2-AMINO-4-HYDROXY-6-FORMYLPTERIDINE, AN INHIBITOR OF PURINE AND PTERINE OXIDASES*

by

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INHIBITION OF XANTHINE OXIDASE BY PTEROYLGLUTAMIC ACID PREPARATIONS

Recent investigations have revealed that a number of pterine compounds exert a marked inhibition of xanthine and xanthopterin oxidase. Addition of a few micrograms of folic acid to xanthine or xanthopterin oxidase reduce the activity of these enzymes markedly[1]. Several commercial pteroyl-glutamic acid (PGA) preparations are capable in concentrations of only 2 μg per ml to reduce the activity of xanthine oxidase to about 10% of that of the uninhibited sample (Table I). The inhibitory potency of such preparations can be eliminated by incubation with xanthine oxidase. After a few hours of incubation the oxidation of xanthopterin is fully restored and that of xanthine has increased markedly (Fig. 1, Table II).

TABLE I

THE INHIBITORY ACTION OF PGA AND OF THE 6-ALDEHYDE ON XANTHINE OXIDASE

Xanthine, $6.7 \cdot 10^{-8}$ mole per ml of 0.2 *M* phosphate buffer, pH 7.6; enzyme, 30 μg of protein per ml. Technique, differential spectrophotometric measurements at $\lambda = 330$ mμ[15]

	Moles of xanthine oxidized in the first 10 minutes after addition of enzyme	Rate of oxidation in per cent of that of the uninhibited sample
Xanthine	$6.0 \cdot 10^{-8}$	100
Xanthine plus PGA, $4.0 \cdot 10^{-9}$ mole per ml	$7.4 \cdot 10^{-9}$	13
Xanthine plus irradiated* PGA $2.3 \cdot 10^{-9}$ mole per ml	$3.3 \cdot 10^{-9}$	5
Xanthine plus 6-aldehyde (H_2SO_3 cleavage prod.) $4.2 \cdot 10^{-10}$ mole per ml	$1.8 \cdot 10^{-9}$	3

* Light source, mercury vapour quarts lamp; time of irradiation, 30 minutes; distance 10 cm; H_2O filter, silica cuvettes

* with the technical assistance of Mrs. I. MØLLER-JENSEN

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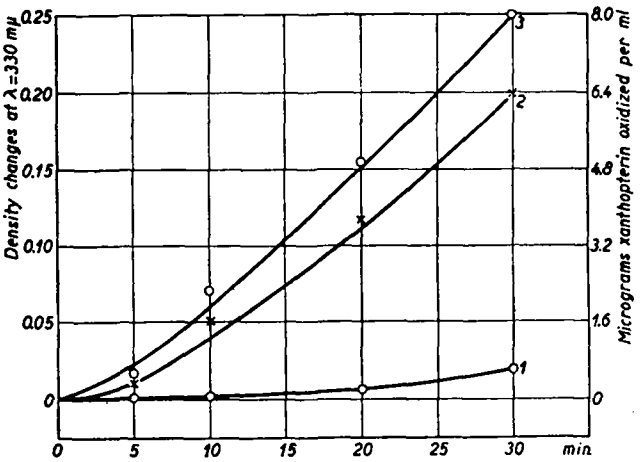

Fig. 1. The ability of preincubation with xanthine oxidase to overcome the inhibitory power of PGA. $1.1 \cdot 10^{-7}$ mole of PGA, xanthine oxidase and xanthine oxidase + $1.1 \cdot 10^{-7}$ mole of PGA were incubated in 4 hours at 37° in 0.5 ml of 0.1 *M* pyrophosphate buffer, respectively. After the incubation period xanthine oxidase was added to the sample previously incubated with PGA alone and at 0 minutes. 2.5 ml of the buffer containing $1.5 \cdot 10^{-7}$ mole of xanthopterin were added to all three samples and the density changes at $\lambda = 330$ mμ were measured.

