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CATABOLISM OF SOME CYTOTOXIC PURINE DERIVATIVES BY XANTHINE OXIDASE AND PEROXIDASE

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Abstract—Some cytotoxic purinyl-6-derivatives of biogenic amines and amino acids as well as the purinyl-6-derivatives of xenobiotic primary and tertiary amines can serve as substrates for xanthine oxidase. This enzymatic oxidation leads to biologically inactive 2,8-dioxy-purinyl-derivatives. The catabolism of these hydroxylated purinyl-derivatives formed by xanthine oxidase can be effected by peroxidase/hydrogenperoxide and by catalase/alkylhydrogenperoxide. The relation between substrate specificity for xanthine oxidase and cytotoxic activity will be discussed.

OUT OF the vast number of known xenobiotic purinyl-6-derivatives there is one group which stands out because of its cytotoxic activity.[1, 2] It was previously demonstrated by Lettré and co-workers that at least some of these cytotoxic purinyl-derivatives can disturb amino acid uptake of cells *in vitro*.[3, 4] Those substances which are selectively cytotoxic to tumour cells[1] such as purinyl-6-histamine, purinyl-6-ε-L-lysine and *bis*-purinyl-ethylene-diamine are, according to Lettré, catabolized and therefore detoxified by normal cells.[3] Lack of this quality in tumour cells may be the cause for destruction of tumour cells.

As there is only little known about the pathway of metabolism of these xenobiotic substances, it was our intention to find out whether these substances might serve as substrates for some isolated enzymes which are supposed to be involved in the metabolism of nonbiogenic purinyl compounds.

These investigations should lead to an understanding of the difference in quality between normal and tumour cells with regard to their ability to catabolize cytotoxic purinyl derivatives. Such a biochemical difference between normal and tumour cells might be another hint of tackling the problem of tumour growth.

In our experiments we examined more thoroughly one of the substances acting selectively *in vitro*—the purinyl-6-histamine—for its behaviour against those enzymes which were of special interest to us. We used this compound to work out a simple screening model which could simulate the possible way of catabolism, and in which the purinyl-6-histamine could be replaced by many other purinyl-6-derivatives.

MATERIALS AND METHODS

Enzymes

Rat liver xanthine oxidase: A crude enzyme fraction was isolated by fractioning the supernatant of a homogenated rat liver (2 vol. bidest. water, 0°, 30 min) with saturated ammonium sulfate solution. We used the fraction between 0·3 and 0·5

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saturation for further purification. It was redissolved in bidest. water and the precipitation was repeated four times.

Milk xanthine oxidase, nucleoside phosphorylase, catalase and peroxidase were commercial products of Firma Boehringer und Söhne, Mannheim.

Purinyl-derivatives

All commercially available purines were purchased from Firma Zellstoff, Mannheim-Waldhof.

Purinyl-6-isotryptamine was a gift from W. Schindler.[5] All other purinyl compounds were synthesized in this institute. Only part of them are published.[1, 2, 6–12] ^{3}H-purinyl-6-histamine was prepared by the Wilzbach method, ^{14}C-purinyl-6-histamine from ^{14}C-6-chloro-purine and ^{14}C-histamine.

Instruments

For spectrophotometrical experiments we used a Beckman DK2-spectrophotometer with time drive attachment, a temperature-regulated cuvette holder and 1 cm cuvettes.

Radiochromatograms were scanned by a Berthold 4π-scanner.

Chromatography

We used ascending chromatography, *n*-propanol/water 75:25, 18 cm, Schleicher und Schüll paper No. 2043 b.

RESULTS

Experiments with xanthine oxidase

The oxidation of adenine[13] and 6-methyl-amino-purine[14] by xanthine oxidase is well known. We expected therefore that this enzyme, because of its low substrate specificity, could also be involved in the catabolism of other 6-alkyl-amino-purines. The incubation mixture of purinyl-6-histamine with a protein fraction of rat liver (first ammonium sulfate fraction) showed after several hours a change of the u.v. spectrum (Fig. 1). A more purified rat liver xanthine oxidase (fourth ammonium sulfate fraction) resulted in the spectral change given in Fig. 2. The shift of the maximum of absorption corresponds with those data given for adenine and 6-methyl-amino-purine when oxidized by xanthine oxidase.

