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Effect of NADH on hypoxanthine hydroxylation by native NAD^+-dependent xanthine oxidoreductase of rat liver, and the possible biological role of this effect

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The course of the reaction sequence hypoxanthine → xanthine → uric acid, catalysed by the NAD^+-dependent activity of xanthine oxidoreductase, was investigated under conditions either of immediate oxidation of the NADH formed or of NADH accumulation. The enzymic preparation was obtained from rat liver, and purified 75-fold (as compared with the 25000 *g* supernatant) on a 5′-AMP–Sepharose 4B column; in this preparation the NAD^+-dependent activity accounted for 100% of total xanthine oxidoreductase activity. A spectrophotometric method was developed for continuous measurements of changes in the concentrations of the three purines involved. The time course as well as the effects of the concentrations of enzyme and of hypoxanthine were examined. NADH produced by the enzyme lowered its activity by 50%, resulting in xanthine accumulation and in decreases of uric acid formation and of hypoxanthine utilization. The inhibition of the xanthine oxidoreductase NAD^+-dependent activity by NADH is discussed as a possible factor in the regulation of IMP biosynthesis by the '*de novo*' pathway or (from unchanged hypoxanthine) by the salvage pathway.

The course of the reaction sequence hypoxanthine → xanthine → uric acid has been investigated with the use of xanthine oxidoreductases from *Veilonella alcalescens* (hitherto known as *Micrococcus lactilyticus*) and bovine milk (Jeżewska, 1973), from avian liver (Priest & Fisher, 1969; Cleere & Coughlan, 1975) and from mouse skeletal muscle (Lalanne & Willemot, 1975), acting with oxygen as an electron acceptor. However, xanthine oxidoreductases mostly occur *in vivo* in the NAD^+-dependent dehydrogenase form (Stirpe & Della Corte, 1969; Joyce & Duke, 1971; Battelli *et al.*, 1972; Francois, 1973; Coughlan, 1980), which, in contrast with the oxidase form, has been found to be inhibited by NADH (Della Corte & Stirpe, 1970). NADH produced during xanthine hydroxylation inhibits the enzyme by about 50% within a few second after the beginning of the reaction (Kamiński & Jeżewska, 1979). Modulation of the enzyme activity by changes in the $NADH/NAD^+$ ratio has been postulated to play a regulatory role in the living cell (Della Corte & Stirpe, 1970). Therefore it seemed of interest to elucidate the effect of NADH on the production of xanthine and uric acid from hypoxanthine by rat liver NAD^+-dependent xanthine oxidoreductase. In the present study, the course of hypoxanthine hydroxylation catalysed by this enzyme, either uninhibited or inhibited by NADH formed during the catalytic process, was compared. Preliminary results have previously been published (Jeżewska & Kamiński, 1980).

Experimental

Animals

Wistar male rats (weight about 300 g) were given standard diet produced by Doświadczalny Zakład Produkcji Pasz, Borów, Poland. They were starved for 24 h (with water *ad libitum*) before being killed.

Reagents

Sources of chemicals were as previously given (Kamiński & Jeżewska 1979). Other chemicals were purchased as follows: hypoxanthine (Serva, Heidelberg, Germany); Trasylol (inhibitor of proteinases; Bayer, Leverkusen, Germany); Hepps [4-(2-hydroxyethyl)-1-piperazinepropanesulphonic acid], A grade (Calbiochem, San Diego, CA, U.S.A.); N^6-(6-aminohexyl)-5′-AMP–Sepharose 4B (Pharmacia, Uppsala, Sweden); KOH, especially pure (UCB, Drogenbos, Belgium). KH_2PO_4, p.A. (Polskie Odczynniki Chemiczne, Gliwice, Poland) was purified from traces of heavy metals as follows:

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a nearly saturated KH_2PO_4 solution was passed through a column (1.5cm × 10cm) packed with Chelex 100 (K^+ form, 100–200 mesh). Redistilled methanol was added to the effluent (2:1, v/v), the precipitate of KH_2PO_4 was separated on Whatman glass-fibre paper GF/C and dried in a vacuum desiccator over P_2O_5.