Curve 1, incubation of PGA alone

Curve 2, incubation of PGA together with enzyme

Curve 3, incubation of enzyme alone

TABLE II

PGA INHIBITION OF XANTHINE OXIDASE WITHOUT AND WITH PREINCUBATION WITH ENZYME

Preincubation experiment: $5.5 \cdot 10^{-7}$ mole of PGA were incubated with 1 (1) ml of 0.1 *M* sodium pyrophosphate, p_H 8.6, or (2) the same buffer plus 50 μl xanthine oxidase, corresponding to about 3 mg protein. After 4 hours at 40° C the incubated mixtures were subjected to 90 min dialysis against 10 ml of 0.067 *M* sodium phosphate, p_H 8, in order to get rid of the enzyme. This was so concentrated that the rate of oxidation of xanthine would be too high to be estimated with the usual differential spectrophotometrical method. The fluid out-side the dialyzing bag was diluted 5 fold with 0.067 *M* phosphate buffer, p_H 7.55. This dilution decreases the PGA concentration to about $7 \cdot 10^{-9}$ mole per ml To 5 ml of the diluted fluid was added 50 μg of xanthine. A third sample (No. 3) was made up of 5 ml of the same buffers and the same amount of xanthine. No density changes occurred at $\lambda = 293$ mμ, until xanthine oxidase was added. Upon addition of xanthine oxidase (about 25 μg protein per ml) xanthine was oxidized in all three samples, but with greatly varying rates.

Sample	Moles of xanthine oxidized in the first 10 minutes
1. PGA preincubated without enzyme	$2 \cdot 10^{-9}$
2. PGA preincubated with enzyme	$7.4 \cdot 10^{-9}$
3. No PGA, no preincubation	$35 \cdot 10^{-9}$

Microbiological assays* showed that incubation of the PGA preparations with xanthine oxidase did not cause any decrease in the amount of growth factor. Apparently there is no relationship between inhibition of oxidase activity and growth factor potency.

* We are indebted to Dr. HOFF-JÖRGENSEN who kindly performed these assays.

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It was therefore felt justified to raise the question whether the enzyme inhibition might be due to an impurity in the PGA preparations used. This interpretation was greatly supported by the fact that a PGA preparation of particularly high purity* possessed less potency as an enzyme inhibitor than preparations of somewhat lower purity. Thus, in order to obtain the same inhibition with the highly purified sample of PGA, about 5 times as high concentrations are required as compared with preparations of ordinary purity. PGA prepared from liver showed also less inhibition. It became likely therefore that most PGA preparations contain an impurity which even in minute traces is able to inhibit the purine and pterine oxidase. This might imply that PGA does not possess any inhibitory activity (or if it is inhibitory the effect becomes manifest only in much higher concentrations) but that the inhibitory action of PGA preparations is due solely to an accompanying substance. Although indications are strong in that direction the question whether pure PGA possesses inhibitory action particularly in higher concentrations cannot be answered definitely at the present time. The question is, however, of much interest in connection with the nutritional studies by KEITH *et al.*[2]. COBB, PEARSON, AND HASTINGS[3] have recently found that the oxidation of pyruvate by brain dispersions is suppressed by addition of PGA preparations. Inasmuch as freshly crystallized PGA was found to be without inhibitory effect the authors are inclined to think that the inhibition is to be ascribed to conversion products of PGA.

THE INHIBITION OF PURINE AND PTERINE OXIDASES BY 2-AMINO-4-HYDROXY-6-FORMYLPTERIDINE

LOWRY, BESSEY, AND CRAWFORD[4] have recently described the existence of a pterine oxidase which catalyzes the oxidation of 2-amino-4-hydroxypteridine to isoxanthopterin. They showed that this enzyme is inhibited strongly by addition of minute amounts of a photo-fission product of PGA. The inhibitory substance was found to be 2-amino-4-hydroxy-6-formylpteridine (6-aldehyde)[5, 6]. This substance was shown to inhibit the oxidation of pterine, xanthopterin and xanthine in concentrations as low as a few millimicrograms per ml[4].

