cessive approximations. By this method we first assume $[HA]/[A^-] = 1$ and determine the approximate degree of dissociation, this gives then a better value of the ratio $[HA]/[A^-]$ which can then be used to correct the ionization constant to a better value. The true value was obtained by repetitions of this procedure, a standard method for the solution of such problems.

All solubility and ionization constant data were obtained with the solutions under an atmosphere of nitrogen and with distilled water.

The maximum error in the average values of the solubilities is about 0.1×10^{-4}; in the values of the ionization constants (final average) 0.2×10^{-4}; and in the value of $K = [A^-]/[B^-]$ about 0.5.

Separation of the Isomers.—In a test case 2.00 g. of HB and 1.00 g. of HA were dissolved in 13.40 ml. of 1 *N* sodium hydroxide (calcd. 13.20 ml.) and varying amounts of 1 *N* hydrochloric acid were added. Each addition of acid was made with stirring while the solution was at 80°. After fifteen minutes the solution was cooled slowly during one hour to 25° and the acid was collected, weighed and dried. Melting points were taken on these fractions without recrystallization. The data are summarized in Table II.

Summary

1. A method of separation of isomeric acids by utilizing the differences in their solubilities and ionization constants is described. The method is applied satisfactorily to the separation of β-(2-naphthoyl)-propionic acid and β-(1-naphthoyl)-propionic acid.

2. Values are given for the solubilities, the ionization constants and the per cent. ionization of these acids in saturated solution at 25°.

COLUMBUS, OHIO RECEIVED MAY 28, 1946

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF PARKE, DAVIS & CO.]

Oxidative Degradation of Vitamin Bc (Pteroylglutamic Acid[1])

BY E. L. WITTLE, B. L. O'DELL, J. M. VANDENBELT AND J. J. PFIFFNER

Of the various methods we investigated as possible degradation routes for determination of the structure of vitamin Bc, oxidation using either neutral permanganate or a sodium chlorate–hydrochloric acid mixture appeared most promising when applied to small amounts of material and was most extensively used. While Angier, *et al.*, have disclosed the structure N-[4-{[(2-amino-4-hydroxy-6-pteridyl)-methyl]-amino}-benzoyl]-glutamic acid (VI) and described a process for the synthesis of this compound, the degradative approach which we used differs from those which they have reported and we feel it is of interest to publish our findings. Oxidation of vitamin Bc gives rise to two main fragments, a pterine and a non-pterine part.

The Pterine Part

Oxidation of vitamin Bc or its dimethyl ester with either permanganate or chloric acid following the procedure of Schöpf and Kottler[2] gave a 35% yield of a yellow, strongly fluorescent microcrystalline solid characterized by its chemical and physical properties as a pterine.[3] It contains a carboxyl group as shown by the formation of a methyl ester and was tentatively labeled acid 1. Because of its solubility properties the compound was very difficult to purify, especially in small quantity. The analytical results on this compound, its methyl ester and hydrochloride are listed chronologically in Table I and the ultraviolet absorption curves are graphed in Fig. 1 A.

As can be seen from Table I it was difficult to determine the formula for acid 1 from the analytical results, with the exception of the last two determinations 15 and 16 which fit excellently the formula $C_7H_5O_3N_5$ now known to be correct. While the first analytical results indicated a formula $C_8H_7O_3N_5$ for the acid, later analytical results, including a Van Slyke amino nitrogen determination and a halogen determination on the crystalline hydrochloride could only be fitted to the formula $C_9H_6O_4N_6$. The analytical values on the vitamin[4] itself when used with the non-pterine fragment ($C_{12}H_{12}O_5N_2Cl_2$) were of no help in deciding the formula of acid 1 since some of the analytical data fit equally well the formula $C_{19}H_{19}O_6N_7$ or $C_{21}H_{20}O_6N_8$ and thus left an N_5 or N_6 formulation possible for acid 1.

With the quantities of acid 1 available, degradation to simpler compounds of known structure was found to be difficult and largely unproductive. The only positive result in this direction was the oxidation with chloric acid from which guanidine, isolated and identified by means of its picrate, was obtained. The formation of this substance pointed to an unsubstituted 2-aminopyrimidine ring and strengthened the view that acid 1 was pterine in nature.

