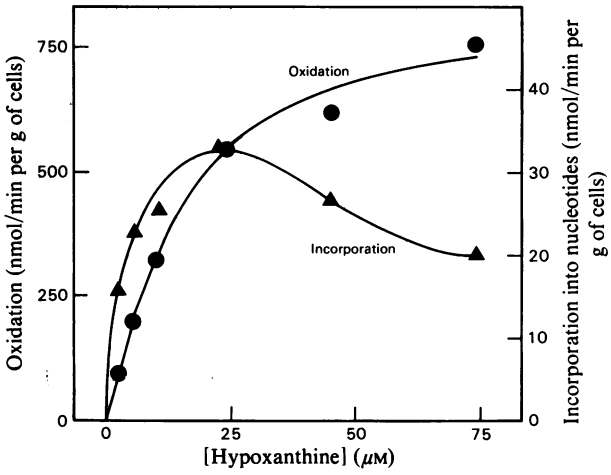


Fig. 2. *Influence of the concentration of hypoxanthine on the initial rates of its conversion into nucleotides and oxidation products*

The initial rates were estimated graphically from time-course experiments performed as depicted in Fig. 1 with various concentrations of [8-^{14}C]-hypoxanthine.

Substantial evidence has been presented that xanthine oxidase acts as a NAD^+-dependent dehydrogenase *in vivo* (Battelli *et al.*, 1972; Waud & Rajagopalan, 1976). Increases in the NADH/NAD^+ ratio, such as those induced by anoxia or ethanol, might thus be expected to modify the metabolism of hypoxanthine. As shown in Fig. 4, preincubation of isolated hepatocytes with ethanol or in a N_2/CO_2 atmosphere induced only slight decreases in the rate of utilization of 10 μM-hypoxanthine (Fig. 4*a*) and in the formation of its oxidation products (Fig. 4*b*). More pronounced effects, which were most marked in anoxic conditions, were observed on the transient conversion into inosine, which was enhanced (Fig. 4*c*), and on the incorporation into the nucleotides, which was decreased (Fig. 4*d*). All these effects were accentuated with higher concentrations of hypoxanthine (results not shown).

The decrease in the rate of incorporation of hypoxanthine into the nucleotides, observed with the various inhibitors of xanthine oxidase, prompted a study of their influence on the concentration of *PP*Rib*P*, the other substrate of HGPRT. Depending on the batch of hepatocytes, the concentration of *PP*Rib*P* varied from 2 to 6 nmol/g of cells. The concentration of *PP*Rib*P* was decreased by approx. 40% after 5 min of incubation with 100 μM-allopurinol, in accordance with data reported for erythrocytes by Fox *et al.* (1970). It decreased by 60% after 20 min of gassing with N_2/CO_2, but was not significantly modified 15 min after the addition of 20 mM-ethanol.

Influence of high concentrations of P_i

The observation that two of the experimental conditions that decreased the rate of incorporation of hypoxanthine into the purine nucleotides, namely incubation with allopurinol and in N_2/CO_2, also decreased the concentration of *PP*Rib*P*, prompted a study of the influence of increasing this substrate on the metabolism of hypoxanthine. The concentration of *PP*Rib*P* can be increased in various types of cells by their incubation with supraphysiological concentrations of P_i (Hershko *et al.*, 1967; Barankiewicz & Henderson, 1977; Bashkin & Sperling, 1978; Raivio *et al.*, 1981). In accordance with those studies, we found that the intracellular concentration of *PP*Rib*P* in isolated hepatocytes increased in a concentration-dependent way on addition of P_i. As depicted in Fig. 5(*b*), these conditions also provoked a concentration-dependent enhancement of the incorporation of hypo-

