[CONTRIBUTION FROM THE CLINICAL PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS SERVICE, GENERAL MEDICINE BRANCH, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

The Oxidation of Certain Pteroylglutamic Acid Analogs[1]

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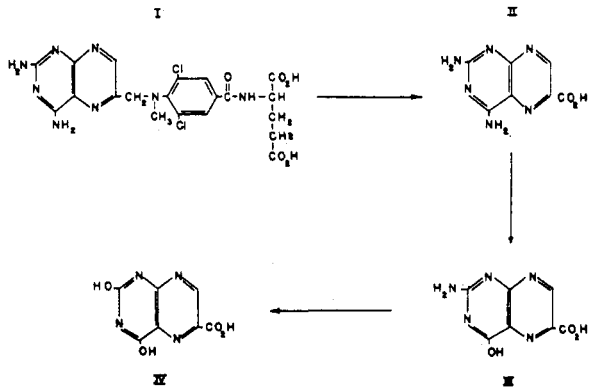
Permanganate oxidation of 4-aminofolic acid analogs has heretofore resulted in deamination at the 4-position because of the high *p*H employed. By maintaining the *p*H at 8–9 during oxidation, deamination was suppressed, and the hitherto undescribed 2,4-diamino-6-pteridinecarboxylic acid formed. Proof of structure was made by stepwise alkaline and acid hydrolysis of the 4- and 2-amino groups. The apparent *p*K's for the diamino acid were determined spectrophotometrically and found to be 1.4 and 5.0.

The chemical determination of folic acid[2] and its derivatives[3] is based on the fact that the oxidation product of these pteridines is much more fluorescent than the original materials. Consequently, within limits, the increase in fluorescence is considered to be a measurement of the original compound. In our pharmacological studies on the folic acid antagonists, it was observed that, on a molar basis, a lower increment in fluorescence after oxidation was obtained with dichloroamethopterin[4a] and amethopterin[4b] than with folic acid.

Zakrzewski and Nichol[5] reported that aminopterin[4c] and amethopterin when oxidized at *p*H 4 by a modification of the permanganate method for folic acid in natural materials, yielded a lower fluorescence than did an equimolar solution of folic acid. However, if the compounds were autoclaved in 1*N* sodium hydroxide prior to oxidation, the fluorescent intensities of the oxidation products become equal to that of the folic acid oxidation product. No attempt was made to isolate and identify the oxidation products obtained without alkaline hydrolysis.

It has been shown that the permanganate oxidation of 2,4-diamino pteridines with a substituted methylene group in the 6 or 7 position, caused the hydrolysis of the 4-amino group and formation of 2-amino-4-hydroxy-6-pteridinecarboxylic acid.[6] The hydrolysis occurred because these oxidations were carried out in highly alkaline solutions at elevated temperatures and the acid formed was initially isolated as the sodium salt from strong alkali.

Using a modification of the Wittle *et al.* technique for the permanganate oxidation of folic acid,[7] dichloroamethopterin (I) was oxidized at approximately *p*H 8. The resultant acid was not isolated as the sodium salt, but was precipitated directly from the reaction mixture as the free acid. This procedure prevented the hydrolysis of the 4-amino group and resulted in the formation of the hitherto undescribed 2,4-diamino-6-pteridinecarboxylic acid (II).



The high nitrogen content and combustion difficulties, characteristic of amino- and hydroxypteridines, made analytical proof of the empirical formula difficult. An indirect proof of structure, involving stepwise hydrolysis of the 2- and 4-amino groups, was therefore decided upon, as both hydrolysis products, 2-amino-4-hydroxy-6-pteridinecarboxylic acid (III)[8] and 2,4-dihydroxy-6-pteridinecarboxylic acid (IV)[9] are known.

Deamination of the 4-amino group, as part of an amidine system in II, proceeded readily in strong alkali at moderate temperatures. As part of a guanidine system, the 2-amino group required considerably more strenuous conditions. Hydroly-

(1) Presented in part at the 137th Meeting of the American Chemical Society, Cleveland, Ohio, April, 1960.

(2) V. Allfrey, L. J. Teply, C. Geffen, and C. G. King, *J. Biol. Chem.*, **178**, 465 (1949).

(3) M. V. Freeman, *J. Pharmacol. Exptl. Therap.*, **120**, 1 (1957).