This observation suggested to us the possibility that the inhibition of xanthine and xanthopterin oxidase by PGA is actually due to 6-aldehyde which might occur in small amounts in most PGA preparations. It was found that the inhibitory potency of PGA preparations is markedly stepped up by subjecting them to irradiation or to sulphurous acid cleavage (see Table I). Both operations have been shown to liberate 6-aldehyde[5, 6].

The 6-aldehyde, isolated from a sulphurous acid digest of PGA and tested in the xanthine oxidase assay, shows a very high inhibitory power. For example 10 to 15 millimicrograms ($0.5–0.8 \cdot 10^{-10}$ mole) of the substance per ml of enzyme substrate mixture (xanthine concentration about $0.7 \cdot 10^{-7}$ mole per ml) are still capable of reducing the enzyme activity about 40 to 50%. After incubation with xanthine oxidase from milk or rat liver the inhibitory power of the 6-aldehyde disappeared.

The 6-aldehyde synthesized by WEYGAND** *et al.*[7] has properties somewhat different from the sulphurous acid cleavage product, but it exerts a marked inhibitory effect on

* Kindly furnished by Dr T. H. JUKES, Lederle Laboratories Division, American Cyanamid Company.

** Kindly furnished by Professor F. WEYGAND, Heidelberg (Germany)

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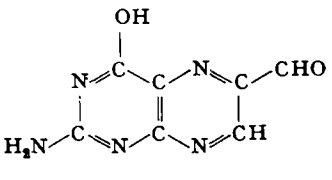
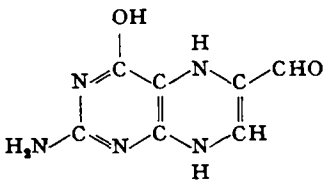
xanthine oxidase, although not to the same extent as the PGA fission product. The enzymatic inactivation of the inhibitory power of this compound occurs more slowly than that of the fission product of PGA.

The formulas proposed by WEYGAND *et al.* are as follows:



synthetic product, 2-amino-4-hydroxy-6-formylpteridine



sulphurous acid cleavage product, 2-amino-4-hydroxy-6-formyl-5,8-dihydropteridine

As soon as this matter is clarified and decisive proof for the above mentioned formulas is furnished the terminology should be altered in accordance with these facts.

THE EFFECT OF THE 6-ALDEHYDE ON VARIOUS ENZYMES

Crude xanthopterin and xanthine oxidases from rat liver are also inhibited markedly by the 6-aldehyde[8]. As a comparison a number of oxidases which are supposed to be more distantly related to xanthine oxidase are neither inhibited by PGA preparations nor by the 6-aldehyde. The following oxidases were tested and proved to be uninfluenced by 6-aldehyde or by PGA preparations: uricase, glucose oxidase (notatin*) and crystalline triosephosphate dehydrogenase.

The inhibition by the 6-aldehyde seems therefore to be confined to a special group of oxidases related to xanthine oxidase. This includes, besides xanthine oxidase, hypoxanthine oxidase (see Table III) and xanthopterin oxidase from milk or liver, the pterin oxidase of LOWRY *et al.*[4], aldehyde oxidase from beef liver, and the closely related liver quinine oxidase from rabbit liver[9]. It should be added that the latter two enzymes are also able to oxidize the 6-aldehyde. The activities of aldehyde and quinine oxidase were estimated by the rate of reduction of methyleneblue under anaerobic conditions.

TABLE III

INHIBITION OF ENZYMATIC OXIDATION OF HYPOXANTHINE TO XANTHINE

Hypoxanthine, $7 \cdot 10^{-6}$ mole per ml; PGA, $4 \cdot 10^{-8}$ mole per ml; phosphate buffer, pH 7.5; enzyme, milk xanthine oxidase. Spectrophotometric measurements at $\lambda = 270$ mμ and $\lambda = 248$ mμ[16]

	Density increase at $\lambda = 270$ mμ during the first 10 minutes	Density decrease at $\lambda = 248$ mμ during the first 10 minutes
Enzyme plus Hypoxanthine	0.046	0.039
The same plus PGA	0.006	0

* Kindly furnished by Dr H. RAISTRICK, London School of Hygiene.

