Det Kongelige Danske Videnskabernes Selskab
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Dan. Biol. Medd. **18,** no. 14 (1951)

THE ENZYMATIC OXIDATION OF 2-AMINO-4-HYDROXY-6-FORMYLPTERIDINE

BY

HANS KLENOW

København
i kommission hos Ejnar Munksgaard
1951

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Introduction.

It has been shown that pteroylglutamic acid (synthetic folic acid) can be converted to an aldehyde which has been proved (1) to be 2-amino-4-hydroxy-6-formylpteridine (6-aldehyde). The transformation can be brought about either by treatment with sulphurous acid (1) or by irradiation with ultraviolet light (2). The conversion product is an extremely effective inhibitor of the oxidation of some purines and pteridines by xanthine oxidase from milk (3 and 4). The inhibitory potency is eliminated by incubation of the 6-aldehyde with xanthine oxidase (3 and 4). From studies of the absorption spectrum of the enzymatic conversion product of the inhibitor LOWRY *et al.* (2) conclude that 2-amino-4-hydroxy-6-pteridine carboxylic acid was formed by an enzymatic oxidation of the corresponding 6-aldehyde.

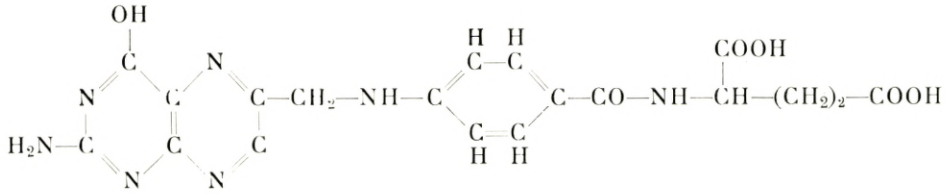
The aim of the present work is to establish that the enzymatic conversion of the inhibitor is 1) of an oxido-reductive nature and 2) that an acid is formed as result of this oxidation.

The relation between the substances mentioned is indicated in the following diagram.

Materials and Methods.

The 2-amino-4-hydroxy-6-formylpteridine used was initially prepared in this laboratory by sulphurous cleavage of folic acid, later Dr. T. H. JUKES, Lederle Laboratories, kindly supplied us with a solid product prepared according to the method of WALLER *et al.* (5). The aldehyde was estimated as the 2,4-dinitrophenylhydrazone according to the method previously described (4).

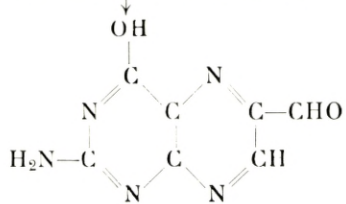
The enzymatic conversion of the aldehyde was followed fluorometrically (4). In a 0.1 M pyrophosphate buffer about pH 9



Pteridine part — p-Amino benzoic acid — Glutamic acid

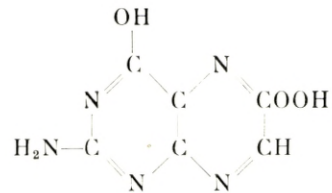
Folic acid

Sulphurous acid or | ultra violet light

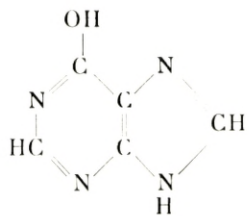


6-Aldehyde

Xanthine oxidase →

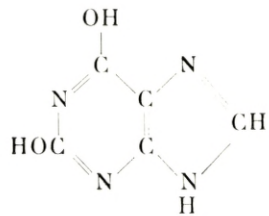


2-Amino-4-hydroxy-6-pteridine carboxylic acid.



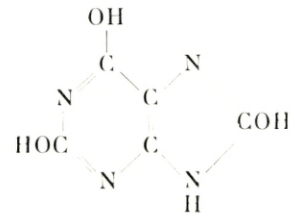
Hypoxanthine

Xanthine oxidase →



Xanthine

Xanthine oxidase →



Uric acid

the increase in fluorescence brought about by the enzymatic reaction was approximately 90 per cent of the original fluorescence. In a 0.1 M glycine buffer about pH 9, however, a decrease in fluorescence took place, corresponding to about 27 per cent of the fluorescence of the original solution of the aldehyde. The fact that the change in fluorescence does not necessarily go in the same direction must be ascribed to a difference in the proportion of the quenching effect of the two buffer solutions on the aldehyde and the corresponding conversion product.

Properties of the Enzyme.