Enzyme preparation

All operations were performed in a cold-room. The procedure was essentially the same as in our previous paper (Kamiński & Jeżewska, 1979), except that the solution for rat liver homogenization was modified and contained 250mM-sucrose, 100mM-Hepps/KOH buffer, pH8.0, 15mM-dithiothreitol and Trasylol (2500 kallikrein-inhibitory units/ml; Kraut *et al.*, 1930). The $(BH_4)_2SO_4$ fraction suspended in 250mM-sucrose (3ml, total xanthine oxidoreductase activity 25nkat) was applied to a 5′-AMP–Sepharose 4B column (Pharmacia Laboratory Column K 15/30, height 20cm), previously equilibrated with 10mM-KH_2PO_4 adjusted to pH6.0 with 1M-KOH (Lowe *et al.*, 1974). After 60min of standing without flow, the column was washed with 200ml of 10mM-KH_2PO_4 adjusted to pH8.6 with 1M-KOH (Greenfield & Pietruszko, 1977). Pooled fractions with the highest NAD^+-dependent activity of xanthine oxidoreductase were stored at −20°C.

The $(NH_4)_2SO_4$ fraction and the enzyme preparation after column chromatography were used in further experiments. The total activity and its distribution between three forms (D, D/O and O) of the enzyme were determined as previously described (Kamiński & Jeżewska, 1979), and expressed in pkat/mg of protein. Protein was determined by the method of Stauffer (1975).

Hypoxanthine, xanthine and uric acid assays

The course of hypoxanthine hydroxylation catalysed by the NAD^+-dependent activity of xanthine oxidoreductase was investigated in the presence of accumulating NADH or under conditions of instantaneous reoxidation of NADH formed. Therefore the standard incubation mixture contained, in a total volume of 3ml: 50mM-Tris/HCl buffer, pH8.0, hypoxanthine at micromolar concentrations, enzyme preparation (20–200pkat/ml), and 175–350μM-NAD^+, or all the above components plus 40nkat of lactate dehydrogenase and 0.5mM-sodium pyruvate. Blanks contained no hypoxanthine.

Reactions were performed at room temperature (20°C) in quartz cells (light-path 1cm) under aerobic conditions. During hypoxanthine hydroxylation with concomitant NADH accumulation, the difference spectra were recorded within the range 250–350nm; when NADH was prevented from accumulation by the action of lactate dehydrogenase, increases in A_{279} and A_{302} were continuously recorded. A Cary 118C spectrophotometer equipped with a repetitive scan was used.

The sum of xanthine and uric acid was determined from the increases in A_{279} [isosbestic point of these purines, differing by 0.5nm from that found previously (Jeżewska, 1973)]. Under the conditions used, the isosbestic point of NAD^+ and NADH occurred at 279nm; thus the changes in their concentrations did not contribute to the increase in A_{279} caused by formation of xanthine and uric acid. Uric acid was determined from an increase in A_{302}; if lactate dehydrogenase and pyruvate were omitted from the incubation mixture, then a correction for NADH absorbance at 302nm was made, as previously described (Kamiński & Jeżewska, 1979). Also appropriate corrections were made to rule out the effect of hypoxanthine disappearance and of the changes in xanthine concentration on the increases in A_{279} and A_{302} respectively. For this purpose the molar absorption coefficients were determined: $\varepsilon_{279}^{Xan} = \varepsilon_{279}^{Uri} = \varepsilon_{302}^{Uri} = 7.58 \times 10^3$, $\varepsilon_{302}^{Xan} = 0.45 \times 10^3$ and $\varepsilon_{279}^{Hyp} = 0.75 \times 10^3$ litre·mol^{-1}·cm^{-1} (abbreviations: Xan, xanthine; Uri, uric acid, Hyp, hypoxanthine). The increases in A_{279} measured during hypoxanthine hydroxylation were caused by changes in the concentrations (C) of three purines:

$$\Delta A_{279} = \varepsilon_{279}^{Xan} \cdot \Delta C_{Xan} + \varepsilon_{279}^{Uri} \cdot \Delta C_{Uri} - \varepsilon_{279}^{Hyp} \cdot \Delta C_{Hyp} \quad (1)$$

In eqn. (1) we substituted $\Delta C_{Hyp} = \Delta C_{Xan} + \Delta C_{Uri}$ and $\varepsilon_{279}^{Xan} = \varepsilon_{279}^{Uri}$, and thus:

$$\Delta A_{279} = (\varepsilon_{279}^{Xan} - \varepsilon_{279}^{Hyp}) \cdot (\Delta C_{Xan} + \Delta C_{Uri}) \quad (2)$$