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PHYSICAL AND CHEMICAL DESTRUCTION OF INHIBITORS

If a solution of the 6-aldehyde in high dilution is subjected to irradiation the inhibitory potency decreases markedly. Incubation with metallic zinc in acid solution[10] likewise brings about a disappearance of the inhibitory effect, presumably due to a reduction of the aldehyde group. The existence of a free aldehyde group seems essential

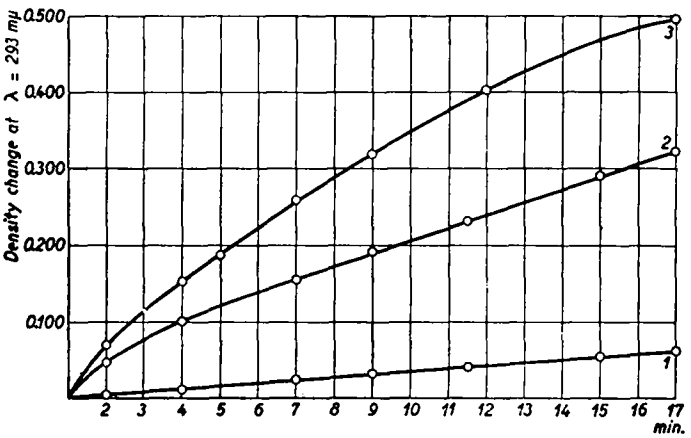

Fig. 2. The ability of preincubation with 2,4-dinitrophenylhydrazine to overcome the inhibitory power of 6-aldehyde

$6.4 \cdot 10^{-8}$ mole of 6-aldehyde, $6.4 \cdot 10^{-8}$ mole of 6-aldehyde plus 2 mg of DPNH and 2 mg of DPNH alone were incubated for 10 minutes in 1 ml of 1 *N* HCl, respectively. After incubation the mixtures were neutralized and made up to 3 ml. Aliquotes of 200 μl were withdrawn from these solutions and added to 3 ml of 0.2 *M* phosphate buffer p_H 8.0 containing $3.3 \cdot 10^{-7}$ mole of xanthine. At 0 minutes xanthine oxidase was added to the mixtures and the density changes at $\lambda = 293$ mμ were measured.

Curve 1, incubation of 6-aldehyde alone

Curve 2, incubation of 6-aldehyde together with DPNH

Curve 3, incubation of DPNH alone

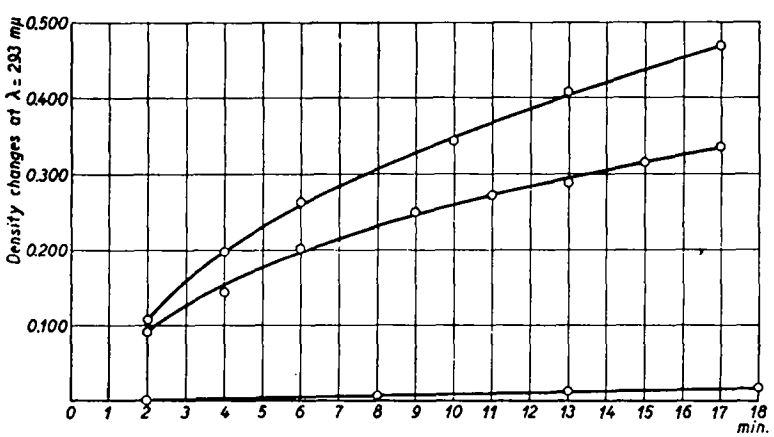

Fig. 3. The ability of preincubation with 2,4-dinitrophenylhydrazine to overcome the inhibitory power of PGA

This experiment was analogous with that in Fig. 2, only did the incubation mixture in this case contain $1.7 \cdot 10^{-7}$ mole of PGA instead of $6.4 \cdot 10^{-8}$ mole of 6-aldehyde.