The differential fluorometric analysis was used as a test for the enzyme. The enzyme was found in milk and the activity always accompanied that of xanthine oxidase prepared from cream. The enzyme was purified as previously described for xanthine oxidase (4). It has not been possible to separate the activity of the two enzymes. About the possible identity of the enzymes the reader is referred to LOWRY *et al.* (3). The enzyme which acts on the aldehyde in question here was practically

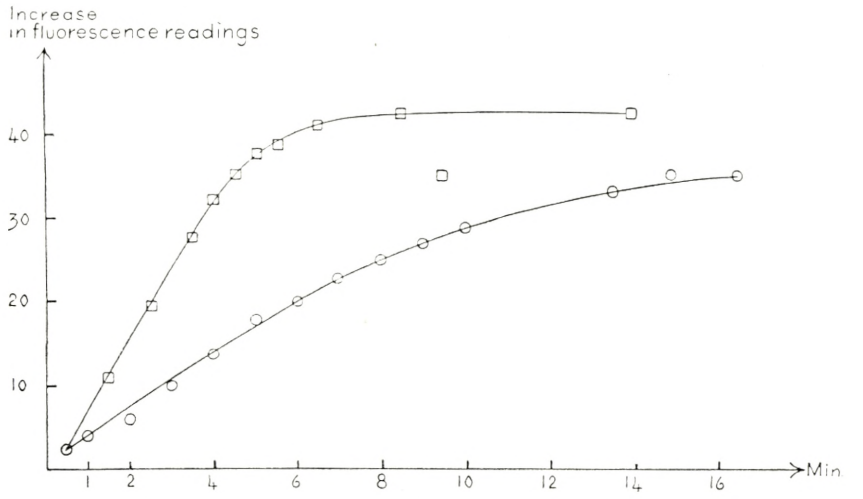


Figure 1. Inhibition of the enzymatic conversion of 2-amino-4-hydroxy-6-formylpteridine by guanine. □ 0.73×10^{-9} moles of the aldehyde per ml of pyrophosphate buffer pH 9. ○ the same with 6.6×10^{-9} moles of guanine added per ml of the mixture. Xanthine oxidase added at zero time. Abscissa: time of incubation. Ordinate: readings at the fluorometer.

inactive at pH 7.5 and the pH optimum of the enzyme was found to be located at about pH 9.

The most purified preparations of the enzyme are able to convert about 1.1×10^{-8} mole of the aldehyde per hour per mg of protein at 20° C. It was found that the activity of the enzyme is inhibited by the presence of guanine. Thus in a sample containing about 0.73×10^{-9} moles of the aldehyde per ml the activity of the enzyme towards the aldehyde is reduced to about 50 per cent of the original activity by the presence of about 6.6×10^{-9} moles of guanine per ml of the mixture (see figure 1).

Type of Reaction Involved in the Enzymatic Conversion of the Aldehyde.

Absorption Spectra.

In figure 2, 3 and 4 the absorption spectra of the aldehyde and of the corresponding oxidation product at different pH values are recorded. It is seen that the characteristics of the shapes of the absorption curves of the conversion product are approximately unchanged at pH ranging from 3 to 9. The absorption curves of the aldehyde at pH 9 is much like those of the conversion products, whereas at pH 3 and 7,7 the curves are almost alike but differ from that at pH 9.

Disappearence of the Aldehyde Group.

The increase in fluorescence during enzymatic conversion of 6-aldehyde can be shown to be directly proportional to the disappearance of the aldehyde as measured by the specific colorimetric method. This is graphically illustrated in figure 5. The experiment was performed in the following way. At fixed time intervals as indicated in the figures, aliquots of the enzyme-aldehyde mixture were deproteinized with perchloric acid. The fluorescence and the hydrazone-color in these aliquots were measured in the usual way. The measurements obtained expressed as percentages of the final values were plotted against the time of incubation.