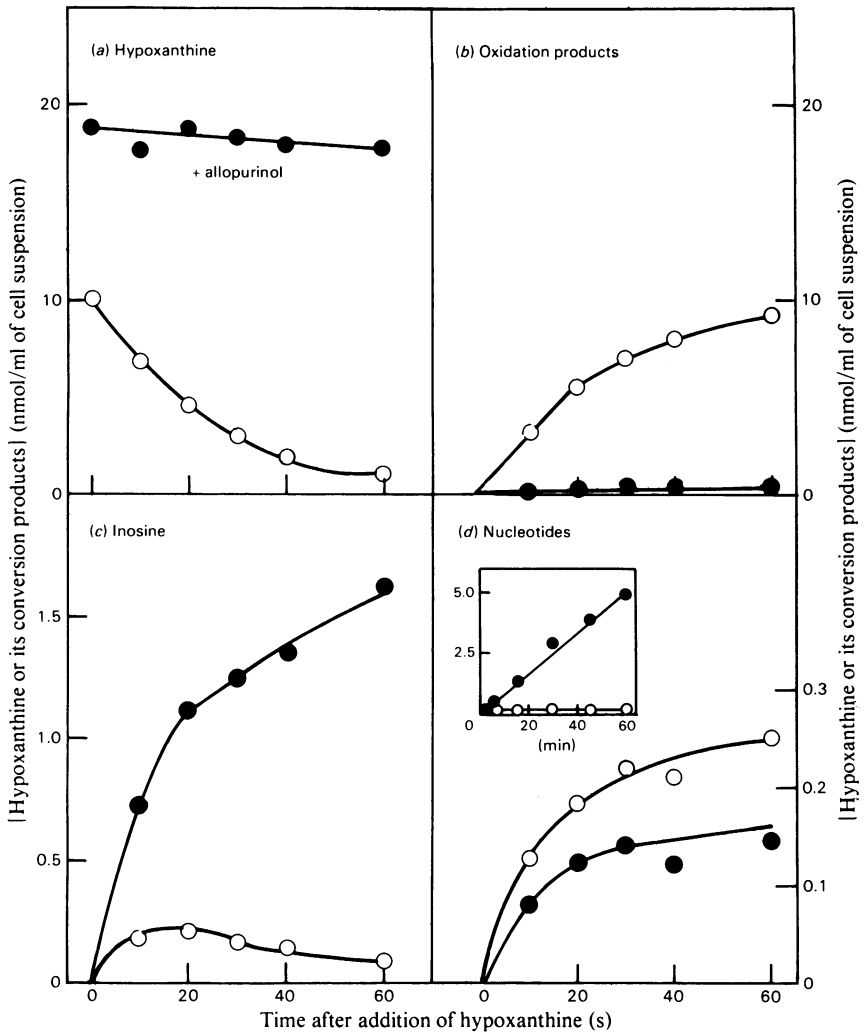


Fig. 3. *Influence of allopurinol on the metabolism of hypoxanthine*

After 5min of incubation without (○) or with (●) 100μM-allopurinol, [8-^{14}C]hypoxanthine (10μM) was added to hepatocyte suspensions. The formation of oxidation products, inosine and purine mononucleotides from labelled hypoxanthine was calculated, taking into account the allopurinol-induced increase in the concentration of hypoxanthine. After a slight decrease during the first 60s (panel *a*), this concentration increased linearly at the rate of 23nmol/min per g of cells and reached the value of 93nmol/per ml of cell suspension at 60min. For further methodological details, see Fig. 1 legend.

xanthine into the purine nucleotides, up to about 3-fold the control value at 25mM-P_i. In contrast, the transient accumulation of inosine recorded after the addition of hypoxanthine was decreased by more than 50% (Fig. 5*a*). The rates of utilization of hypoxanthine and of its conversion into allantoin were not significantly modified (results not shown). The effect of 25mM-P_i to increase the concentration of *PP*Rib*P* and the rate of incorporation of hypoxanthine into the nucleotides was also observed in the presence of allopurinol. It resulted in a complete conversion of the purine base into nucleotides within 15min (results not shown).