With both the analytical and degradative routes proving difficult, structure determination turned to the synthesis of probable pterine compounds. From a consideration of ultraviolet absorption curves it was possible to rule out pterines having a tautomeric oxygen atom at position 6 or 7 such

(1) Angier, *et al.*, *Science*, **103**, 667 (1946). A comparison of crystalline vitamin Bc isolated from liver and yeast with a synthetic sample of liver *L. casei* factor generously supplied by the Lederle Laboratories has demonstrated the identity of the compounds. When we reported the first successful isolation of a pure crystalline chick antianemia factor from liver, (*ibid.*, **97**, 404 (1943)) the designation vitamin Bc suggested by Hogan and collaborators for the chick antianemia factor was retained for the pure natural compound pending elucidation of its structure. Angier, *et al.* have achieved a chemical synthesis and suggested a suitable chemical name, pteroylglutamic acid, for this substance.

(2) Schöpf and Kottler, *Ann.*, **539**, 128 (1939).

(3) Schöpf, *Naturwissenschaften*, **30**, 368 (1942).

(4) THIS JOURNAL, **69**, 1476 (1947).

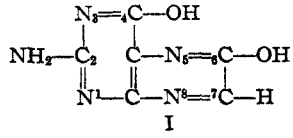
TABLE I

ANALYTICAL RESULTS ON THE PTERINE PART (ACID 1)

	C	H	N[a]	OMe	ML[b]
Acid 1					
1	43.83[c]	2.92	D 31.95		4.6
2	43.35	2.64	D 31.09		4.2
3	41.55	3.07	D 31.88		3.4
4	41.94	2.79	K 31.77		3.8
5	41.80	2.47		0.00	5.0
6	41.82	3.05	SK 32.38		3.3
7	41.91	2.65	D 33.16		3.9[d]
8	41.38[e]	2.73			2.8
9[h]	41.93	2.76			5.6
10[i]	42.60	2.94	D 30.11		4.6
11	41.37	2.49	SK 32.40		3.8
12	41.19	2.72	D 30.57		3.2
13	41.28	2.78	K 30.74		3.2
14[j]	40.18	2.66	K 32.69		2.7
15[j]	40.46	2.75	SK 33.61		2.2
16[j]	40.51	2.97			2.2
$C_8H_7O_3N_5$	43.44	3.19	31.67		
$C_9H_6O_4N_6$	41.23	2.31	32.06		
$C_7H_5O_3N_5$	40.58	2.43	33.81		
Acid 1 Methyl Ester					
17	43.93	3.31	K 31.05		3.0
18	43.09	3.72	D 29.03		5.2
19	42.24	3.49	SK 30.18	11.34	5.3
20	44.24	3.51	SK 29.37	12.61	4.9
$C_9H_9O_3N_5$	45.96	3.86	29.78	13.16	
$C_{10}H_8O_4N_5$	43.48	2.92	30.43	11.24	
$C_8H_7O_3N_5$	43.44	3.19	31.67	14.02	
Acid 1 Hydrochloride					
21	34.24	3.42	SK 26.82	11.47	2.2
22	34.16	3.39			
$C_8H_{10}O_4N_5Cl$	34.85	3.66	25.41	12.86	
$C_9H_9O_3N_6Cl$	34.13	2.86	26.54	11.20	
$C_7H_8O_4N_5Cl$	32.13	3.08	26.77	13.56	
$C_7H_6O_3N_5Cl$[g]	34.51	2.48	28.75	14.56	

[a] D is Dumas nitrogen determination; K is Kjeldahl and SK is a Kjeldahl nitrogen with a special digestion process. [b] ML is % moisture loss at 145° and 10^{-3} mm. mercury to constant weight; unless otherwise stated all dryings were done under these conditions. [c] We are unable to account for these first two high carbon values. [d] Dried at 175°, 10^{-3} mm., compound turned orange. [e] Corrected for 0.8% ash. [f] Dried at 56°, 10^{-3} mm. to constant weight. [g] Calculated without a molecule of water. [h] Acid 1 from hydrolysis of methyl ester. [i] Crystallized from aqueous acid. [j] Crystallization of the sodium salt.