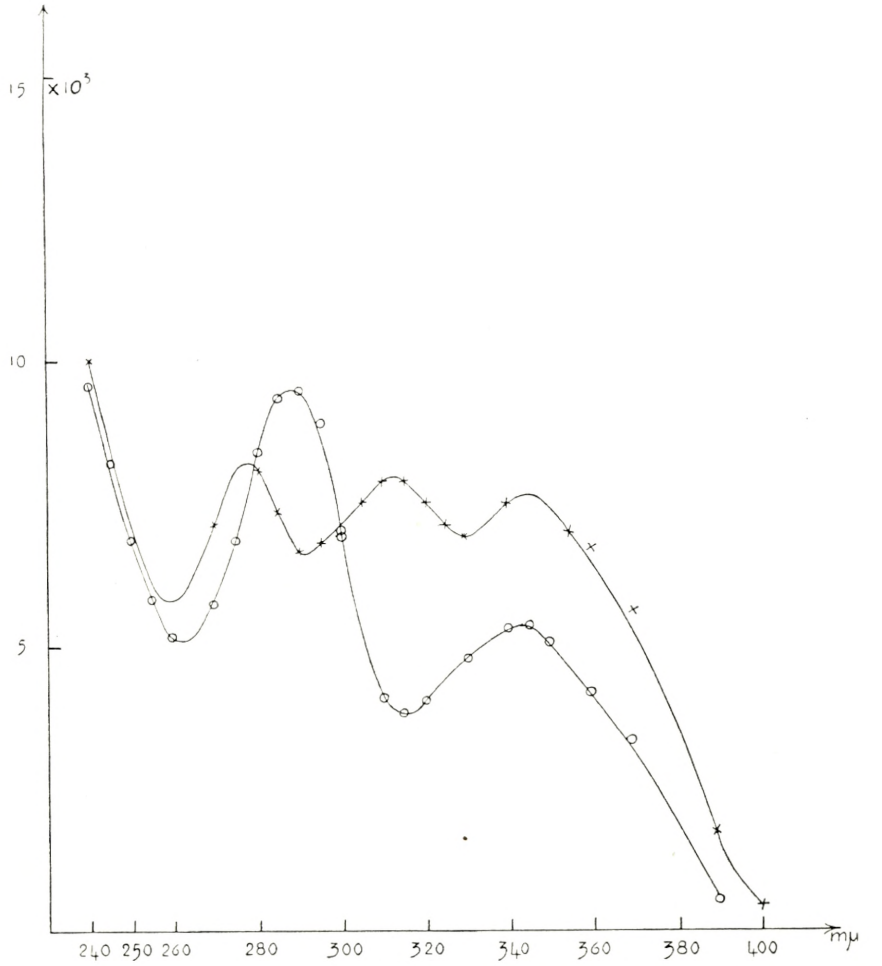


Figure 2. Molar extinction curve of 2-amino-4-hydroxy-6-formylpteridine and of the enzymatic conversion product. M/5 glycine buffer pH 3.0. × 2-amino-4-hydroxy-6-formylpteridine. ○ Enzymatic conversion product. Abscissa: Wavelength in mμ. Ordinate: Molar extinction.

Formation of an Acidic Group.

It seemed probable that the enzymatic conversion of the aldehyde group was due to an oxidation or a dismutation. It was not, however, possible to demonstrate the formation of an acidic group by means of pH indicators. The appearance of an acidic group was established by estimating the distribution coefficient between aqueous buffer solutions and butanol of the aldehyde

