PHOTOLYTIC AND ENZYMATIC TRANSFORMATIONS OF PTEROYLGLUTAMIC ACID

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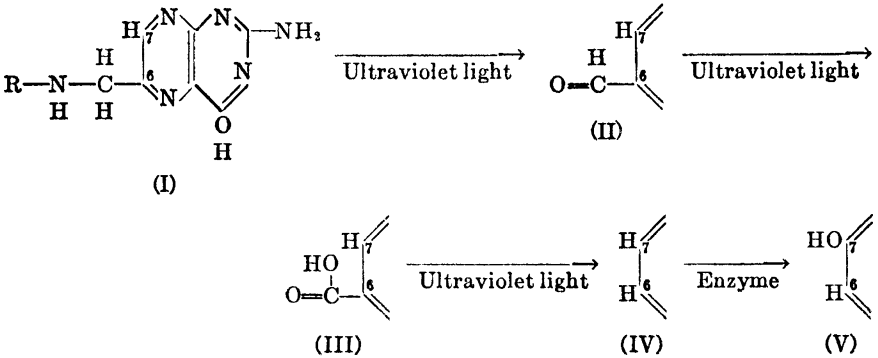
Bloom *et al.* (1) found that ultraviolet light converted pteroylglutamic acid (PGA) which is not fluorescent into a fluorescent material. Stokstad, Fordham, and de Grunigen (2) observed that sunlight caused the liberation of a diazotizable amine, indicating that cleavage had occurred between the pterine and the *p*-aminobenzoylglutamic acid components. As part of a search for a sensitive analytical reaction for PGA, this photolytic effect has been further investigated, and a rather interesting sequence of transformations has been found to occur. Evidence to be presented below indicates that when PGA (I) is irradiated with ultraviolet light it is first converted to 2-amino-4-hydroxy-6-formylpteridine (II) and a diazotizable amine. With further irradiation the aldehyde is converted to the corresponding 6-carboxylic acid derivative (III) and finally to the decarboxylated 2-amino-4-hydroxypteridine (AHP) (IV). This, in turn, is susceptible to oxidation to isoxanthopterin (V) by an enzyme present in cream, which is probably identical with xanthine oxidase and xanthopterin oxidase.



(I) (II) (III) (IV) (V)

It seems possible that either the increase in fluorescence on irradiation or the more specific change in fluorescence resulting from the enzymatic oxidation of the final irradiation product might prove analytically useful. The aldehyde formed as the first photolytic product has proved to be a

remarkably effective inhibitor of the oxidative enzyme. As little as 2×10^{-4} γ per ml. causes definite inhibition.

EXPERIMENTAL[1]

It was observed that a solution of 20 γ of PGA per ml. in 0.01 N acetic acid became very fluorescent (Table I) when irradiated with a source of the 365 mμ mercury line (a General Electric B-H4 lamp).[2] The development of fluorescence was accompanied by the appearance of diazotizable amine with a molar yield of about 80 per cent (compared to *p*-aminobenzoic acid).[3] When this fluorescent solution was diluted 4-fold with 0.05 N acetic acid and subjected to more intense irradiation (General Electric A-H4 or A-H5 lamp), there was little change in fluorescence as measured in borate buffer at pH 9, but the fluorescence in 0.1 M phosphate buffer of pH 6.8 fell appreciably, suggesting that there had been further change (Table I, lower section). The occurrence of such change could be confirmed by treating the irradiated solutions with a preparation of "xanthine oxidase" from cream. This enzyme was without effect on the products of mild irradiation, but when incubated with the products of more intense irradiation, the enzyme effected a large increase in the fluorescence in phosphate buffer (Table I, Column 5). The enzyme concentrate was prepared according to Ball (4). The question of its identity with xanthine oxidase or the xanthopterin oxidase of Wieland and Liebig (5) and Kalckar and Klenow (6) will be discussed in a separate paper (7).

Thus two distinct fluorescent irradiation products appeared to be formed from PGA. That still a third substance was produced early in the reaction was indicated by the presence of material inhibitory to the above enzyme. This inhibitory substance appeared and then disappeared before the enzyme-sensitive product was formed (Table I, Column 7).