The increases in A_{302} measured during hypoxanthine hydroxylation were caused by changes in concentrations of two purines:

$$\Delta A_{302} = \varepsilon_{279}^{Xan} \cdot \Delta C_{Xan} + \varepsilon_{302}^{Uri} \cdot \Delta C_{Uri} \quad (3)$$

By introducing the values obtained for molar absorption coefficients and by solving eqns. (1), (2) and (3) for changes in the concentrations of hypoxanthine, xanthine and uric acid, we obtain:

$$-\Delta C_{Hyp} = 146.5 \Delta A_{279}$$

$$\Delta C_{Xan} = 155.6 \Delta A_{279} - 140.2 \Delta A_{302}$$

$$\Delta C_{Uri} = 140.2 \Delta A_{302} - 9.2 \Delta A_{279}$$

The calculated amounts of the three purines were expressed in nmol/ml of the incubation mixture.

Results

The $(NH_4)_2SO_4$ fraction and the fraction eluted from the 5′-AMP–Sepharose column were purified 6- and 75-fold, respectively, as compared with the

25000*g* supernatant. The former fraction at first showed only the NAD^+-dependent activity of xanthine oxidoreductase related to the presence of the dehydrogenase and dehydrogenase/oxidase forms (Kamiński & Jeżewska, 1979); during storage, the O_2-dependent activity of the oxidase form appeared. The latter preparation eluted with commercial KH_2PO_4 showed up to 30% of the O_2-dependent activity. However, after removal of traces of heavy metals from KH_2PO_4, the NAD^+-dependent activity accounted for 100% of the total xanthine oxidoreductase activity of the enzyme preparations. On storage at pH 8.6 and −20°C, the total and NAD^+-dependent activities remained unchanged for only 3 days.

In the presence of lactate dehydrogenase and pyruvate, no difference in the course of the reaction sequence hypoxanthine → xanthine → uric acid was found, irrespective of whether the $(NH_4)_2SO_4$ fraction or other fraction after column chromatography was used as the enzyme source. However, the use of the more purified preparation afforded more precise spectrophotometric data. A change in the NAD^+ concentration from 175 to 350 μM had no effect on the reaction course. When lactate dehydrogenase and pyruvate were omitted from the incubation mixture, 350 μM-NAD^+ was found to be saturating for hydroxylation of 50 μM-hypoxanthine until its total transformation into uric acid. At lower NAD^+ concentrations the utilization of hypoxanthine decreased.

On account of the much longer reaction time under conditions of NADH accumulation even at saturating NAD^+ concentrations, we verified whether the observed decrease in the hypoxanthine utilization rate was due only to enzyme inhibition by NADH or also to the enzyme inactivation during prolonged incubation. This was done by observation of a set of progress curves, in which all parameters except enzyme concentration were kept constant, and by plotting the results with the use of an abscissa not of time but of time multiplied by enzyme concentration (Selwyn, 1965). Under conditions of NADH reoxidation and for incubation times up to 60 min, the curves of hypoxanthine and xanthine utilization (Figs. 1*a* and 1*b* respectively), obtained at different enzyme concentrations, were superimposed. This result, together with the observation that the total enzymic activity did not change during the transformation of the dehydrogenase form to the oxidase form (Kamiński & Jeżewska, 1979), showed that xanthine oxidoreductase was not inactivated during the experiment.

The time-course of hypoxanthine hydroxylation to intermediate xanthine and then to uric acid under NADH-reoxidation conditions (Figs. 2*a* and 2*b*, curves with open symbols) was found to be independent of the initial hypoxanthine concentration; the reaction-progress curves of hypoxanthine utilization and of xanthine and uric acid formation, obtained for initial 23.2 μM, 45.0 μM- and 85.0 μM-hypoxanthine, were superimposable when the dimensions of both axes were properly selected. The comparison of the initial hypoxanthine hydroxy-