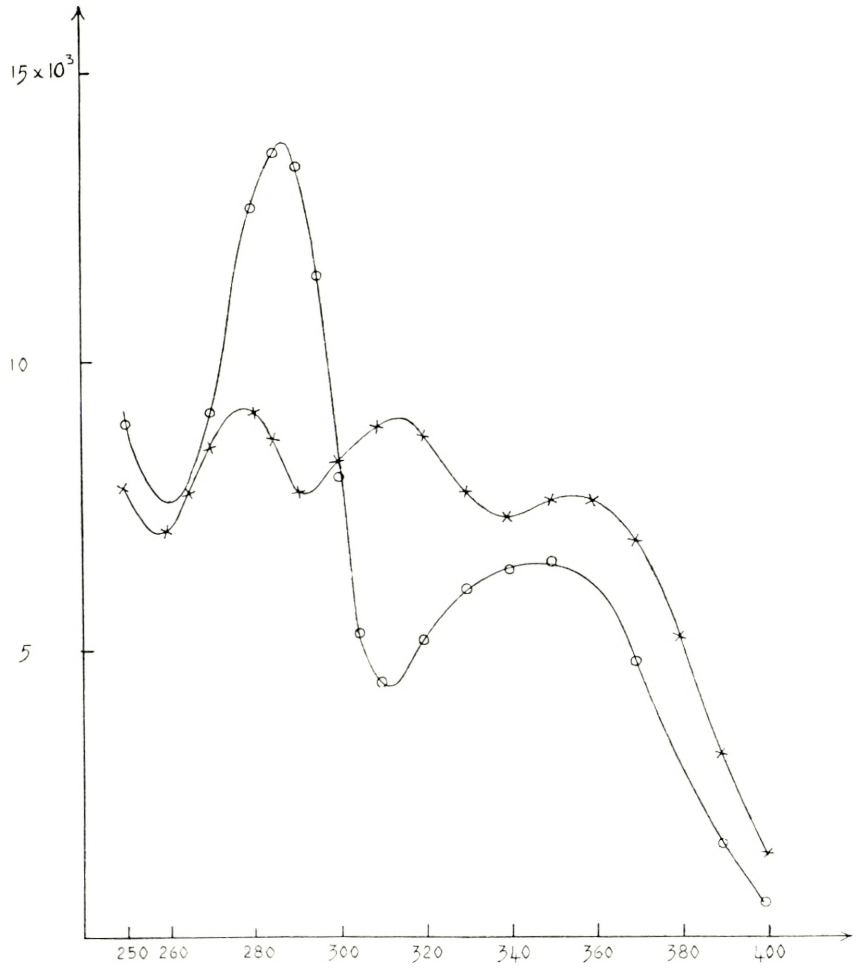


Figure 3. Molar extinction curve of 2-amino-4-hydroxy-6-formylpteridine and of the enzymatic conversion product. M/5 glycine buffer pH 7.7. × 2-amino-4-hydroxy-6-formylpteridine. ○ Enzymatic conversion product. Abscissa: Wavelength in mμ. Ordinate: Molar extinction.

and that of the enzymatic conversion product. The two pH values selected were pH 3 and pH 7. This shift in pH should not affect the distribution coefficient of the aldehyde. However, an acidic group of a pK between 3 and 7 should be extracted much more into the butanol layer at pH 3 than at pH 7 since it is dissociated at the latter pH.

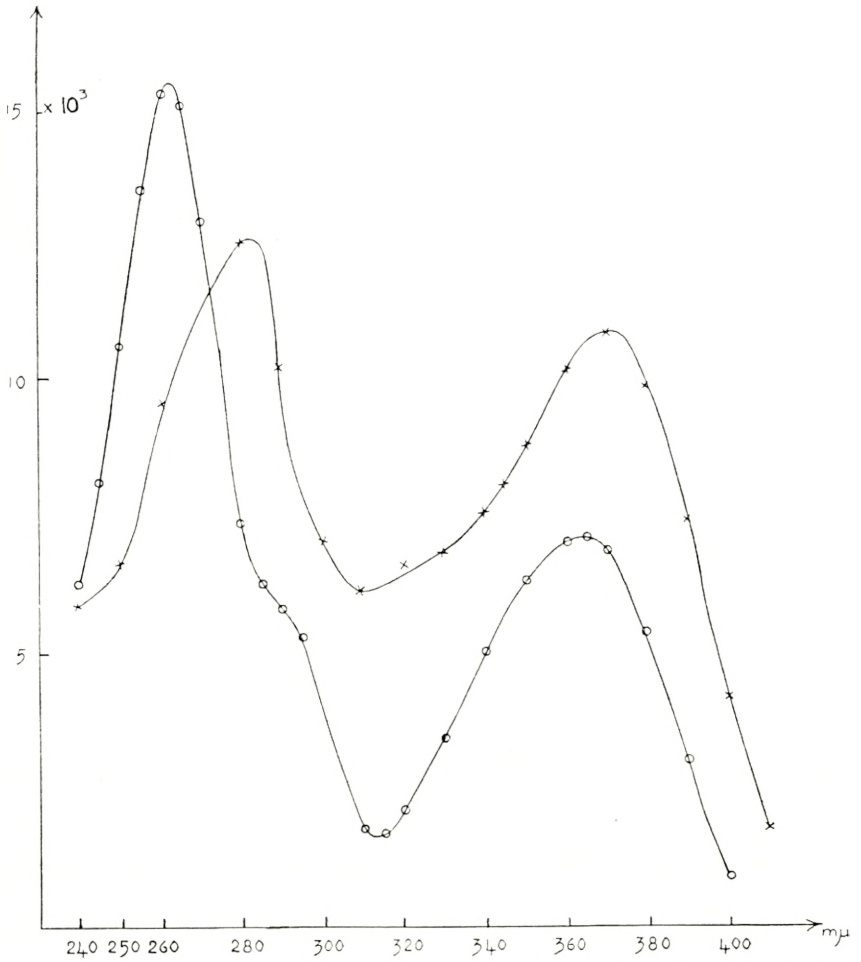


Figure 4. Molar extinction curve of 2-amino-4-hydroxy-6-formylpteridine and of the enzymatic conversion product. M/5 pyrophosphate buffer pH 9.0. × 2-amino-4-hydroxy-6-formylpteridine. ○ Enzymatic conversion product. Abscissa: Wavelength in mμ. Ordinate: Molar extinction.

The distribution coefficients of the compounds were obtained by measuring the decrease in fluorescence in the buffer solutions during a number of extractions with butanol.