as in xanthopterine (I), isoxanthopterine, leucopterine or their substituted analogs, and synthetic



I

work became centered on pterines having alkyl substitutents at these positions. Of the compounds which were prepared, 2-amino-4-hydroxy-6-carboxy-7-methylpteridine (IV) obtained by

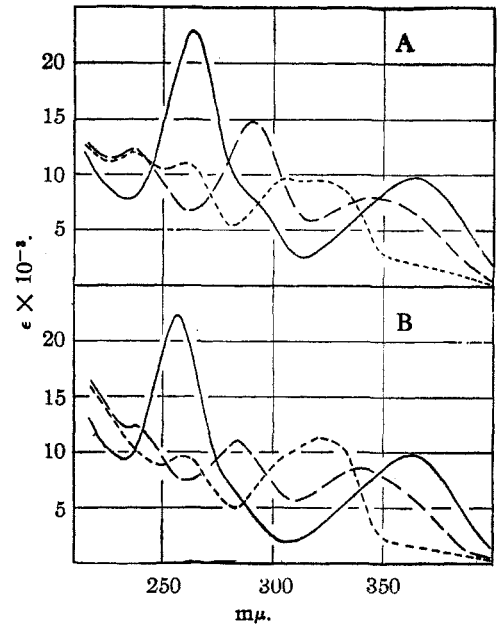
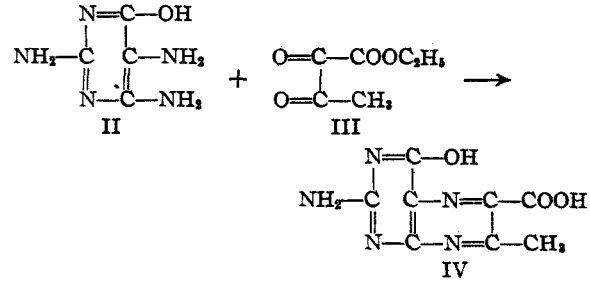


Fig. 1.—Ultraviolet absorption spectra of: A, acid 1 (2-amino-4-hydroxy-6-carboxypteridine) (V); ——, at *p*H 11; — —, at *p*H 3; - - - -, at *p*H 1; B, 2-amino-4-hydroxy-6-carboxy-7-methylpteridine (IV); ——, at *p*H 11; —·—, at *p*H 3; - - - -, at *p*H 1.

condensing ethyl α,β-diketobutyrate (III) with 2,4,5-triamino-6-hydroxypyrimidine (II) was the most similar to acid 1. The ultraviolet absorption curves of this compound are shown in Fig. 1B and it will be seen that they differ from those of acid only in the curve at *p*H 1 where a band at 236 mμ is lacking.

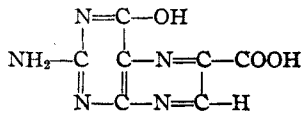


II III IV

While the respective positions of the carboxyl and methyl groups at 6 and 7 in this compound have not been proven, the directional influence of the more reactive α-carbonyl in ethyl α,β-diketobutyrate and the more basic 5-amino group in 2,4,5-triamino-6-hydroxypyrimidine would strongly indicate the structure shown in (IV). All attempts to reverse this condensation and obtain the 6-methyl-7-carboxy isomer were unsuccessful as were other methods of syntheses. However, the possibility that acid 1 was the 6-methyl-7-carboxy compound was ruled out not only by later analyses but also by oxidation. Treatment of either (IV) or 2-amino-4-hydroxy-6,7-dimethylpteridine with alkaline permanganate gave the

corresponding 6,7-dicarboxypteridine in contrast to acid 1 which was unchanged by this treatment.

It was crystallization of the sodium salt of acid 1 from alkaline solution which finally yielded a specimen which gave analytical results (15 and 16, Table I) fitting the formula $C_7H_5O_3N_5$, but before synthetic work could be carried further the structure of the vitamin and acid 1 was disclosed by Angier, *et al.*[1] Acid 1 was shown to be 2-amino-4-hydroxy-6-carboxypteridine (V).



V

Our results are in agreement with this structure.