From this analogy we concluded that our purinyl-6-histamine is also oxidized by this enzyme in 2- and 8-position of the purine ring. The spectral change in time revealed that this oxidation is not a one-step-reaction. There is only an isospestic point near the end of the reaction (285 mμ), and the time drive curve (305 mμ) is not linear (Fig. 3). The accumulation of an intermediate (reaction type A $\rightarrow$ B $\rightarrow$ C) is obvious. The result of low enzyme activity (crude rat liver xanthine oxidase, (Fig. 1)) is the end of the reaction after the intermediate has been formed.

Wyngaarden and Dunn[15] have demonstrated that adenine is first oxidized in 8-position, then in 2-position. The u.v. spectrum of our intermediate also corresponds with the spectrum of an 8-oxy-purine-derivative[15] rather than with that of a 2-oxy-purine. Purinyl-6-histamine is therefore obviously transformed by xanthine oxidase via 8-hydroxy-purinyl-6-histamine into 2,8-dihydroxy-purinyl-6-histamine.

The formation of an intermediate has also been demonstrated by radiochromatography of this reaction (Fig. 4).

Milk xanthine oxidase (Boehringer) effected the same spectral changes of our substance (Fig. 2). This fact made it possible for us to test about 80 purinyl-6-derivatives for their behaviour against this enzyme.

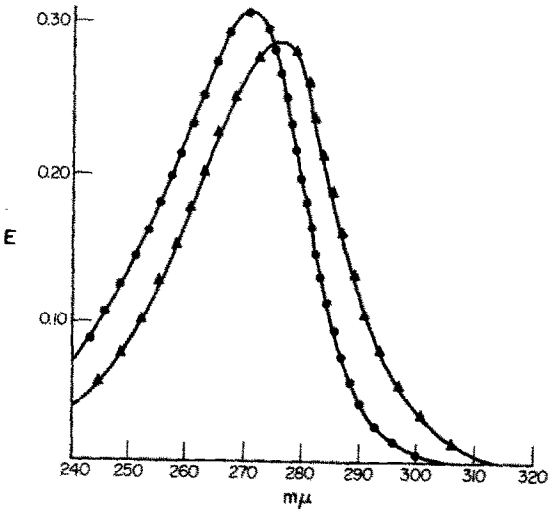

FIG. 1. Conversion of purinyl-6-histamine ● into 8-hydroxy-purinyl-6-histamine ▲ after 10 hr incubation with crude rat liver xanthine oxidase. 5×10^{-8} M substrate, 350 μg protein in m/15 phosphate buffer pH 7·4, total vol. 2·4 ml, at 20°.

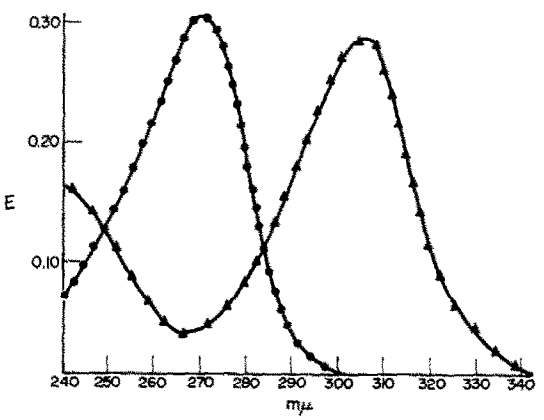

FIG. 2. Conversion of purinyl-6-histamine ● into 2,8-dihydroxy-purinyl-6-histamine ▲ by purified rat liver xanthine oxidase or by milk xanthine oxidase.

Rat liver xanthine oxidase: 5×10^{-8} M substrate, 350 μg protein in m/15 phosphate buffer pH 7·4, total volume 2·4 ml, at 35°. Reaction time 4 hr.