Effects of the inhibition of HGPRT

As an additional assessment of the role of HGPRT in the metabolism of hypoxanthine, either added to the hepatocyte suspension or generated during normal purine catabolism, experiments were performed with 6-mercapto-9-(tetrahydro-2-furyl)purine, an inhibitor of this

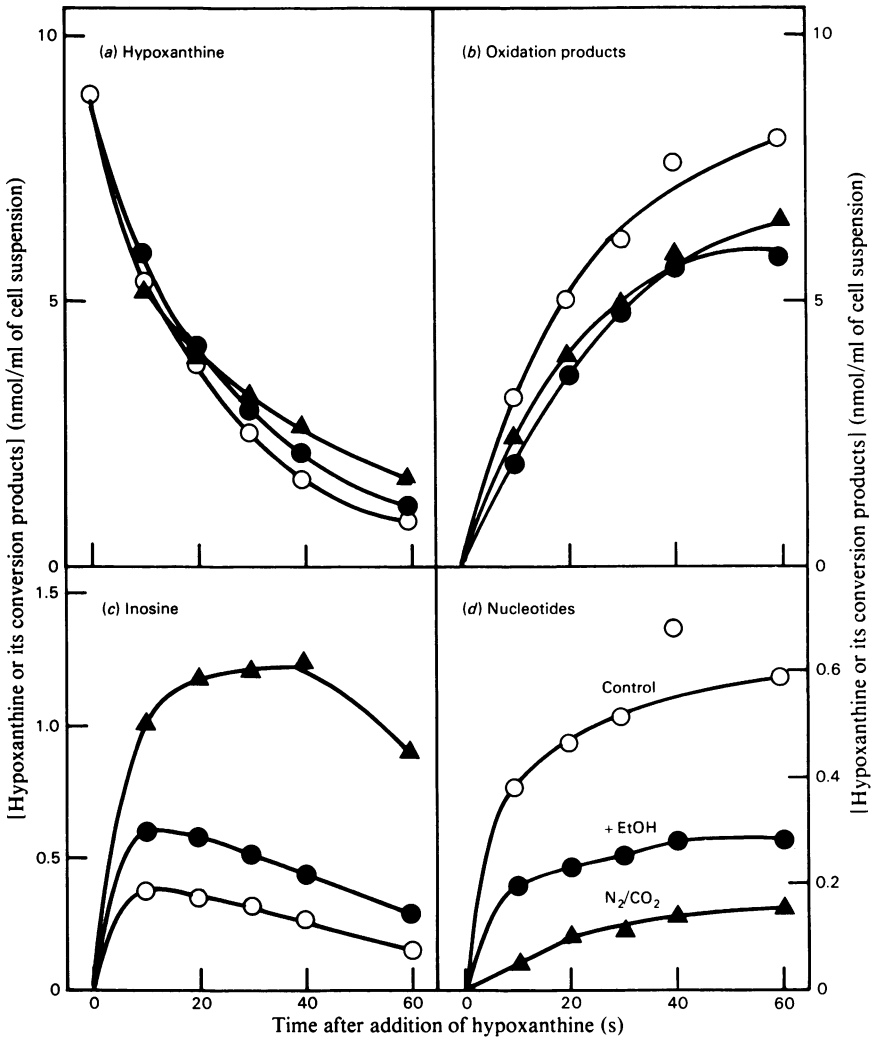


Fig. 4. *Influence of ethanol and anoxia on the metabolism of hypoxanthine*
[8-^{14}C]Hypoxanthine (10μM) was added to control cells (○) or to hepatocytes that had been preincubated for 15min in the presence of 20mM-ethanol (●) or for 20 min in N_2/CO_2 (19 : 1) (▲). For further methodological details, see Fig. 1 legend.

enzyme in human erythrocytes and mouse ascites-tumour cells (Jadhav *et al.*, 1979). It was verified that this compound also inhibited purified HGPRT from rat liver (see below). The incorporation of 20μM-hypoxanthine into nucleotides was decreased in a dose-dependent manner, to 40% of the control value at 0.5–1mM inhibitor, without significant modification of the concentration of *PP*Rib*P* (results not shown). 6-Mercapto-9-(tetrahydro-2-furyl)purine also induced a small dose-dependent decrease in the utilization of hypoxanthine and a parallel increase in the accumulation of inosine (results not shown). Prelabelling of the adenine nucleotide pool of the cells with 1μM-[^{14}C]adenine allows the subsequent observation of a linear decrease in the radioactivity in the adenine nucleotides, mirrored by an equivalent increase in the radioactivity in allantoin, which reflects the basal rate of adenine nucleotide catabolism (Van den Berghe *et al.*, 1980). Neither parameter was modified during a 60min incubation of the cells with 6-mercapto-9-(tetrahydro-2-furyl) purine at 0.8mM (results not shown).