[1] This study was greatly simplified by having available all of the 6-substituted derivatives of 2-amino-4-hydroxypteridine. The PGA, pteroyldiglutamic acid, pteroyltriglutamic acid, 2-amino-4-hydroxy-6-formylpteridine, and 2-amino-4-hydroxy-6-carboxypteridine were generously furnished by Dr. E. L. R. Stokstad and Dr. T. H. Jukes of the Lederle Laboratories Division, American Cyanamid Company. The 2-amino-4-hydroxy-6-hydroxymethylpteridine, 2-amino-4-hydroxypteridine, xanthopterin, and isoxanthopterin were kindly supplied by Dr. G. H. Hitchings of Burroughs Wellcome and Company.

[2] The fluorescence was measured with either the Coleman fluorometer model 12 or the very sensitive Farrand micro fluorometer (3). The light filter combination used consisted of a primary of Corning glass 5860 (transmits Hg line 365 mμ), and a secondary of Corning glass 4308, Wratten gelatin film 2A, and Corning glass 3389 in this order receding from the sample tube. This is similar to the filter combination usually employed with thiochrome, but is modified to reduce the optical blank (3).

[3] The diazotizable amine disappears completely (Table I) when irradiated with the powerful A-H5 lamp which has a clear glass envelope and emits some light at wavelengths shorter than 365 mμ.

It seemed worth while to explore the possibility that all three of these products were 2-amino-4-hydroxypteridines, substituted in the 6 position. The 6-methyl and the 6-hydroxymethyl derivatives were ruled out as major products, since they were both stable to ultraviolet irradiation and were unaffected by the cream enzyme. Xanthopterin (the 6-hydroxy

TABLE I

Effect of Ultraviolet Irradiation on Pteroylglutamic Acid

Time of irradiation	Fluorescence, galvanometer divisions				Diazotizable amine	Enzyme inhibitor
	Before enzyme		After* enzyme	Change due to enzyme		
	Borate, pH 9	Phosphate, pH 6.8	Phosphate, pH 6.8			
(1)	(2)	(3)	(4)	(5)	(6)	(7)
Irradiation 2 in. from arc of B-H4† lamp (in 0.01 N acetic acid)						
min.					*per cent theoretical*	*per cent theoretical‡*
0	1	0	1	1	2	1.6
15	12	5	5	0	25	14
30	52	27	26	−1	78	29
45	60	30	30	0	78	7
60	66	33	34	1	78	0.3
120	67	32	36	4	78	0.3
Irradiation continued 2.8 in. from arc of A-H5† lamp						
15	66	28	51	23	40§	
30	67	25	60	35	22§	
60	66	20	73	53	4§	
180	63	17	69	52		

* Enzyme treatment after irradiation.

† General Electric Company mercury arc lamps. The 100 watt B-H4 lamp has a filter jacket which passes primarily the 365 mμ line. The 250 watt A-H5 lamp has a clear glass jacket, and hence passes wave-lengths down to about 300 mμ.

‡ Calculated as per cent of the possible 6-aldehyde which could be formed from the PGA present. The inhibitor was measured by its ability to inhibit the enzymatic oxidation of 2-amino-4-hydroxypteridine, and was compared with known amounts of 6-aldehyde (7).

§ Interpolated from a separate experiment.

derivative) could be eliminated by its behavior with the cream enzyme. This oxidase converts xanthopterin into leucopterin (5, 6) which, instead of being more fluorescent, is non-fluorescent in the neutral pH range. This left three other possibilities, the 6-aldehyde, the 6-carboxylic acid, and the 6-hydrogen derivative or AHP. These compounds proved to have the properties of the three successive photolytic products of PGA. Thus

the aldehyde is a strong inhibitor of the cream enzyme, and both the aldehyde and the carboxylic acid are converted by irradiation into AHP, which was in turn found to be the only member of the series readily oxidized by the cream enzyme. More complete evidence for the identity of these materials follows.