In the case of the enzymatically transformed aldehyde the experiments were performed in the following way. The buffer solutions (saturated with butanol) containing the enzymatic conversion product of the aldehyde were extracted about ten

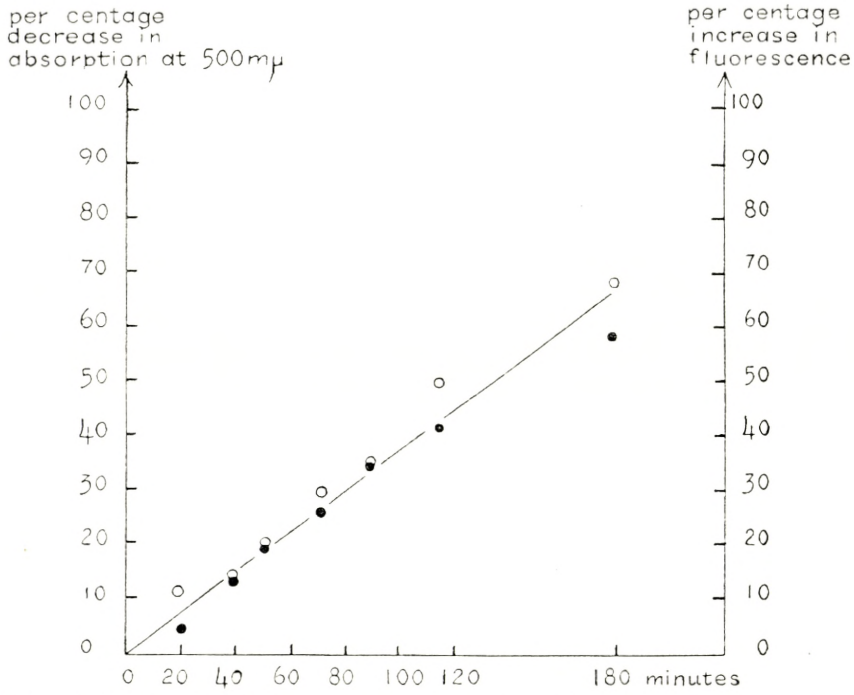


Figure 5. The relation between the disappearance of the aldehyde group and the increase in fluorescence during incubation with 2-amino-4-hydroxy-6-formylpteridine with xanthine oxidase. Abscissa: time of incubation. Ordinate: increase in the fluorescence of aldehyde solution (Ō) and decrease in absorption (at 500 mμ) of alkaline phenylhydrazone (●), expressed in percentages of terminal changes (after 24 hours).

times with the equal volumes of butanol (saturated with the appropriate buffer solution). After each extraction small samples were taken from the aqueous layer and were blown out into 0.1 M pyrophosphate buffer pH 9.0 and the fluorescence of these solutions was measured. The logarithm of the fluorescence was plotted against the number of extractions (cf. fig. 6).

The same technique was used in the extraction of the unreacted aldehyde. In this case, however, also a determination of the concentrations of the aldehyde present in the final pyrophosphate solutions was performed by enzymatic differential fluorometry. When the logarithm of these concentrations is plotted against the number of extractions a straight line is obtained. The slope of this line gives a specific value for the distribution coefficient