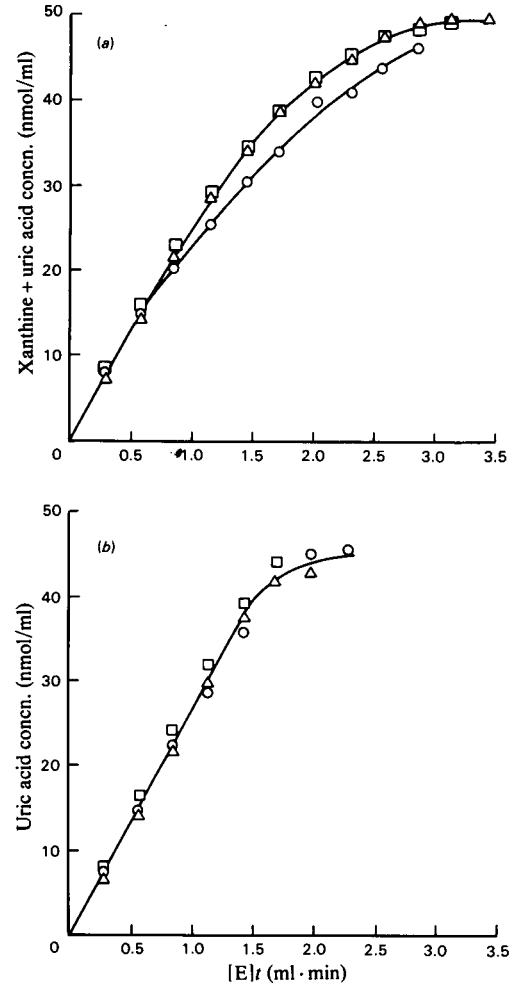

Fig. 1. *Reaction-progress curves of hypoxanthine and xanthine utilization plotted against time multiplied by activity of the NAD^+-dependent forms of xanthine oxidoreductase*

Methods are described in the Experimental section. The incubation mixture contained 350 μM-NAD^+, lactate dehydrogenase + pyruvate, xanthine oxidoreductase (△, 0.2 ml; □, 0.1 ml; ○, 0.05 ml) and (*a*) 50 μM-hypoxanthine or (*b*) 45 μM-xanthine. Utilization of hypoxanthine was measured as the sum of the products, xanthine + uric acid, and that of xanthine as uric acid formation.

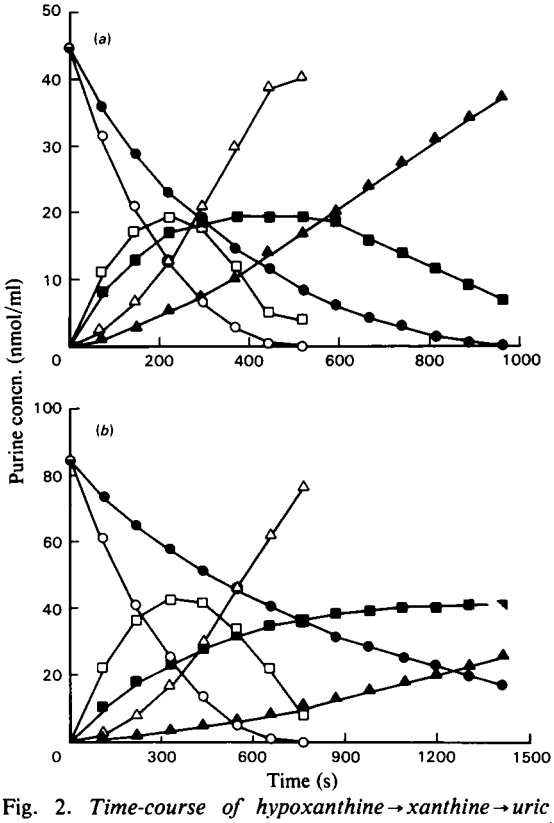

Fig. 2. *Time-course of hypoxanthine→xanthine→uric acid hydroxylation by the NAD⁺-dependent activity of xanthine oxidoreductase, uninhibited or partly inhibited by NADH*

Methods are described in the Experimental section. The incubation mixture, with or without lactate dehydrogenase and pyruvate, contained: (*a*) 45 μM-hypoxanthine, 350 μM-NAD^+ and 167 pkat of enzymic activity/ml; (*b*) 85 μM-hypoxanthine, 175 μM-NAD^+ and 220 pkat of enzymic activity/ml. Open symbols, oxidized NADH; closed symbols, accumulating NADH. ○, ●, Hypoxanthine; □, ■, xanthine; △, ▲, uric acid.