Activity of HGPRT in liver extracts

In high-speed supernatants prepared from homogenates of either rat liver or isolated hepatocytes, the activity of HGPRT, measured at

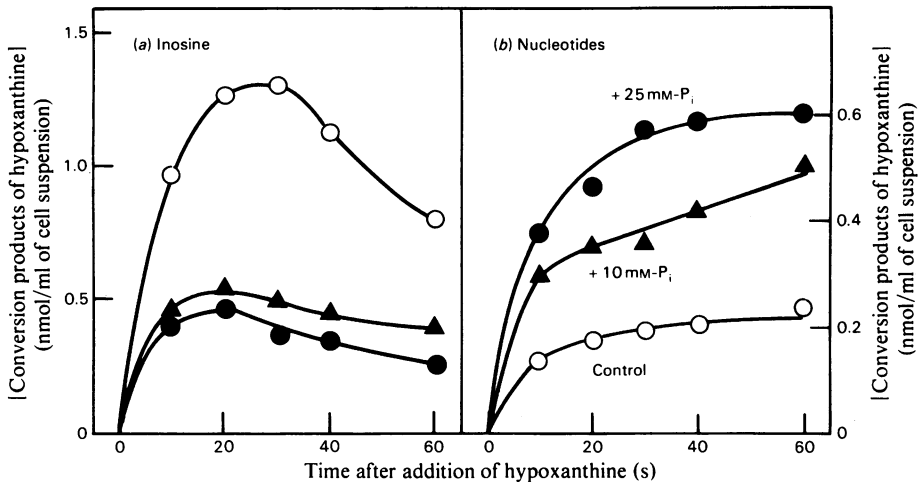


Fig. 5. *Influence of high concentrations of P_i on the conversion of hypoxanthine into inosine and nucleotides*
Hepatocytes were prepared with Krebs–Henseleit bicarbonate buffer containing only 0.55mM-$CaCl_2$ and pre-incubated for 20min without (○) or with addition of (▲) 10mM- or (●) 25mM-Na_2HPO_4, pH 7.4. At zero time, the concentration of *PP*Rib*P* (nmol/g of cells) was 2.1 in control conditions (P_i = 1.12mM), 12.7 with addition of 10mM-P_i and 26.9 with addition of 25mM-P_i. Hypoxanthine was then added at a final concentration of 10μM. For further methodological details, see Fig. 1 legend.