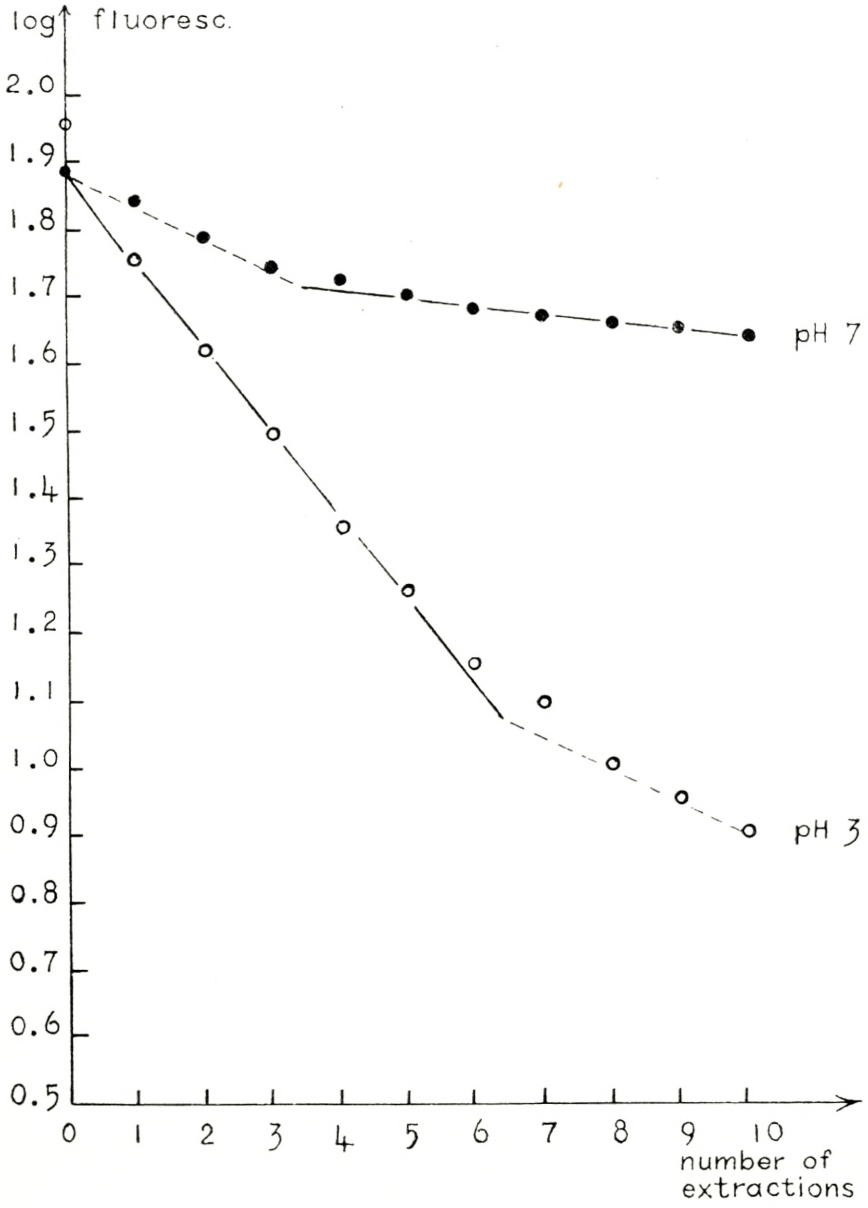


Figure 6. Butanol extraction of buffer solutions containing enzymatic digest of 2-amino-4-hydroxy-6-formylpteridine (about 1×10^{-6} moles per ml). ○ extraction at pH 3.0 (citrate buffer). ● extraction at pH 7.0 (phosphate buffer). Abscissa: number of extractions with equal volume of butanol. Ordinate: the logarithm of the fluorometer readings of the aqueous phase at pH 9.0 (pyrophosphate buffer).