(4) These are generic names for: (a) *N*-[4-{[(2,4-diamino-6-pteridyl)methyl]-*N*-methylamino}-3′,5′-dichlorobenzoyl]-glutamic acid; (b) *N*-[4-{[(2,4-diamino-6-pteridyl)methyl]-*N*-methylamino}benzoyl]glutamic acid, also known as methotrexate; (c) *N*-[4-{[(2,4-diamino-6-pteridyl)methyl]-amino}benzoyl]glutamic acid.

(5) S. F. Zakrzewski and C. A. Nichol, *J. Biol. Chem.*, **205**, 361 (1953).

(6) D. Seeger, D. Cosulich, J. Smith, Jr., and M. Hultquist, *J. Am. Chem. Soc.*, **71**, 1753 (1949).

(7) E. L. Wittle, B. L. O'Dell, J. M. Vandenbelt, and J. J. Pfiffner, *J. Am. Chem. Soc.*, **69**, 1786 (1947).

(8) J. H. Mowat, J. H. Boothe, B. L. Hutchings, E. L. Stokstod, C. W. Waller, R. B. Angier, J. Somb, D. B. Cosulich, and Y. SubbaRow, *J. Am. Chem. Soc.*, **70**, 14 (1948).

(9) R. B. Angier, J. H. Boothe, J. H. Mowat, C. W. Waller, and J. Semb, *J. Am. Chem. Soc.*, **74**, 408 (1952).

sis of the 2-amino group was attempted by means of nitrous acid,[9] without success.

Taylor and Cain[10] conducted a deamination study on various substituted pteridines and found that refluxing in 6*N* hydrochloric acid for thirty hours successfully deaminated the 2- and 4-amino groups. This method was therefore chosen to deaminate successfully the 2-position of III to form 2,4-dihydroxy-6-pteridinecarboxylic acid (IV).

EXPERIMENTAL[11]

2,4-Diamino-6-pteridinecarboxylic acid (II). A solution of 1.0 g. of I in 40 cc. of 0.1*N* sodium hydroxide and 30 cc. of water was adjusted to approximately *p*H 8 with dilute hydrochloric acid. While stirring, 30 cc. of a saturated potassium permanganate solution was added dropwise in 15 min. The solution was raised to 75° and an additional 20 cc. of the permanganate solution was added dropwise over 2 hr. The solution was maintained at 75° for an additional 1.5 hr. After cooling to room temperature and standing overnight with stirring, the excess permanganate was reduced with sodium sulfite. The manganese dioxide was centrifuged and washed with two 25-cc. portions of 0.05*N* sodium hydroxide. The washings were added to the bulk solution, which was acidified with 2*N* hydrochloric acid to *p*H 2. A very pale yellow flocculent precipitate formed immediately. This mixture was digested at 70° for 1 hr. On cooling, the precipitate was centrifuged and redissolved in a minimal amount of 0.1*N* sodium hydroxide. After dilution to 200 cc. with water, the solution was adjusted to *p*H 2 with 1*N* hydrochloric acid. The digestion, centrifugation, and subsequent recrystallization from dilute acid was repeated. Following the final recrystallization, the suspension was refrigerated overnight and the pale yellow precipitate washed twice with 20-cc. portions of acetone; yield 295 mg. (69%); m.p., above 300°. For analysis the compound was dried over phosphorus pentoxide at 100° *in vacuo* for 5 hr.

Anal. Calcd. for $C_7H_6N_6O_2$: C, 40.78; H, 2.93; N, 40.77. Found: C, 40.29; H, 2.92; N, 40.44.

The compound was very hygroscopic. Moisture gained on equilibration after drying was 7.93%. Calculated for the monohydrate; 8.03%. In 0.1*M* sodium hydroxide, II showed maxima at 267 and 370 mμ and log ε of 4.37 and 3.95, respectively; in 0.2*M* acetate buffer, *p*H 4.1, maxima were 252 and 336 mμ and log ε of 4.15 and 3.87, respectively; in 0.1*N* hydrochloric acid, maxima were at 257 and 335 mμ and log ε of 4.28 and 4.05.

Apparent ionization constants of II. Because of the poor aqueous solubility the solutions used for spectrophotometric measurements were made from a stock solution prepared by dissolving 2.5 mg. of II in a minimal amount of 0.1*N* sodium hydroxide, followed by adjustment to approximately *p*H 7 and diluted to 5.0 cc. with water. Aliquots of 0.20 ml. of the stock solution were then diluted to 25.0 cc. with buffers, dilute acids or bases. The ionic strength was 0.1 and the *p*H varied from 0.2 to 13 (0.1*N* sodium hydroxide).