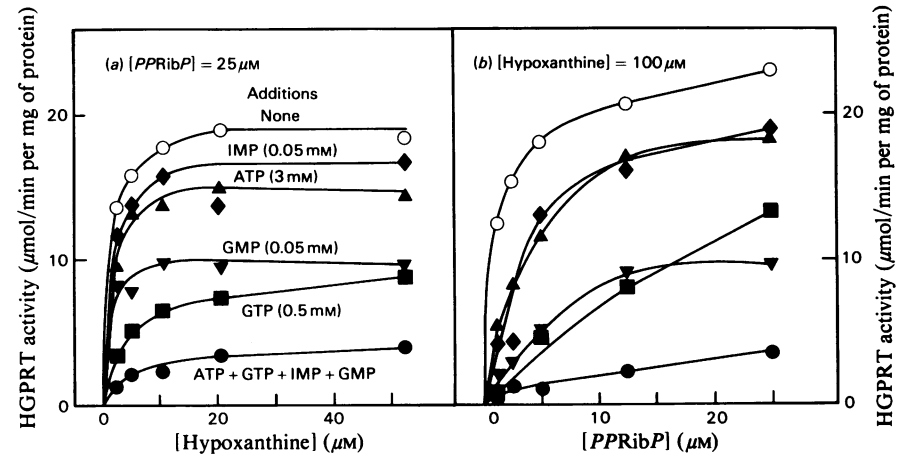


Fig. 6. *Influence of purine mononucleotides on the saturation curves of purified HGPRT for hypoxanthine and PPRibP*
The concentrations of the effectors used are those present in control liver (Clifford *et al.*, 1972; Vincent *et al.*, 1982). The same symbols are used in parts (*a*) and (*b*).

micromolar concentrations of hypoxanthine (10μM) and *PP*Rib*P* (5μM), reached approx. 0.5μmol/min per g of tissue at 37°C. Neither was significantly modified by filtration on Sephadex G-25. Substrate-saturation curves showed a K_m of approx. 2μM for hypoxanthine and a progressive inhibition of enzymic activity above 50μM. K_m for *PP*Rib*P* was approx. 2μM. Addition of a variety of purine mononucleotides at their physiological concentrations in the liver cells revealed that several of them, most notably GMP and GTP, were exerting a potent inhibition on the enzymic activity. These results prompted a detailed study of their influence on a purified preparation of liver HGPRT.

Studies with purified HGPRT

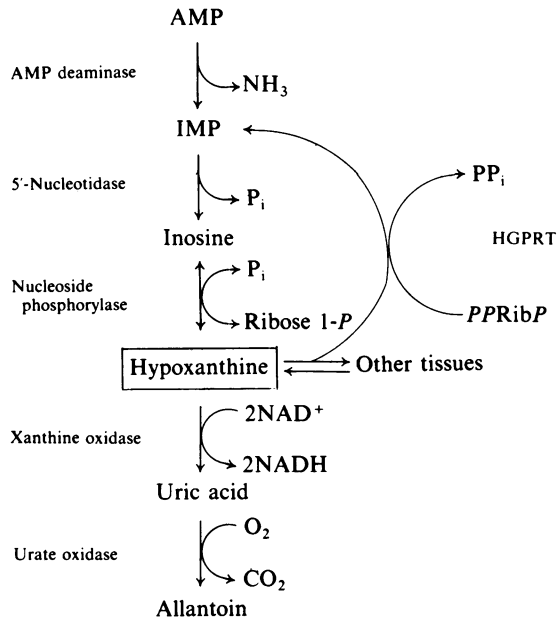
Fig. 6(*a*) depicts the influence of various purine mononucleotides on the substrate-saturation curve for hypoxanthine of purified HGPRT from rat

liver, assayed in the presence of a saturating concentration of *PP*Rib*P*. Under all conditions the saturation curves were hyperbolic, the half-maximal velocity being obtained at 1 μM-hypoxanthine under control conditions. Inhibition by high concentrations of substrate (up to 500 μM) was not observed. Physiological concentrations of ATP, IMP, GTP and GMP exerted mixed inhibitions that were most pronounced with the guanine nucleotides. A mixture of the four nucleotides, at the concentrations prevailing in control liver cells, increased the K_m for hypoxanthine of the enzymic preparation 4-fold and decreased its $V_{max.}$ 6-fold.

Substrate-saturation curves for *PP*Rib*P*, obtained in the presence of a saturating concentration of hypoxanthine, were also hyperbolic, with K_m approx. 2.5 μM for *PP*Rib*P* in the absence of additions (Fig. 6*b*). The order of potency of physiological concentrations of the inhibitory nucleotides and their effect on the $V_{max.}$ were comparable with those depicted in Fig. 6(*a*). Their influence was, however, more pronounced on the K_m for *PP*Rib*P* than on the affinity for hypoxanthine. The physiological mixture of nucleotides increased the K_m of the enzyme for *PP*Rib*P* approx. 10-fold. Measurements of the activity of purified HGPRT at concentrations of hypoxanthine and *PP*Rib*P* in the physiological range (5 and 2.5 μM respectively) revealed that it was 95% inhibited by the physiological mixture of nucleotides. The same degree of inhibition was observed in the presence of mixtures of nucleotides at concentrations prevailing in the liver cells in anoxia (Vincent *et al.*, 1982) (results not shown). Other nucleotides, namely ADP and AMP, as well as P_i and PP_i, were without effect when added at physiological concentrations. The activity of purified HGPRT was inhibited by 70% by 0.5 mM-6-mercapto-9-(tetrahydro-2-furyl)purine and was not influenced by glycerol 3-phosphate up to 10 mM.