The Non-Pterine Part

Except for acid 1 and urea, the products from permanganate oxidation were quite intractable.

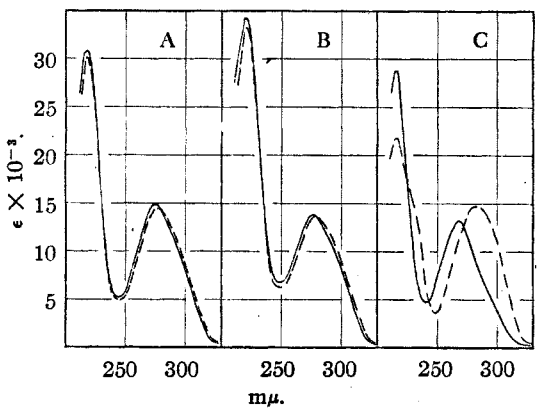


Fig. 2.—Ultraviolet absorption spectra of: A, 3,5-dichloro-4-aminobenzoylglutamic acid (VII); —, at *p*H 11; – – –, at *p*H 3 and 1; B, 3,5-dibromo-4-aminobenzoylglutamic acid; —, at *p*H 11; – – –, at *p*H 3 and 1; C, 3,5-dichloro-4-aminobenzoic acid (VIII); —, at *p*H 11; – – –, at *p*H 3 and 1.

Extraction of the aqueous solution with ethyl acetate after removal of acid 1 gave a 10% yield of yellow oil which could not be obtained crystalline. A small amount of oil obtained from the salt after evaporation of the aqueous solution could not be characterized.

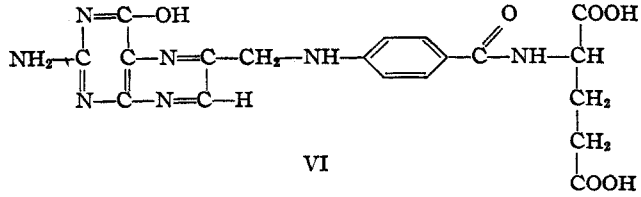
Chloric acid oxidation, however, was more successful. After the removal of acid 1, an ethyl acetate extract of the aqueous solution gave a dark red oil from which a 15% yield of crystalline solid could be obtained, first by extraction with ethyl acetate and then by recrystallization from the same solvent. The pure compound which was obtained as needles melted at 177–179° and was found by analysis and molecular weight determinations to have the formula $C_{12}H_{12}O_5N_2Cl_2$. Four of the oxygen atoms were shown to be located in two carboxyl groups by the formation of a dimethyl ester, either with methanolic hydrogen chloride or diazomethane. The formula of this ester, $C_{14}H_{16}O_5N_2Cl_2$, substantiates the formula for the free acid. A similar oxidation using sodium bromate–hydrobromic acid solution instead of chloric acid gave analogously a compound, m. p. 205–208°, the formula $C_{12}H_{12}O_5N_2Br_2$. The ultraviolet absorption curves of these compounds are shown in Fig. 2A and B. Thus, this portion of the molecule shows a very ready halogenation tendency.

On heating either the dichloro or dibromo compound above the melting point, about 210–240°, a decomposition takes place and two lower molecular weight pieces sublime from the melt at atmospheric pressure. The two products from the decomposition of the dichloro compound were readily separated and isolated in pure form by crystallization from dilute methanol. The one compound is quite insoluble in dilute methanol and by recrystallization from this solvent is obtained as crystals which sublime on the hot stage microscope up to 250° without melting and melt in a capillary tube at 290–293°. This compound was found to have the formula $C_7H_5O_2NCl_2$ and the ultraviolet absorption curves shown in Fig. 2C.

The second compound obtained from the aqueous mother liquors was obtained crystalline from acetone. It also sublimes on the hot stage microscope but melts at 182–183° and has no ultraviolet absorption.

Subtraction of the formula of the first decomposition product ($C_7H_5O_2NCl_2$) from that of the original dichloro compound, $C_{12}H_{12}O_5N_2Cl_2$, indicated a formula of $C_5H_7O_3N$ for this fragment. This was found to be the case by elementary analysis and the compound was identified as *dl*-pyrrolidonecarboxylic acid (IX) by mixed melting point with a synthetic sample prepared from glutamic acid.