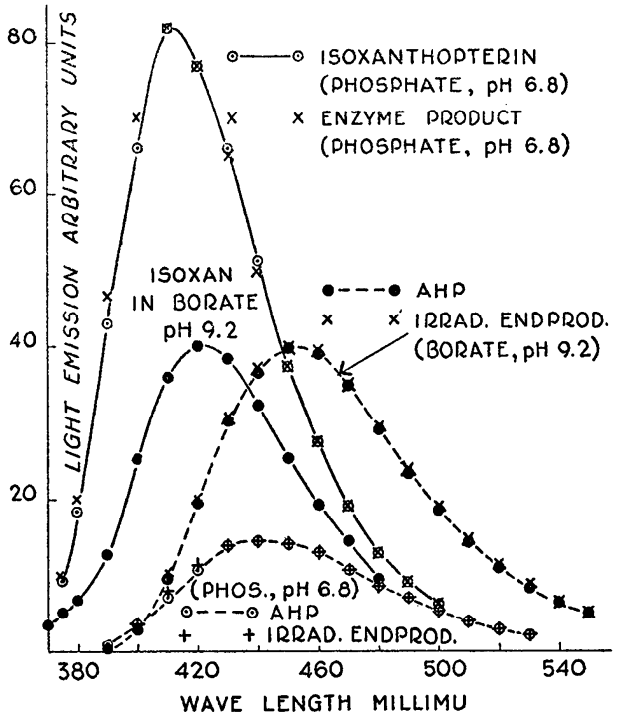


FIG. 1. Fluorescence spectra of known and suspected 2-amino-4-hydroxypteridine (AHP) and isoxanthopterin. The irradiation end-product is the third successive compound resulting from ultraviolet irradiation of pteroylglutamic acid. The enzyme product is the substance formed when "xanthine oxidase" is allowed to act on this irradiation end-product.

The product of enzyme action on the end-product of photolysis of PGA appears to be isoxanthopterin, *i.e.* 2-amino-4,7-dihydroxypteridine. Its fluorescence spectrum[4] (Fig. 1) coincides with that of synthetic isoxanthop-

[4] The fluorescence spectra were measured by exciting with the 365 mμ mercury line, passing the fluorescence through the Beckman spectrophotometer, and measuring the feeble output with an electron multiplier phototube. The emission spectra were corrected for phototube sensitivity and light losses through the instrument on the basis of calibration with a tungsten lamp. The calibration was made in a manner similar to that described by Burdett and Jones (8). The fluorescence spectra were measured with a resolution of 3 to 5 mμ and concentrations of 5 to 10 γ of pteridine per ml.

terin. None of the other pteridines of this series have fluorescence spectra in this region. The fluorescence spectrum of synthetic AHP similarly was found to coincide with the product of intense irradiation of PGA (Fig. 1). This agreement, however, could not alone identify the compound, (1) since some of the other pteridines have fluorescent spectra which are very similar and (2) the 6-methyl and 6-hydroxymethyl analogues show a similar quantitative change between phosphate and borate buffers.