Discussion

In the liver cell, hypoxanthine is formed from inosine by nucleoside phosphorylase and can be either reutilized by HGPRT to form IMP, or oxidized to the terminal product of hepatic purine catabolism, uric acid or allantoin according to the species (Scheme 1). In addition, the possibility exists that HGPRT, together with the cytoplasmic 5'-nucleotidase and nucleoside phosphorylase, may allow a futile recycling of metabolites, as shown by Barankiewicz *et al.* (1982) in human mononuclear cells and fibroblasts. The balance between the activities of HGPRT and xanthine oxidase inside the liver cell derives its importance from the fact that it may constitute a supplementary control of hepatic adenine nucleotide catabolism, located



Scheme 1. *Pathways of formation, oxidation and phosphoribosylation of hypoxanthine in the liver*

distally from AMP deaminase, the limiting enzyme of this process (for a review see Van den Berghe, 1981). It could also potentially play a role in the regulation of the purine economy of the body as a whole, since it may control the fate of hypoxanthine, provided to the liver by tissues in which the latter compound constitutes the end-product of purine catabolism. Finally, the sum of the activities of HGPRT and xanthine oxidase may determine the supply of purines, in the form of hypoxanthine, to other organs. Indeed, the liver is often considered the major source of purine available to other tissues for salvage (for a review see Murray, 1971).

In the present work, information about the three routes of hypoxanthine metabolism has been obtained by combining studies of the fate of labelled hypoxanthine added to suspensions of isolated rat hepatocytes with a kinetic investigation of purified HGPRT from rat liver.

Oxidation of hypoxanthine

In accordance with findings by other authors (Smith *et al.*, 1977; Lalanne & Lafleur, 1980), we found that hypoxanthine added to suspensions of isolated rat hepatocytes was rapidly metabolized, mainly to allantoin (Fig. 1). The maximal velocity of this process reaching 750 nmol/min per g of cells (Fig. 2), is in close agreement with the $V_{max.}$ of xanthine oxidase, determined in rat liver extracts (Stirpe & Della Corte, 1969; Hashimoto, 1974).

The concentration of hypoxanthine providing the half-maximal rate of oxidation (20 μM) was about 10-fold higher than the K_m for xanthine of the purified rat liver enzyme (Waud & Rajagopalan, 1976). The nearly complete inhibition of the oxidation of hypoxanthine towards allantoin, observed in isolated hepatocytes in the presence of allopurinol (Fig. 3*b*), is in agreement with the findings of Lalanne & Lafleur (1980). The decrease in the rate of oxidation of hypoxanthine in the presence of ethanol and in anoxia (Fig. 4*b*), and its dependence on the concentration of the purine base (M. F. Vincent, unpublished work), can be explained by an inhibition of the dehydrogenase (type D) form of xanthine oxidase by NADH, as shown in rat liver extracts by Della Corte & Stirpe (1970) and with the partially purified enzyme by Kaminski & Jezewska (1981). It is indeed postulated that xanthine oxidase is predominantly in the dehydrogenase form intracellularly, as also evidenced by our previous finding (Vincent *et al.*, 1982) that anoxic hepatocytes continue to form uric acid, although, in accordance with the oxygen-dependence of urate oxidase (Keilin & Hartree, 1936), their production of allantoin is inhibited. In comparison with the marked elevation of the $NADH/NAD^+$ ratio recorded under the influence of ethanol and anoxia, and with the low K_i (about 3 μM) of the inhibition of xanthine dehydrogenase by NADH (Della Corte & Stirpe, 1970), the decrease in the metabolism of hypoxanthine nevertheless appears strikingly limited. This may be explained by the existence of multiple cytoplasmic pools of NAD^+, which are influenced to different degrees by modifications of the redox state of the hepatocyte, as demonstrated by Berry *et al.* (1980).