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for the inhibitory action of 6-aldehyde. This is illustrated by the fact that addition of 2,4-dinitrophenylhydrazine (DNPH) to a solution of 6-aldehyde eliminates the inhibitory potency of the pterine compound (see Fig. 2). The effect of DNPH can also be demonstrated on folic acid solutions which exert an inhibition on xanthine oxidase (see Fig. 3). Likewise the inhibition brought about by highly purified PGA preparations was also eliminated by DNPH. This effect was interpreted as a further indication that the inhibitory component of the PGA preparations used is to be ascribed to the presence of the 6-aldevyde.

QUANTITATIVE DETERMINATION OF THE 6-ALDEHYDE

The determination of the 6-aldehyde is based on the observation that the 2,4-dinitrophenylhydrazone has an intense red colour in alkaline solution with an absorption maximum at $\lambda = 500$ mμ. The method is very convenient and sensitive with a molar extinction coefficient equal to about $40 \cdot 10^8$, *i.e.* a density $E_{1\,cm} = 0.204$ per μg aldehyde per ml (see Table IV). It is carried out without removing the excess of DNPH since the molar extinction of this compound is negligible in strong alkaline solution. The aldehyde colour formed is not completely stable but decreases 5–10% per hour. The determination is performed in the following way: to a water or buffer solution of aldehyde is added an equal volume of 0.1% DNPH in 2*N* HCl, the mixture is heated on waterbath a few minutes at 70–100° C then cooled to room temperature and a NaOH solution is added to make a final concentration of 0.1 to 1*N* with respect to NaOH. This solution is measured in the spectrophotometer at $\lambda = 500$ mμ. As the hydrazone is rather insoluble the final concentration should not exceed about 1 μg per ml. The method is not restricted to 6-aldehyde alone but gives colours with other aldehydes[11] with somewhat variable absorption maxima and stability.

TABLE IV

Aliquots of up to 400 μl of a 6-aldehyde solution were added to 200 μl of 0.1% of 2.4 dinitrophenylhydrazine in 1 *N* HCl. Water was added to bring the final volume to 600 μl. After heating the volumes were brought to 3 ml with 0.1 *N* NaOH.

Amount of 6-aldehyde μg per ml	Extinction at $\lambda = 500$ mμ
0.000	0.016
0.185	0.061
0.370	0.095
0.555	0.127
0.740	0.168
0.925	0.198
1.110	0.233
1.480	0.285

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ENZYMATIC INACTIVATION OF INHIBITOR

If the 6-aldehyde is incubated with xanthine oxidase preparations from milk the aldehyde compound slowly undergoes a conversion which manifest itself by a disappearance of the inhibitory activity[8] (cf. Fig. 1 and Table II). The nature of this enzymatic conversion will be described in detail in a subsequent article[12]. The enzymatic conversion can be measured not only by a decrease in inhibitory potency but also by an increase in fluorescence and a change in ultraviolet absorption spectrum (at pH 7 a marked difference exists in the ultraviolet absorption spectrum of the aldehyde and in that of the conversion product) and, finally, by a gradual disappearance of aldehyde groups and an appearance of an acidic group (cf.[12]). The most sensitive methods for this enzymatic conversion proved to be the estimation of the oxidase inhibition and the determination of the changes in fluorescence. It appears from Table V that the gradual increase in fluorescence which takes place when the 6-aldehyde is incubated with xanthine oxidase is accompanied by a corresponding decrease in inhibitory power.

TABLE V

THE EFFECT OF ENZYMATIC INCUBATION OF 6-ALDEHYDE ON FLUORESCENCE AND ON INHIBITION

0.05 *M* pyrophosphate buffer p_H 9.3 containing $1.3 \cdot 10^{-9}$ mole of the 6-aldehyde per ml was incubated with the enzyme at 23° C. At time intervals as indicated in the Table, aliquotes of 1 ml were withdrawn and added to 3 ml of 0.2 *M* phosphate buffer p_H 7.4 containing 33 μg of xanthine per ml. The rate of the oxidation of xanthine in the aliquotes was immediately estimated with the usual differential spectrophotometric method[15], and at the same time the fluorescence of the aldehyde-enzyme mixture was measured.