Milk xanthine oxidase: Protein 100 μg, reaction time 4 hr at 20°. Other data are the same as above.

All substrates showed the same typical shift of the maximum of absorption (about 30–50 mμ) to higher wave lengths.

The reaction times differed from 1 to 20 hr. (For comparison: hypoxanthine 20 min, adenine 10 hr.) As substrates for xanthine oxidase could serve all purinyl-

6-derivatives of biogenic and xenobiotic primary amines (such as histamine, tryptamine, isotryptamine, 5- and 6-fluoro-tryptamine, phenyl-ethylamine, 2-pyridyl-ethylamine, ethanol-amine and picolyl-amines) and of some amino acids (such as ε-L-lysine, L-asparagine and L-carnosine).

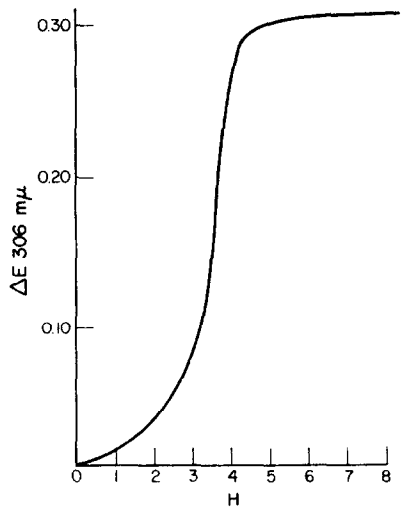

FIG. 3. Time drive curve of the reaction demonstrated in Fig. 2 (milk xanthine oxidase)

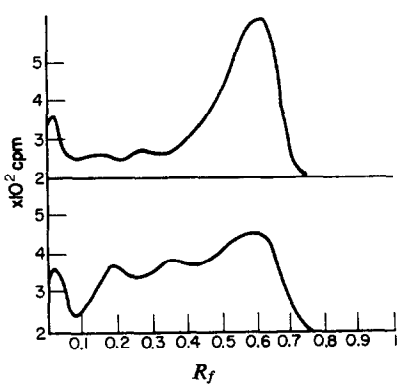

FIG. 4. Radiochromatography of the oxidation of ^{3}H-purinyl-6-histamine by milk xanthine oxidase. Incubation mixture contained 5×10^{-7} M substrate, 100 μg protein, 2 ml m/15 phosphate buffer of pH 7·4. The reaction was carried out at room temperature. Upper curve: Radiochromatogram of the incubation mixture just after mixing. There can be noticed the 'run off' peak near the starting line and the peak of the starting marerial (R_f — 0·6).

Lower curve: Radiochromatogram of the same mixture after 7 hr at room temperature. There can be noticed two additional peaks, resulting from formed intermediate (R_f = 0·35) and endproduct (R_f — 0·2).

6-C-substituted purinyl-6-derivatives could also serve as substrates provided that the C-chain was shorter than C_3 (such as 6-methyl, 6-ethyl, 6-carboxylic acid and 6-cyano). Other substrates were the *bis*-purinyl-6-derivatives of all tested diamines, the 6-alkoxy-derivatives of purine, the 6-histamine and 6-ε-L-lysine derivatives of

8-aza-purine and all purinyl-6-derivatives with an immonium structure such as purinyl-6-pyridinium-chloride and related compounds.

Those compounds which could not serve as substrates were all 9-substituted purine derivatives (R = methyl, acetyl, tetrahydropyranyl, ribosyl), dialkylated adenine-derivatives, purinyl-6-derivatives of neutral amino acids, and purinyl-6-derivatives with a quarternary ammonium structure. (Purinyl-6-trimethyl-ammonium-chloride.)

These tests for substrate specificity were all carried out according to the details given in Fig. 2.