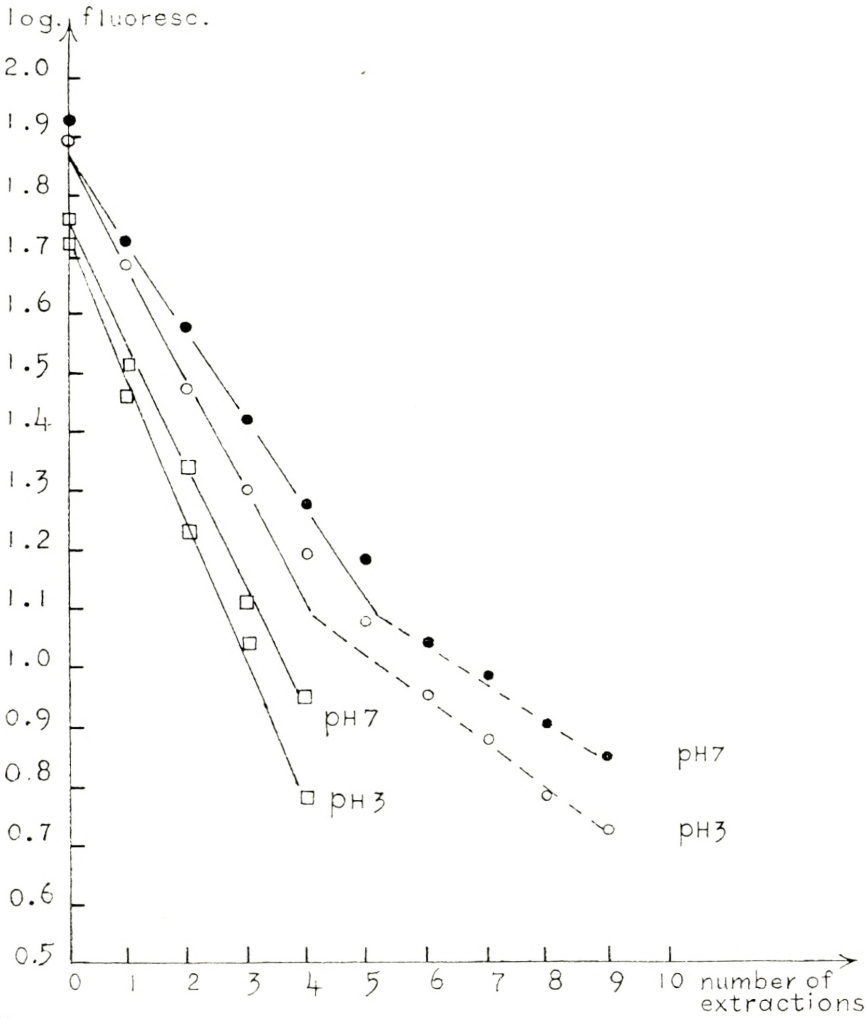


Figure 7. Butanol extraction of buffer solutions containing 6-amino-4-hydroxy-6-formylpteridine (about 1×10^{-6} mole per ml). ○ extraction at pH 3.0 (citrate buffer). ● extraction at pH 7.0 (phosphate buffer). Abscissa: number of extractions with equal volume of butanol. Ordinate: the logarithm of the fluorometer readings of the aqueous phase at pH 9.0 (pyrophosphate buffer). □ the amount of the aldehyde present in the aqueous phase during extraction (at pH 3.0 and at pH 7.0, respectively) as measured by increase in fluorescence due to enzymatic conversion. Ordinate: the logarithm of the increase in the fluorescence of the aqueous phase at pH 9.0 (pyrophosphate buffer).

of the aldehyde, independent of the possible presence of other fluorescent compounds in the solution (cf. fig. 7).

The curve obtained from the extraction experiments can be

interpreted on the following assumption. When the logarithm of the fluorescence of a solution of the aldehyde is plotted against the number of extractions, according to the law of constant distribution, a straight line should be obtained. The slope (a) of the line gives a value for the distribution coefficient (K), which can be calculated from the equation: $K = \frac{1}{10^{-a} - 1}$.

As is evident from the figures, some of the curves obtained may conceivably have been made up of two straight lines. This indicates that the corresponding solutions contain two fluorescent components, the distribution coefficients of which can be read from the slopes of the two lines, respectively.

It can be concluded from the extraction curves of the experiment with the aldehyde that the preparation contained two fluorescent components both of which have about the same solubility in butanol at pH 3 and at pH 7. It also appears that the fluorescent component which is most soluble in butanol and which corresponds to the curves with the greatest slope is identical with the aldehyde, as these curves have about the same slope as the curves obtained from the enzymatic estimation of the amount of aldehyde present in the final solutions. The occurrence of the curves having the slightest slope may possibly be ascribed to the presence of fluorescent impurities.

The solution containing the enzymatic conversion product of the aldehyde also contains two fluorescent components, as appears from fig. 6. From the points of intersection of the lines with the ordinate the proportion between the fluorescence of the two components in the original solution can be calculated thus, the fluorescence of the substance which is most soluble in the aqueous layer at pH 7 (K is about 44) and the fluorescence of that which is least soluble in the aqueous layer pH 3 (K is about 2.5) are calculated to amount in both cases to about 70 per cent of the fluorescence present in the original solutions. These two components, consequently, must be identical. The proportion of solubilities of this component is characteristic of an organic acid with $3 < pK < 7$. The other fluorescent substance present in these solutions is supposed to be identical with the non-aldehyde component found in the aldehyde preparation since these two compounds exhibit about the same solubility in the two solvents.

It appears from these extraction experiments that by enzymatic conversion of 6-aldehyde only one fluorescent substance is formed and that this has acidic properties.

Reduction of Methylene Blue.

Using the methylene blue technique as previously mentioned (6) the amount of hydrogen released during the conversion of the 6-aldehyde was measured. The experiment was performed under strictly anaerobic conditions in 0.1 M pyrophosphate buffer pH 9. The following controls were run: 1) Aldehyde plus methylene blue 2) enzyme plus methylene blue. From figure 8 it is seen that on the assumption that two hydrogen atoms are taken up per mole of the methylene blue preparation used, about 0.75 moles of hydrogen are transferred per mole of aldehyde when the aldehyde is converted into the acid.

Conclusion and Discussion.

From the experiments mentioned above it appears that when 2-amino-4-hydroxy-6-formylpteridine is enzymatically converted the aldehyde group disappears and an acidic group is formed. From the methylene blue experiment it appears, moreover, that the reaction is a dehydrogenation. The fact that apparently not 1.0 but only about 0.75 moles of hydrogen are transferred per mole of aldehyde might be partly due to common experimental errors and partly to impurities in the methylene blue and in the aldehyde preparation used. The reaction therefore is supposed to be an aldehyde dehydrogenation. In conformity with LOWRY *et al.* (2) the reaction product is concluded to be 2-amino-4-hydroxy-6-pteridine carboxylic acid.