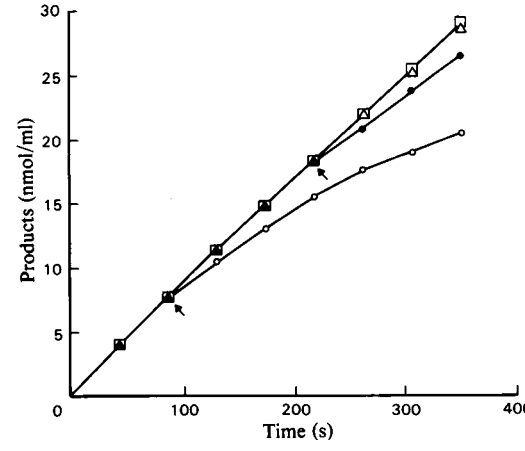

Fig. 3. *Comparison of hypoxanthine and xanthine hydroxylation by the NAD⁺-dependent forms of xanthine oxidoreductase at saturating concentrations of substrates*

Methods are described in the Experimental section. The incubation mixture contained: 350 μM-NAD^+, lactate dehydrogenase + pyruvate, enzyme (83 pkat/ml) and either hypoxanthine (○, 23.2 μM; ●, 46.4 μM; △, 92.8 μM) or xanthine (□, 93.0 μM). Hydroxylation of both purines was measured as in Fig. 1. Curves representing hypoxanthine hydroxylation became non-linear when the hypoxanthine/xanthine ratio fell to about 2.6 (arrows).

lation rates at various substrate concentrations (Fig. 3) indicated that all three hypoxanthine concentrations used were initially saturating under experimental conditions and that an excess of hypoxanthine exerted no inhibitory effect.

The plot of $1/v$ versus $1 + (\alpha/S)$ (Yun & Suelter, 1977), corresponding to a single reaction progress curve of hypoxanthine utilization, showed a non-linear convex shape; therefore the apparent Michaelis constant for hypoxanthine could not be determined by this method. The hyperbolic shape of the reaction-progress curve of hypoxanthine utilization (Figs. 2*a* and 2*b*), under conditions of NADH reoxidation (curves with open symbols), has suggested that this process may follow first-order kinetics (Jeżewska & Kamiński, 1980), as in the model of the time-course plot for two consecutive reactions discussed by Gutfreund (1972, pp. 123–124). However, in the present studies the procedure described by Guggenheim (1926) gave a non-linear plot of $\ln(S_i - S'_i)$ versus time (Gutfreund, 1972, p. 118). An analysis of the data obtained showed that the half-time of hypoxanthine utilization decreased with time. It is difficult to interpret this fact. Hypoxanthine and xanthine are competitive substrates for xanthine oxidase from mouse muscle (Lalanne & Willemot, 1975). Thus xanthine released into the reaction medium (Figs. 2*a* and 2*b*) may inhibit hypoxanthine utilization, and moreover hypoxanthine may inhibit further xanthine hydroxylation to uric acid; these interrelations are complicated by the continuous changes in the hypoxanthine/xanthine ratio in the incubation mixture. This problem may probably be simplified somewhat by the fact that the rates of hydroxylation of both purines seem to be the same. When the hypoxanthine concentration was high enough (92.8 μM) for the accumulating xanthine to be unable to

compete for the enzyme, the reaction-progress curve was initially identical with that obtained for hydroxylation of xanthine used at the same concentration (Fig. 3).

In the presence of accumulating NADH, the nature of curves representing the two-step hypoxanthine hydroxylation (Figs. 2*a* and 2*b*, curves with closed symbols) remained unchanged. However, the process was slower and the same stages of purine-ring hydroxylation were attained after a longer time. At saturating concentration of NAD^+, accumulating NADH inhibited hypoxanthine utilization by about 35%. This inhibition was lower than that found with xanthine as substrate (about 50%; Kamiński & Jeżewska, 1979); however, when both consecutive reactions (i.e. the total number of the hydroxyl groups introduced into the purine ring of hypoxanthine) were taken into account, the enzyme inhibition approached 50%. The inhibition by NADH mainly decreased utilization of hypoxanthine and lowered the formation of uric acid, whereas xanthine concentration remained high (Fig. 2*a*). If the NAD^+ concentration was not saturating for the given concentration of hypoxanthine, then hypoxanthine utilization and uric acid formation decreased even more, but the xanthine concentration was still high (Fig. 2*b*).