Though the pH optimum for xanthine oxidase with purines as substrates is between pH 7 and pH 8, it can be demonstrated by Fig. 5 that the initial rate of oxidation of

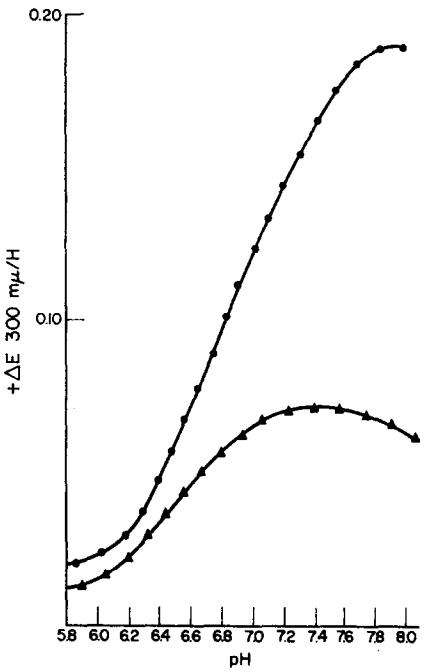

FIG. 5. pH-Dependence of initial rate oxidation of two different purinyl-6-derivatives by milk xanthine oxidase. For concentrations see Fig. 2. Purinyl-6-histamine ●; Purinyl-6-tryptamine ▲.

purinyl-6-histamine is pH dependent to a much higher degree than is the case with purinyl-6-tryptamine. This effect will be discussed in relation to the selective action of the purinyl-6-histamine.

The competitive inhibition of this enzyme by purinyl-6-histamine and purinyl-6-tryptamine is striking (Table 1) and will also be discussed in relation with the biological action of these substances.

Experiments with peroxidase and catalase

Further catabolism of the 2,8-dihydroxy purines should be possible by the same enzymes which are involved in the catabolism of uric. acid. However we could not observe any action of the very specific uricase on the 2,8-dihydroxy purines examined by us. But according to Canellakis and co-workers[16] uric. acid is catabolized by those

organisms which do not have any uricase by catalase/alkyl hydrogen peroxide and by peroxidase/hydrogen peroxide to alloxane, allantoin, alloxanic acid and urea.

By Figs. 6 and 7 it is demonstrated that our 2,8-dihydroxy-purines can serve as substrates for the same peroxidative systems.

TABLE 1

Inhibitor	10^{-7} M	2×10^{-7} M	5×10^{-7} M
Purinyl-6-histamine	19%	41%	50%
Purinyl-6-tryptamine	58%	75%	80%
Allo purinol (22)	90%	—	—

Percentage inhibition of purified milk xanthine oxidase by purinyl-6-histamine (selectively cytotoxic) and purinyl-6-tryptamine (cytotoxic), for comparison allo purinol (not cytotoxic). Substrate was hypoxanthine (10^{-7} M), 100 μg protein, phosphate buffer of pH 7·4, total vol. 2·8 ml, at 20°.

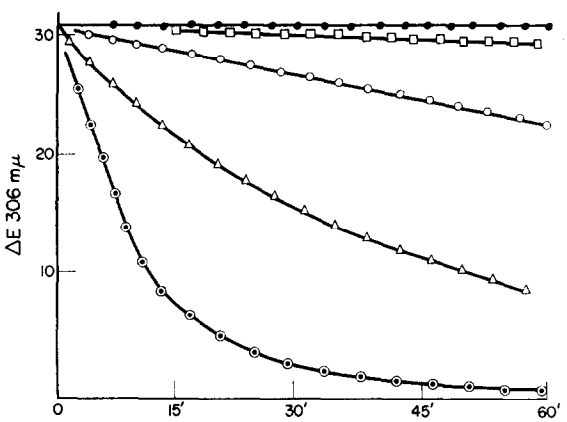

FIG. 6. Decomposition curves of 2,8-dihydroxy-purinyl-6-histamine (enzymatically formed according to the data given in Fig. 2 after addition of: ● 3×10^{-7} M hydrogen peroxide; □ 3×10^{-7} M tertiary butyl hydrogen peroxide; ○ 3×10^{-7} M tertiary butyl hydrogen peroxide/100 μg catalase; M 3×10^{-7} M peracetic acid; W 3×10^{-7} M hydrogen peroxide/20 μg peroxidase; Reactions in m/15 phosphate buffer pH 7·4 at 20°.