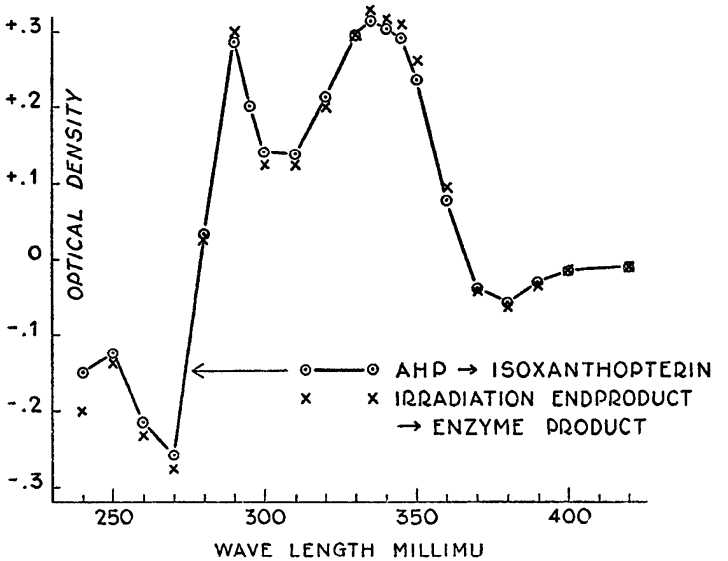


FIG. 2. Change in absorption spectra of known and suspected AHP with enzyme treatment. A solution containing 8.8 γ per ml. of 2-amino-4-hydroxypteridine (AHP) was converted to isoxanthopterin with "xanthine oxidase;" the resultant change in the absorption spectrum is recorded. Similarly, the end-product of irradiation of pteroylglutamic acid was treated with the same enzyme; the comparable difference spectrum is given.

Stronger evidence is furnished by the change in the absorption spectra (Fig. 2) which occurred when synthetic AHP and presumptive AHP (from irradiation) were treated with cream enzyme in the Beckman spectrophotometer. The concordance of the two difference spectra could scarcely be fortuitous. The difference spectra are given rather than the spectra themselves, since the irradiated specimen contained ultraviolet-absorbing degradation products of *p*-aminobenzoylglutamic acid, as well as the pteridine. The actual absorption spectrum obtained after treatment of known AHP with enzyme agrees with that of known isoxanthopterin. There seemed to be little question left that the final irradiation product was AHP, and that the enzyme product was isoxanthopterin.

All the evidence indicated that the precursor of AHP was 2-amino-4-

hydroxy-6-carboxypteridine. The precursor could be formed by irradiating a solution of PGA for several hours with a B-H4 lamp (Table I, upper section). Such a solution was neither very inhibitory for the cream enzyme, nor did it contain appreciable enzyme substrate. That is, the fluorescent material present was neither the 6-aldehyde nor AHP. The precursor had the same distribution coefficient between water and organic solvents as known 6-carboxylic acid. Both substances were convertible into AHP at identical rates by irradiation. The changes in absorption spectrum resulting from this further irradiation were quite similar for both known 6-carboxylic acid and for presumptive 6-carboxylic acid from irradiated PGA. The two difference spectra did not coincide exactly because of concomitant change in the *p*-aminobenzoylglutamic acid present in the sample from irradiated PGA.[3] A small amount of suspected 6-carboxylic acid was obtained by differential elution of a sample of irradiated pteroyltriglutamic acid from Florisil, and the absorption spectrum concurred with that of known 6-carboxylic acid.

The precursor of 6-carboxylic acid, and apparently the first fission product of PGA, is almost certainly 2-amino-4-hydroxy-6-formylpteridine. At low substrate concentrations (10^{-6} to 10^{-7} M), oxidation of AHP to isoxanthopterin is markedly inhibited by less than 10^{-3} γ of 6-aldehyde per ml. (7) or by an equal amount of presumptive aldehyde from short irradiation of PGA. No other inhibitory substance with activity approaching this potency has been found. A small amount of this early irradiation product was isolated from a strong solution of irradiated PGA (1 mg. per ml.). The amount of dissolved oxygen was apparently inadequate for the complete oxidation of such a strong solution, and as a result there was an accumulation of the aldehyde. Comparison of the absorption spectrum of this material with that of known 6-aldehyde (Fig. 3) confirmed the identity of this initial photolytic product.

The three pteridine photolytic products from PGA and the enzymatic derivative of the last of these would seem to be reasonably well identified. Perhaps the strongest evidence is furnished by the chain of reactions as a whole. One may start with any member of the series and obtain in the predicted manner the next members of the sequence.

Factors Affecting Photolysis—The oxidative photolysis, as expected, proceeds very slowly in the complete absence of dissolved oxygen. The slow reaction which does occur is presumably made possible by photolytic release of oxygen from the water.

The rate of reaction is maximal at pH 3, although it has been carried to completion both at pH 1 and pH 7. At pH 9 the reaction has not been studied thoroughly. Fluorescence develops, but much more slowly than in the acid range.