The dependence of the two-step hypoxanthine hydroxylation on the NAD^+-dependent xanthine oxidoreductase concentration under conditions of NADH reoxidation and NADH accumulation is presented in Fig. 4 (curves with open and with closed symbols respectively). The decrease in hypoxanthine utilization caused by NADH accumulation was nearly the same within the enzyme concentration range of 65–150 pkat/ml. Within this enzyme concentration range, in the presence of NADH accumulating, xanthine was maintained at the same concentration, in contrast with a decrease in xanthine concentration occurring with an increase of enzyme concentration under conditions of NADH reoxidation. When NADH accumulated, the higher the initial enzyme concentration used, the greater was the decrease in uric acid formation.

The effect of the concentration of hypoxanthine on its hydroxylation by the NAD^+-dependent xanthine oxidoreductase activity is presented in Fig. 5. The changes in concentrations of three purines in the reaction medium under conditions of NADH reoxidation (curves with open symbols) and of

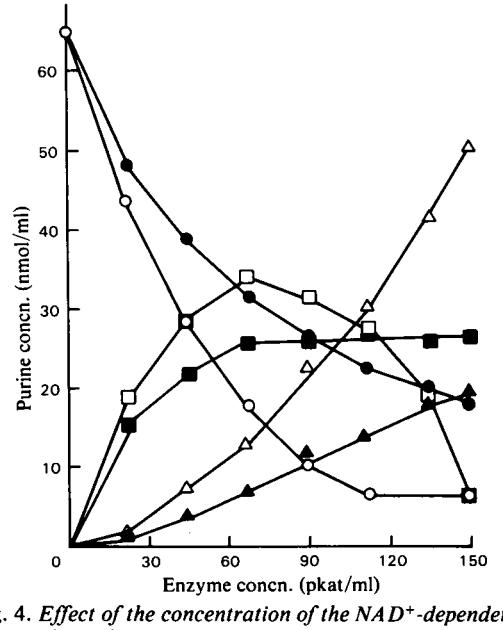

Fig. 4. *Effect of the concentration of the NAD^+-dependent forms of xanthine oxidoreductase, uninhibited or partly inhibited by NADH, on hypoxanthine→xanthine→uric acid hydroxylation*

Experimental conditions and symbols are as for Fig. 2; 65 μM-hypoxanthine and 175 μM-NAD^+ were used. Reaction time was 10 min.

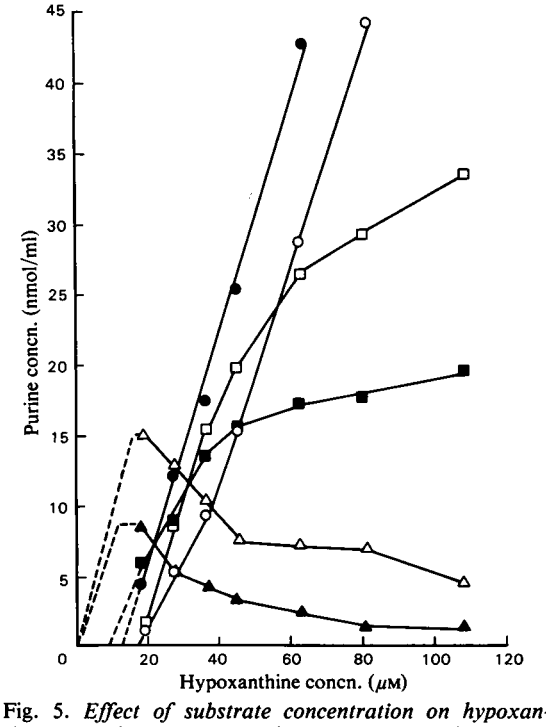

Fig. 5. *Effect of substrate concentration on hypoxanthine→xanthine→uric acid hydroxylation by the NAD^+-dependent forms of xanthine oxidoreductase, uninhibited or partly inhibited by NADH*

Experimental conditions and symbols are as in Fig. 2; 175 μM-NAD^+ and an enzymic activity of 107 pkat/ml were used. Reaction time was 5 min.