Incubation with enzyme (minutes)	Fluorescence readings at the galvanometer scale	Increase in fluorescence expressed in per cent of terminal increase	Rate of the oxidation of xanthine expressed in per cent of the uninhibited sample
0	65		13
25	79	44	35
50	89	74	51*
100	94	90	93

* *i.e.* apparently one sixteenth of $1.3 \cdot 10^{-9}$ mole/ml of $0.8 \cdot 10^{-10}$ mole/ml of 6-aldehyde still capable of suppressing the xanthine oxidase activity to half.

As mentioned above a similar transformation is obtained when the 6-aldehyde is incubated with aldehyde oxidase from liver. If a small amount of antabuse (tetraethylthiuramdisulphide)[13] is added to the incubation mixture the liver aldehyde oxidase is strongly inhibited, and the transformation of 6-aldehyde will cease[14] (Table V).

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TABLE VI

INHIBITION OF THE OXIDATION OF 6-ALDEHYDE BY ANTABUSE

1 ml of 0.1 *M* phosphate buffer, p_H 7.0 with $5.3 \cdot 10^{-9}$ mole per ml of 6-aldehyde. 0.1 ml rabbit liver enzyme (2 mg protein) incubated 10 minutes at 23° C, fluorescence measured after 0 and 10 minutes. Aldehyde concentration determined after 10 minutes, as the 2.4-dinitrophenylhydrazone.

	Increase in fluorescence in the first 10 minutes	Density at $\lambda = 500$ mμ of the hydrazone in NaOH sol.
Complete system	18	0.015
Complete system + antabuse final conc. $5 \cdot 10^{-5}$ mole/ml	3	0.065
Controle without enzyme		0.065

SUMMARY

Most folic acid preparations are found to exert an inhibitory effect on xanthopterin and xanthine oxidase. This seems to be due to the presence of an impurity in the preparations.

The inhibitor is supposed to be 2-amino-4-hydroxy-6-formylpteridine. The inhibitory activity is destroyed by irradiation with light, reduction with metallic zinc or by binding of the aldehyde group with 2,4-dinitrophenylhydrazine.

A colorimetric method for the estimation of the aldehyde is given.

By incubation of the aldehyde with xanthine oxidase the inhibitory activity almost completely disappears. The enzymatic conversion of the aldehyde can be followed both by the changes in the fluorescence and in the absorption spectrum of the compound.

RÉSUMÉ

Nous avons trouvé que la plupart des préparations d'acide folique excercent un effet inhibiteur sur la xanthoptérine et la xanthine oxidase. Cet effet semble être dû à une impureté dans les préparations.

Nous supposons que l'inhibiteur soit la 2-amino-4-hydroxy-6-formylptéridine. L'activité inhibitrice est détruite par irradiation par la lumière, par réduction au moyen du zinc métallique ou par bloquage du groupe aldéhydique par la 2,4-dinitrophénylhydrazine.

Nous présentons une méthode d'évaluation colorimétrique de l'aldéhyde.

L'activité inhibitrice disparaît presque complètement par incubation de l'aldéhyde avec de la xanthine oxidase. La transformation enzymatique de l'aldéhyde peut être suivie par les changements de la fluorescence et du spectre d'absorption du composé.

ZUSAMMENFASSUNG

Die meisten Folinsäurepräparate hemmen Xanthopterin und Xanthin-oxidase. Dies scheint durch eine Verunreinigung in den Präparaten verursacht zu werden.

Es wird angenommen, dass der Hemmstoff 2-Amino-4-hydroxy-6-formylpteridin ist. Die Hemmwirkung wird durch Lichtbestrahlung, durch Reduktion mit metallischem Zink und durch Blockieren der Aldehydgruppe mit 2,4-Dinitrophenylhydrazin zerstört.

Eine kolorimetrische Bestimmungsmethode für das Aldehyd wird angegeben.

Nach Inkubation des Aldehydes mit Xanthinoxidase verschwindet die Hemmwirkung fast vollständig. Die enzymatische Umwandlung des Aldehydes kann mit Hilfe der Veränderungen der Fluoreszenz und des Absorptionspektrums der Verbindung verfolgt werden.

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