The apparent ionization constants were calculated from the extinction coefficients at 267 mμ, by the method of Rosenblatt.[12]

2-Amino-4-hydroxy-6-pteridinecarboxylic acid (III). A 50-mg. sample of II was dissolved in 35 cc. of 2*N* sodium hydroxide and the solution warmed at 70° for 2.5 hr. The evolution of ammonia was observed. The solution was diluted to 200 cc. with water and then acidified with 4*N* hydrochloric acid to *p*H 2. A white flocculent precipitate formed slowly. The mixture was digested at 70° for 30 min. and refrigerated overnight. The precipitate was treated in the same manner as II; yield 43 mg. (86%); m.p. above 300°. For analysis, the compound was dried *in vacuo* at 100° over phosphorus pentoxide for 5 hr.

Anal. Calcd. for $C_7H_5N_5O_3$: C, 40.58; H, 2.43; N, 33.81. Found: C, 40.71; H, 2.59; N, 33.80.

In 0.1*N* sodium hydroxide, III showed maxima at 263 and 363 mμ and log ε of 4.40 and 4.00; in acetate buffer, *p*H 4.1, maxima were at 237, 287, and 344 mμ and log ε of 4.06, 4.20, and 3.89, respectively; in 0.1*N* hydrochloric acid, maxima were at 234, 304, and 318(s) mμ and log ε of 4.07, 4.05, 4.00, and 3.99, respectively.

2,4-Dihydroxy-6-pteridinecarboxylic acid (IV). A 20-mg. sample of III was refluxed for 30 hr. with 5 cc. of 6*N* hydrochloric acid. The white microcrystalline precipitate that formed on cooling was centrifuged, dissolved in a minimal amount of 1*N* sodium hydroxide and diluted to 5 cc. with water. Upon acidification to *p*H 2 with 2*N* hydrochloric acid, IV precipitated as a white microcrystalline product. The precipitate was treated the same way as previously described; yield, 17 mg. (85%); m.p. above 300°. For analysis it was dried *in vacuo* at 100° over phosphorus pentoxide for 5 hr.

Anal. Calcd. for $C_7H_4N_4O_4$: N, 26.92. Found: N, 26.29.

In 0.1*N* sodium hydroxide IV showed maxima at 266 and 369 mμ and log ε of 4.35 and 3.98, respectively; in 0.1*N* hydrochloric acid, maxima were 237, 262, and 327 mμ and log ε of 4.11, 4.14, and 4.05; in 0.1*N* ammonium hydroxide, maxima were at 241, 287, and 352 mμ and log ε of 4.18, 4.24, and 3.90, respectively.

DISCUSSION

The apparent ionization constants for 2,4-diamino-6-pteridinecarboxylic acid were, pK_1 1.4 and pK_2 5.0. The former was assigned to the N_3 ring nitrogen and the latter to the carboxyl group. The N_3 assignation seems somewhat anomalous in view of the fact that there are two primary amino groups available. However, it has been shown[13] that in amino derivatives of π-deficient nitrogen heterocyclics on formation of a cation, the absorption maxima exhibit no shift or a slight bathochromic shift as the proton addition is to a ring nitrogen. In contrast, the addition of a proton to an exocyclic nitrogen, such as an aniline, results in a hypsochromic shift. These facts are in accordance with our data in which the maxima of the neutral molecule shifts from 252 mμ at *p*H 4 to 257 mμ in 0.1*N* hydrochloric acid. The N_3 ring nitrogen was assigned rather than N_1, as it has been shown to be the more basic of the two in amino pteridines.[14]

It will be noted that the spectra of the diamino and dihydroxy acids in 0.1*N* sodium hydroxide are virtually identical. This constitutes additional

(10) E. C. Taylor and C. K. Cain, *J. Am. Chem. Soc.*, **71**, 2538 (1949).

(11) All *p*H measurements were made with a Beckman Model H-2 *p*H meter. A Cary Model 14PM Recording Spectrophotometer was used for all spectral measurements.

(12) D. H. Rosenblatt, *J. Phys. Chem.*, **58**, 40 (1954).

(13) A. Albert, *Heterocyclic Chemistry*, Oxford University Press, Inc., New York, N. Y., 1959, p. 49.