Any 2,8-dihydroxy-purine shows a rapid decrease in absorption when incubated with one of these systems (Fig. 6).

When a purinyl-6-derivative (substrate for xanthine oxidase) was simultaneously incubated with xanthine oxidase and peroxidase, a decrease of absorption of the maximum which is characteristic for the substrate was observed. The increase of absorption which is characteristic for the formation of a 2,8-dihydroxy-purine was not as striking as shown in Fig. 2 (Fig. 7). The hydrogen peroxide formed as a by-product of the hydroxylation is utilized by peroxidase for further peroxidation.

This oxidative decomposition has also been studied by radiochromatography (Fig. 8). The curves I → II → III are illustrating the course of the oxidative decomposition of purinyl-6-histamine which has been demonstrated by spectrophotometrical methods in Fig. 6. The curves II → IV are elucidative for Fig. 7. This means that the

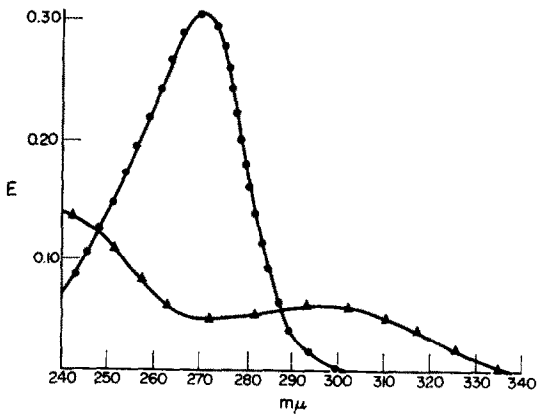

FIG. 7. Purinyl-6-histamine (5×10^{-8} M) when incubated simultaneously with 100 μg milk xanthine oxidase and 20 μg peroxidase in m/15 phosphate buffer (pH 7·4) phosphate buffer (pH 7·4) at 20°. Total vol. 2·4 ml. Absorption curve of the reaction mixture at zero time ● and after 4 hr ▲

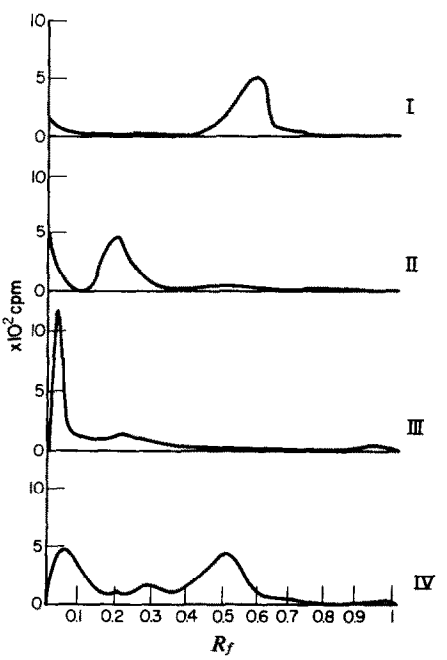

FIG. 8. Radiochromatography of the oxidation of ^{14}C-purinyl-6-histamine by milk xanthine oxidase and peroxidase. Incubation mixture: 4·3 × 10–6 M substrate in 1 ml of phospahte buffer of pH 7·4 at 35°. Total vol. for all mixtures 1.3 ml. Curve I: Without any enzyme. Curve II: 15 hr after addition of 1 mg milk xanthine oxidase. Curve III: 15 hr after addition of 1 mg milk xanthine oxidase and 0·2 mg of peroxidase. Curve IV: 15 hr after addition of 0·2 mg of peroxidase and 10^{-5} M hydrogen peroxide to the incubation mixture resulting in curve II.

peroxidation with endogeneously formed hydrogen peroxide leads to one single product (Fig. 8, curve III) while excessive hydrogen peroxide results in more than one endproduct (Fig. 8, curve IV). In this curve the peak with the $R_f = 0{\cdot}5$ seems to result from formed histamine. The R_f-value is identical with that of histamine (*n*-propanol/water) and the u.v. spectrum after eluation by 0·5 *n* HCl shows a maximum of absorption at 212 mμ which is exactly in agreement with an identical sample. The peaks with R_f-values of 0·06, 0·2 and 0·3 are not yet identified.