Fluorescence does not appear if samples are irradiated in the presence

of alcohols. This is apparently not due to inhibition of fission, but due to a light-catalyzed reaction between the alcohols and the liberated pteridines. The fluorescence of all three pteridines concerned is greatly decreased if they are irradiated for a short time in the presence of a 1 per cent aqueous solution of benzyl alcohol. Methyl, ethyl, butyl, and benzyl alcohol, in this order, show increasing capacity to inhibit the appearance of fluorescence on irradiation of PGA. This phenomenon would need to be considered in any attempt to use the above sequence of reactions for analytical purposes.

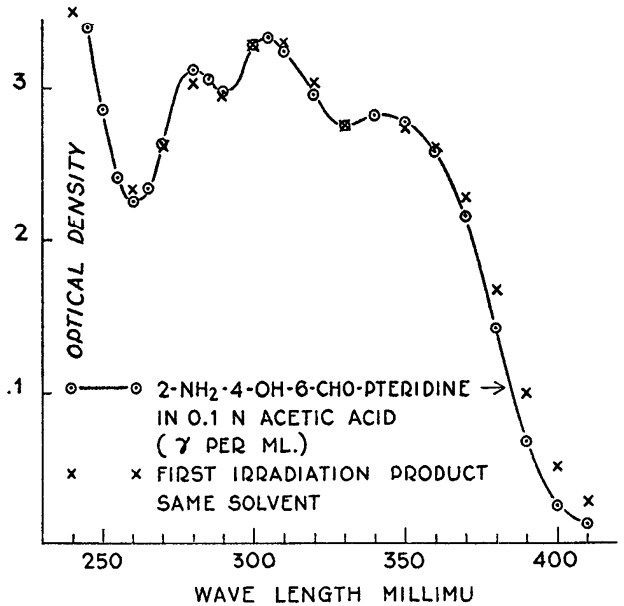


FIG. 3. Absorption spectra 2-amino-4-hydroxy-6-formylpteridine and first irradiation product of pteroylglutamic acid.

Fluorescent Properties of Pteridines—The pteridines are fluorescent over a wide pH range, but the fluorescence is markedly influenced by the composition of the medium. Thus the amount and character of emitted light vary markedly with pH (Fig. 1) (see also Jacobson and Simpson (9)). Particularly striking is the degree to which a number of anions quench the fluorescence of pteridines (Figs. 4 and 5). The fluorescence of AHP or xanthopterin is 90 to 95 per cent quenched in 0.3 M phosphate at pH 6.8 (Fig. 4). Isoxanthopterin is much less affected. For this reason the conversion of AHP to isoxanthopterin is accompanied by a large increase in fluorescence if the reaction takes place in 0.1 M or stronger phosphate buffer or in certain other buffers (Fig. 5). In dilute salt solutions AHP and xanthopterin differ only a little in the intensity of their fluorescence. Since there is a linear relationship between the reciprocal of the fluorescence and

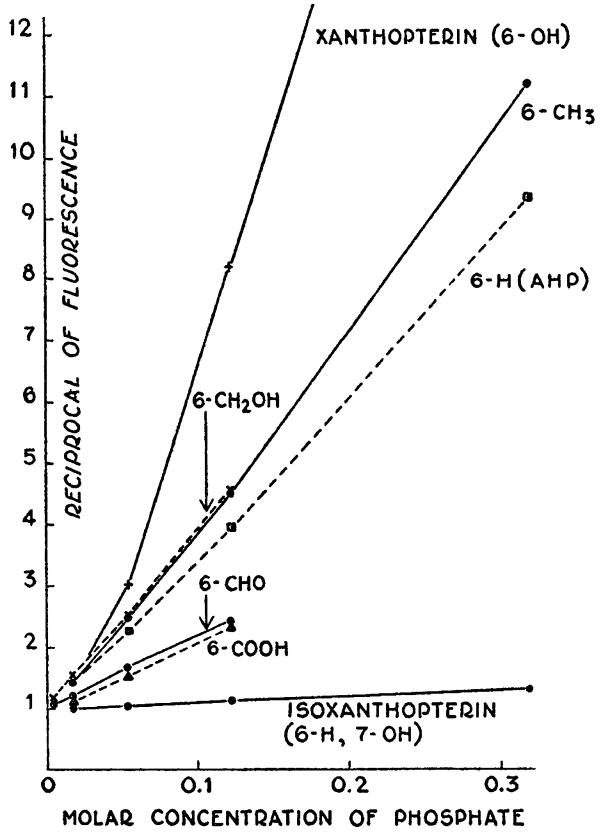


FIG. 4. Quenching of fluorescence of pterines by phosphate. The compounds represented are all 2-amino-4-hydroxypteridines with the indicated substituents in the 6 or 7 position. Reciprocal of fluorescence = (fluorescence without salt)/(actual fluorescence).