Conversion of hypoxanthine into inosine

The transient increase in the concentration of inosine, observed after the addition of 10 μM-hypoxanthine to the hepatocyte suspension (Figs. 1, 3*c*, 4*c* and 5*a*), has also been observed with higher concentrations of the purine base by Lalanne & Lafleur (1980). As discussed by those authors, it could be explained by a reversal of the nucleoside phosphorylase reaction or/and by the hydrolysis of IMP formed from hypoxanthine. This hydrolysis involves the cytoplasmic 5'-nucleotidase, which, according to our previous studies (Van den Berghe *et al.*, 1977*b*; Bontemps *et al.*, 1983), is the only 5'-nucleotidase that intervenes in the catabolism of intracellular hepatic 5'-nucleotides. From the observation that the accumulation of inosine was decreased when the hepatocytes were incubated in supraphysiological concentrations of P_i (Fig. 5*a*), Lalanne & Lafleur (1980) had concluded that it resulted from the ribosylation of hypoxanthine. Indeed, the K_m for P_i of nucleoside phosphorylase reaches approx. 15 mM (Lewis & Glantz, 1976). However, the inhibitory effect of P_i on the cytoplasmic 5'-nucleotidase (Van den Berghe *et al.*, 1977*b*) could also have curtailed a formation of inosine from IMP. Nevertheless, two additional reasons indicate that the accumulation of inosine results mainly from a reversal of the nucleoside phosphorylase reaction. Firstly, partial inhibition of HGPRT by 6-mercapto-9-(tetrahydro-2-furyl)purine increased rather than decreased the accumulation of inosine recorded after the addition of hypoxanthine. Secondly, the accumulation of inosine was greatly enhanced by both allopurinol (Fig. 3*c*) and anoxia (Fig. 4*c*), two conditions that have in common a decrease in the concentration of *PP*Rib*P*, and are thus expected to decrease the activity of HGPRT. The effect of allopurinol can be explained by the accumulation of hypoxanthine in the cell suspension. The effect of anoxia remains unexplained, particularly since it provokes a 2–3-fold elevation of the concentration of P_i inside the hepatocytes (Vincent *et al.*, 1982).

Phosphoribosylation of hypoxanthine

The discrepancy between the high activity of HGPRT in hepatocyte extracts and the low rate of incorporation of hypoxanthine in intact cells has also been noted by Smith *et al.* (1977). It is also found in mouse brain (Wong & Henderson, 1972), human leucocytes (Henderson *et al.*, 1974), Ehrlich ascites-tumour cells (Henderson *et al.*, 1975), erythrocytes of several mammalian species (Lalanne & Willemot, 1980) and rat hepatoma cells (Wohlhueter *et al.*, 1982). Its explanation requires an evaluation of the kinetic properties of HGPRT in the conditions prevailing inside the cells. By allowing a quantitative estimate of hypoxanthine salvage, our results also provide an insight into the physiological significance of this process in the liver.

Kinetic properties of HGPRT. Inhibition of HGPRT from different tissues by several mononucleotides, including IMP and GMP, products of the reaction, has been reported (Murray, 1966; Krenitsky & Papaioannou, 1969; Olsen & Milman, 1974). Its physiological significance had, however, not been evaluated. Our results show that a mixture of inhibitory nucleotides, at the concentrations prevailing in the liver cell, markedly affects some of the kinetic parameters of the enzymic reaction. In particular, the $V_{max.}$ of the enzyme is profoundly decreased, its K_m for hypoxanthine being elevated about 4-fold and its K_m for *PP*Rib*P* increased approx. 10-fold. The 95% decrease, by the mixture of inhibitory nucleotides, of the reaction rate of the purified enzyme, measured at concentrations of hypoxanthine and *PP*Rib*P* in the physiological range is in close