NADH accumulation (curves with closed symbols) were parallel, except that xanthine appeared at lower hypoxanthine concentration and that hypoxanthine was utilized to a smaller extent when the enzyme was inhibited by NADH. Within the range of low hypoxanthine concentrations this substrate was entirely transformed into uric acid within a reaction time shorter than the 5 min used in the present experiments (see extrapolation of curves representing xanthine concentration in Fig. 5).

The time course of hypoxanthine hydroxylation by the NAD^+-dependent form of xanthine oxidoreductase from rat liver, and the dependence of this process on the enzyme activity and substrate concentration under NADH-reoxidation conditions, resemble those found with the oxidase form of the enzyme from milk (Jeżewska, 1973, 1974). However, in progress curves the lag phase of uric acid formation seems to be shorter for the NAD^+-dependent form; the cause of this difference remains unexplained.

Discussion

As shown in the present paper, the inhibition of the NAD^+-dependent xanthine oxidoreductase activity by NADH at physiological (Tischler *et al.*, 1977) micromolar concentrations resulted in a decrease in hypoxanthine hydroxylation and uric acid production, as well as in accumulation of xanthine. It seems that this effect of NADH may provide a sensitive system of temporary saving of hypoxanthine in mammalian organisms. Changes in the $NADH/NAD^+$ ratio, which occur in the living cells (Gumaa *et al.*, 1971; Schultz & Lowenstein, 1978), exert a regulatory effect on many metabolic processes, including purine biosynthesis (Gumaa *et al.*, 1971). It is well known that during transient hypoxia, when the energy charge falls and the $NADH/NAD^+$ ratio rises, hypoxanthine concentration increases (Wichert *et al.*, 1972; Gerlach & Zimmer, 1976; Saugstad *et al.*, 1976; Schultz & Lowenstein, 1978; Harkness *et al.*, 1980). In view of the data presented, it seems that the increase in hypoxanthine concentration could occur not only by enhanced purine nucleotide catabolism, but also by xanthine oxidoreductase inhibition by NADH. The increased amount of hypoxanthine may enhance hypoxanthine salvage and, in turn, decrease the availability of 5-phosphoribosyl 1-pyrophosphate, a common substrate in this process and in purine biosynthesis *de novo*. The excessive purine production in the case of a purine nucleoside phosphorylase (EC 2.4.2.1) deficiency, in which the catabolism of purine nucleotide is ended at the nucleoside step (Cohen *et al.*, 1976), points to the important role of hypoxanthine in the limitation of purine biosynthesis *de novo*. At the same time, the effect of a decrease in the xanthine oxidoreductase activity on the limitation of this pathway, probably by saving hypoxanthine for the salvage pathway, is suggested by the diminished oxypurine excretion in some patients with xanthinuria (Watts *et al.*, 1964) and in patients treated with allopurinol, a xanthine oxidoreductase inhibitor (Yü & Gutman, 1964; Kelley *et al.*, 1968; Skupp & Ayvazian, 1969). However, in the latter case the interpretation is complicated owing to the possible inhibition of biosynthesis *de novo* by the increased nucleotide concentration. More recently, an enhancement of the hypoxanthine salvage pathway under conditions of xanthine oxidoreductase inhibition by allopurinol has been shown in isolated rat liver cells by Lalanne & Lafleur (1980). These authors claim that, in the presence of allopurinol, up to 46% of available phosphoribosyl pyrophosphate is used for incorporation of exogenous hypoxanthine into nucleotides (the incorporation of endogenous hypoxanthine was not evaluated by those authors), whereas in the absence of allopurinol hypoxanthine is catabolized nearly quantitatively to allantoin.

The proposed role of the inhibition of native xanthine oxidoreductase by NADH for maintenance of an equilibrium between both pathways of IMP biosynthesis could be eliminated by factors transforming the NAD^+-dependent enzyme form into the O_2-dependent form (e.g. by drugs acting as thiol reagents: Stirpe & Della Corte, 1969, 1972; Waud & Rajagopalan, 1976; Krenitsky & Tuttle, 1978). Since the latter form is not inhibited by NADH, this transformation of the enzyme could accelerate the catabolism of hypoxanthine, attenuate the salvage pathway and enhance the IMP biosynthesis *de novo*.

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