Experiments with phosphoribose pyrophosphorylase and nucleoside phosphorylase

Using purinyl-6-histamine as a potential substrate for the phosphoribose pyrophosphorylase/phosphoribose pyrophosphate system[17] we have been unable to detect nucleotide formation.

Another way towards nucleotide formation would be via nucleoside. But purinyl-6-histamine could neither serve as substrate for nucleoside phosphorylase. Purinyl-6-histamine-riboside, prepared by chemical methods, showed no spectral change when incubated simultaneously with nucleoside phosphorylase and xanthine oxidase.

From these facts we conclude that purinyl-6-histamine cannot be incorporated into nucleic acids. The mechanism of action seems therefore to be different from other xenobiotic purinyl-6-derivatives such as 6-mercapto-purine.[17]

DISCUSSION

The results of our experiments *in vitro* are encouraging us to the conclusion that the pathway of metabolism of some xenobiotic purinyl-6-derivatives might also be effected in living systems in two steps*: Oxidation of purinyl-6-derivatives into the corresponding 2,8-dihydroxy compounds via monohydroxylated intermediates by xanthine oxidase, followed by a peroxidation effected by catalase or peroxidase.

Furthermore, there is obviously a relation between substrate specificity against xanthine oxidase and cytotoxic action. All substances having this action belong to the group of substrates for this enzyme. There is only one cytotoxic purinyl-6-derivative (purinyl-6-trimethyl-ammonium-chloride) which could not serve as substrate in our *in vitro* experiments. It could be derived from this fact that the oxidized products formed by xanthine oxidase could be the biologically active substances. But as the 2,8-dihydroxy-purinyl-6-histamine did not show the same effect on cells in tissue culture as was observed in the case of unoxidized purine derivative, we must conclude that oxidation is equivalent to detoxification.

The selective cytotoxic effect of purinyl-6-histamine on tumour cells could be explained by the low xanthine oxidase activity in tumour cells.[18] By normal cells the drug is oxidized and therefore detoxified while in tumour cells it remains unchanged for a longer period. In addition, there is a strong pH dependence in the oxidation of purinyl-6-histamine (Fig. 5). The pH in tumour cells which is lower than in normal cells (glycolysis) increases this effect.

If we consider those cytotoxic compounds which are toxic in normal and tumour cells (e.g. purinyl-6-tryptamine) we should expect that these compounds should fully develop their toxic character before possible catabolism can start.

Though the pathway of catabolism and the selective action of purinyl-6-histamine is hereby explained, the mechanism of toxic action remains still unknown.

* Preliminary notes on this subject have been published by us earlier.[19, 20]

Feigelson and co-workers[21] have discussed a relation between the carcinostatic action of some purine derivatives and inhibition of xanthine oxidase by these substances. We also found that the cytotoxic purinyl-6-derivatives are inhibitors for this enzyme (Table 1), but since there are known much stronger inhibitors (e.g. allo purinol[22]) without any action on cells in tissue culture, one cannot conclude that inhibition of this enzyme is the reason for cytotoxic or carcinostatic action of these compounds.

Elion *et al.*[23] have demonstrated that inhibitors for this enzyme are increasing the carcinostatic effect of 6-mercapto-purine. In this case rapid catabolism of the drug is avoided by inhibitors and 6-mercapto-purine is transformed at a higher degree into the nucleotide, the true biologically active substance.

Another possibility of interaction of xanthine oxidase with the cytotoxic purinyl-6-derivatives might be the fixation of the latter at a special region of the surface of the cell where they might disturb normal membrane permeability. Encouraging work has been started in this field.

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