(14) A. Albert, *Quart. Rev.* (*London*), **6**, 197 (1952).

proof of structure as their spectra should be nearly the same, since the electronic configurations are similar under the existing conditions.[15]

(15) R. N. Jones, *J. Am. Chem. Soc.*, 67, 2127 (1945).

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Protein Binding of Model Quinone Imides. III. Preparation of N^{ϵ}-(1-Hydroxy-2-acetamido-4-fluorenyl)-DL-lysine[1]

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The synthesis of an amino acid derived from a metabolite [*N*-(1-hydroxy-2-fluorenyl)acetamide] of the carcinogen, *N*-2-fluorenylacetamide, is described. The amino acid, N^{ϵ}-(1-hydroxy-2-acetamido-4-fluorenyl)-DL-lysine, was obtained by reaction of 1,2-fluorenoquinone-2-acetimide with N^{α}-tosyl-DL-lysine benzyl ester, with subsequent removal of the protecting groups of the lysine moiety.

It has been suggested that the proximate metabolite of the carcinogen *N*-2-fluorenylacetamide which is bound to cellular proteins is the *o*-quinoneimine, 1,2-fluorenoquinone-2-imine.[4] More recently, in studies on the reaction of 1,2-fluorenoquinone-2-acetimide with crystalline bovine serum albumin,[5] the ϵ-amino group of the lysine residues of the protein was shown to be involved in the binding reaction by way of 1,4-addition to the *o*-quinone imide. Since the unequivocal identification of the adduct, *N*-(1-hydroxy-2-amino-4-fluorenyl)-L-lysine or its hydrochloride salt (V), in hydrolysates of tissue proteins obtained from rats after feeding *N*-2-fluorenylacetamide would require the availability of an authentic sample of V, the synthesis of this adduct was undertaken.

Reaction of N^{α}-tosyl-DL-lysine benzyl ester (I) with 1,2-fluorenoquinone-2-acetimide (II) gave a product which was homogeneous upon paper chromatography. Since primary amines react with quinone imides either by 1,2-addition or by 1,4-addition,[6,7] the isolated product should have either structure VI or III. The elemental analysis eliminated structure VI and, by inference, structure III has been assigned to the product. III was further characterized as its hydrochloride salt.

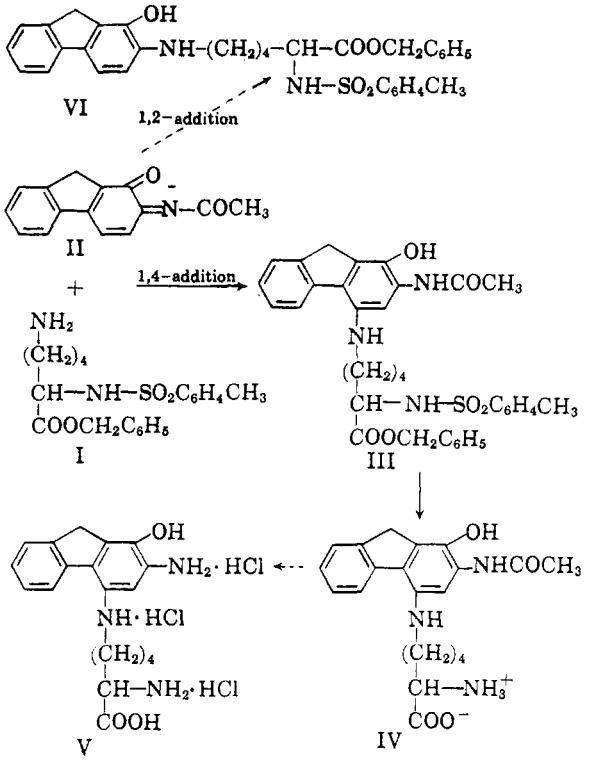

Fig. 1. Preparation of N^{ϵ}-(1-hydroxy-2-acetamido-4-fluorenyl)-DL-lysine (IV)

Removal of the protecting groups on the lysine moiety of III with 37% hydrogen bromide in glacial acetic acid[8] and subsequent removal of hydrogen bromide with an anion exchange resin yielded

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(5) C. C. Irving and H. R. Gutmann, *J. Biol. Chem.*, 234, 2878 (1959).

(6) R. Adams and K. A. Schowalter, *J. Am. Chem. Soc.*, 74, 2597 (1952).

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(8) K. Poduška, J. Rudinger, and F. Šorm, *Collection Czechoslov. Chem. Commun.*, 20, 1174 (1955).