From the absorption spectra of the 2-amino-4-hydroxy-6-formylpteridine it might be assumed that it exists in two tautomeric forms, the prevailing form being dependent on the pH of the solution in which the compound is dissolved. It seems reasonable to assume that the tautomerism is an enol-keto shift at position 4 in the pteridine molecule. The enol form then should be present at pH 9 and at more alkaline reactions. As the curves

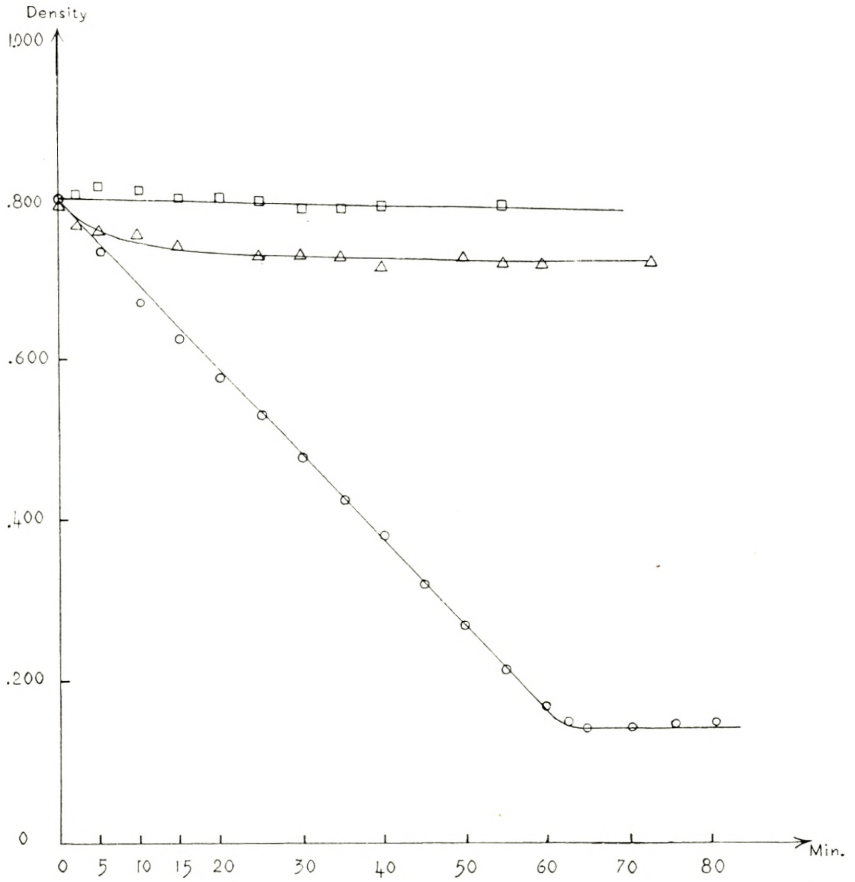


Figure 8. Reduction of methylene blue under anaerobic conditions. 2-amino-4-hydroxy-6-formylpteridine: 1×10^{-8} mole per ml, methylene blue: 1×10^{-8} mole per ml, xanthine oxidase: about 1.5 mg of protein per ml, buffer: M/10 pyrophosphate pH 9.0. ○ Enzyme plus aldehyde plus methylene blue. △ Enzyme plus methylene blue. □ Aldehyde plus methylene blue. Abscissa: time of incubation in minutes. Ordinate: density at $\lambda = 660$ mμ. Measured in a Coleman spectrophotometer.

of the acid have about the same characteristics as that of the aldehyde at pH 9 (and this is even more pronounced at more alkaline reaction) it is possible that the acid is present only in the enol form at pH 3 and at more alkaline reaction.

I am greatly indebted to Dr. H. M. KALCKAR for valuable suggestions and guidance in the performance of the experiments and for the preparation of the manuscript.

Summary.

1. 2-amino-4-hydroxy-6-pteridylpteridine(6-aldehyde) is oxidized by xanthine oxidase from milk to the corresponding acid. The oxidation is characterized partly by the disappearance of the aldehyde group and partly by the formation of an acid group and finally by the reduction of methylene blue under anaerobic conditions.
2. The aldehyde is supposed to exist in two tautomeric forms (in an enol and in a keto form) according to the pH of the solvent.

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Institute of Cytophysiology,
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