agreement with the limited incorporation of hypoxanthine into the nucleotides of the intact cells. Whereas the K_m for hypoxanthine of purified HGPRT (Fig. 6*a*) was also in good agreement with the concentration of the purine base providing its half-maximal rate of incorporation into the cellular purine nucleotides, namely 2 μM (Fig. 2), we have no explanation as yet for the decrease in the latter process observed at hypoxanthine concentrations above 25 μM. The inhibition of HGPRT by an excess of substrate which we have found in crude liver extracts confirms data for the same preparation by Wohlhueter (1975). It has, however, not been reported for the purified enzyme. An increased feedback inhibition, caused by an elevation of the concentration of IMP, appears unlikely in view of the limited incorporation of hypoxanthine into the nucleotides.

The mixture of inhibitory nucleotides increased the K_m for *PP*Rib*P* of purified liver HGPRT from approx. 2.5 μM, which is in the range of the hepatic concentration of this metabolite, to about 25 μM. The decrease in the velocity of the incorporation of hypoxanthine into the nucleotides induced by allopurinol and anoxia, as well as its increase in the presence of supraphysiological concentrations of P_i, can thus be explained by the modification of the concentration of *PP*Rib*P* recorded in these conditions. The slower but long-lasting incorporation of hypoxanthine into the nucleotides recorded in the presence of allopurinol (Fig. 3*d*), is in accordance with the 10-fold augmentation of the incorporation of hypoxanthine into rat liver nucleic acid purines observed 17h after the administration of allopurinol *in vivo* (Pomales *et al.*, 1963). We have no explanation as yet for the decreased incorporation of hypoxanthine into the nucleotides provoked by ethanol, since this compound did not modify the concentration of *PP*Rib*P*. The activity of purified liver HGPRT was also not influenced by concentrations of glycerol 3-phosphate in the range of those recorded after ethanol administration (results not shown).

Physiological significance of hypoxanthine salvage. Previous work from this laboratory has led to the conclusion that AMP deaminase constitutes the initial step as well as the limiting enzyme of the catabolism of the adenine nucleotides in the liver (Van den Berghe *et al.*, 1977*a*,*b*, 1980; Vincent *et al.*, 1982). A continuous production of adenosine by the cytoplasmic 5'-nucleotidase, discovered by Bontemps *et al.* (1983), was shown not to contribute to the basal production of allantoin because of the recycling of adenosine by adenosine kinase. Our present work was aimed at an evaluation of the role of HGPRT in the control of the hepatic production of allantoin from hypoxanthine derived from this organ or from other tissues.

In theory, the exact rate of reutilization of hypoxanthine by the liver in physiological conditions, as well as the existence of a futile cycle between IMP and hypoxanthine, could be assessed by the increase in the turnover rate of the adenine nucleotides and of the production of allantoin induced by a complete inhibition of the activity of HGPRT. However, because of the low potency of the HGPRT inhibitor 6-mercapto-9-(tetrahydro-2-furyl)purine, no conclusion could be drawn from the observation that neither parameter was modified by the addition of this compound. From the data depicted in Fig. 2, it can be calculated that at concentrations of hypoxanthine around that observed in rat liver (below 1 μM in basal conditions) the rate of reutilization of the purine base is proportional to its concentration and reaches 20–30% of its rate of oxidation. Increases in the concentration of hypoxanthine, such as recorded in human plasma during intense exercise (Harkness *et al.*, 1983), will thus result not only in an increase in the rate of oxidation of the purine base, but also in an enhancement of its rate of reutilization, thereby limiting to a certain extent the loss of purines known to occur under these conditions (Sutton *et al.*, 1980). In so far as they can be extrapolated to human pathology, our results indicate that the liver contributes little to the overproduction of uric acid caused by the deficiency of HGPRT, characterizing the Lesch–Nyhan syndrome (Seegmiller *et al.*, 1967), as well as by other kinetic abnormalities of the enzyme (for a review see Wilson *et al.*, 1983).

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