the concentration of the quenching anion (Figs. 4 and 5), it seems probable that the quenching can be ascribed to the formation of a dissociable non-fluorescent combination between the particular ion and the pteridine.[5]

[5] If anion (A) + pterine $(P) \rightleftarrows AP$, then

$$\frac{(A)(P)}{(AP)} = K$$

$$A = \frac{K(AP)}{(P)} = K\left[\frac{(\text{total pterine}) - (P)}{(P)}\right] = K\left[\frac{(\text{total pterine})}{(P)} - 1\right]$$

If P alone is fluorescent then

$$(A) = K\left[\frac{\text{fluorescence without salt}}{\text{actual fluorescence}} - 1\right]$$

When fluorescence = 50 per cent, $A = K$. The amount of bound anion is ignored in the above equation, since it is negligible.

The apparent dissociation constant of this complex is given by the salt concentration necessary to reduce the fluorescence to 50 per cent, *e.g.* 0.043 mole of acetate per liter for AHP. Weil-Malherbe has discussed a similar situation with other fluorescent materials (10). When xanthopterin solutions are diluted with phosphate buffer, the fluorescence does not immediately reach a stable value, but falls somewhat for a period of minutes.

The quenching by anions is much less marked at more alkaline pH values (7.5 or greater), but the fluorescence of certain pteridines, notably that of xanthopterin, is enhanced at slightly alkaline pH by a variety of di- and trivalent cations, in some cases at very low cation concentrations.

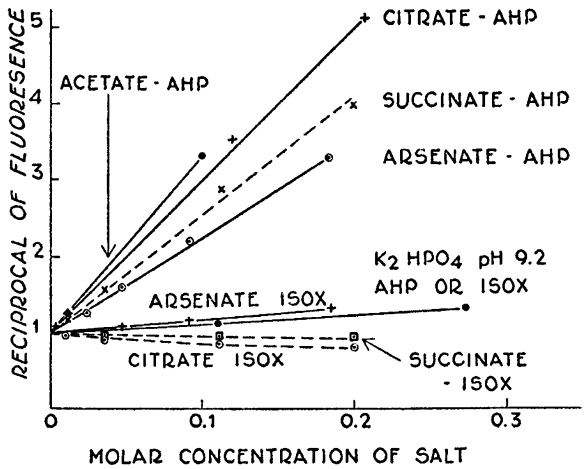


FIG. 5. Quenching of the fluorescence of 2-amino-4-hydroxypteridine (AHP) and isoxanthopterin (ISOX) by various anions. Reciprocal of fluorescence = (fluorescence without salt)/(actual fluorescence). Unless indicated, the pH is approximately 7.

Because of the marked susceptibility of the fluorescence of the pteridines to quenching or enhancement, fluorescent measurements with the pteridines must be controlled with internal standards if quantitative concentration values are desired.

SUMMARY

It has been observed that, when pteroylglutamic acid is irradiated with ultraviolet light in slightly acid solution, it undergoes oxidative cleavage to yield in succession 2-amino-4-hydroxy-6-formylpteridine, 2-amino-4-hydroxy-6-carboxypteridine, and 2-amino-4-hydroxypteridine. The final photolytic product is susceptible to oxidation to isoxanthopterin by "xanthine oxidase" from cream. The four compounds were identified chiefly

by their fluorescence spectra, absorption spectra, and behavior toward xanthine oxidase. The aldehyde, which is the first photolytic product, is an extremely active inhibitor of xanthine oxidase. As little as 2×10^{-4} γ per ml. produces demonstrable inhibition.

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