Here again the structure of the vitamin was disclosed before the remainder of this work was completed and the structures of the C_{12} and C_7 compounds were readily seen from the published structure of pteroylglutamic acid (VI).

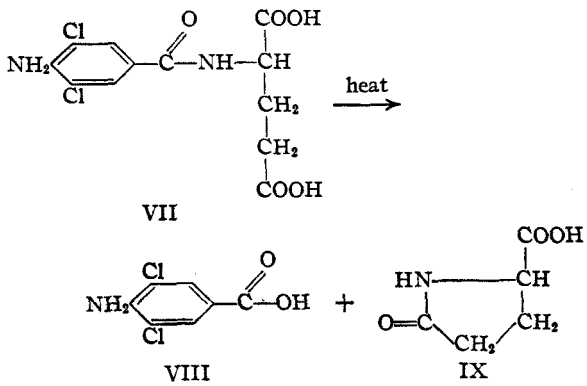


VI

The C_{12} dichloro compound should be 3,5-dichloro-4-aminobenzoylglutamic acid and this was shown to be the case by comparing it with a synthetic sample of 3,5-dichloro-4-aminobenzoylglutamic acid prepared from 4-amino-benzoylglutamic acid[5] and chloric acid. The C_7 compound was shown by a mixed melting point and ultra-

(5) Anhagen, *Z. physiol. Chem.*, 277, 208 (1943).

violet absorption curves (Fig. 2C) to be identical with 3,5-dichloro-4-aminobenzoic acid, prepared according to Müller and Tietz.[6] Thus the dichloro peptide (VII) decomposes on heating to form 3,5-dichloro-4-aminobenzoic acid (VIII) and pyrrolidonecarboxylic acid (IX). Our degradation results are thus in agreement with the published structure for pteroylglutamic acid.



VII

VIII IX

We wish to thank Dr. E. S. Bloom for his advice and suggestions on this problem and Mr. A. W. Spang for some of the microanalytical determinations which were made.

Experimental

Oxidation of Vitamin Bc with Sodium Chlorate in 2 *N* Hydrochloric Acid Solution.—A mixture of 500 mg. of Vitamin Bc and 107 cc. of distilled water was placed in a 200-cc. flask and 20 cc. of concentrated hydrochloric acid was added to the mixture. The flask was fitted with a buret, and with a stream of nitrogen bubbling through the solution to effect stirring, it was heated in a water-bath to 80°. The vitamin soon dissolved and 14.3 cc. of *M*/8 sodium chlorate was then added dropwise to the solution at 80° over a period of one hour. The solution was kept at 80° for twenty minutes and then allowed to cool to 35° over a period of one hour. A solution of 6 *N* sodium hydroxide was added slowly with cooling until the solution was neutral (approximately 40 cc.) and the solution was then made acid (*p*H 2–3) with a small amount of hydrochloric acid and allowed to stand for several hours. The solid which precipitated and which was named acid 1 was centrifuged from the solution. It was washed twice with 5 cc. of water by stirring and centrifuging and then dried over phosphorus pentoxide in a vacuum desiccator; yield 175–200 mg., 35–40% of the starting product. The acid 1 was purified by dissolving it in 35 cc. of 0.1 *N* sodium hydroxide, centrifuging the solution to remove insoluble matter and precipitating the acid 1 by acidification with 2 *N* hydrochloric acid to *p*H 2–3. The solution was warmed in a water-bath at 80° for one half hour to coagulate the acid 1, cooled, and the acid 1 separated from the solution by centrifuging. This process was repeated and the product was then washed several times (with 5–10 cc. portions of warm distilled water) until it started to become colloidal. It was dried over phosphorus pentoxide in a vacuum desiccator. Acid 1 as obtained by this process is a pale yellow or tan solid, micro crystalline when viewed under the microscope, and having a blue-violet fluorescence in solution when viewed under ultraviolet light. It is insoluble in common organic solvents and has no definite melting point; it darkens at 300° and chars but does not melt below 360°. This sample gave analyses such as 3–7 in Table I. For the amino nitrogen determination the micro apparatus described by Van Slyke[7] was used but the reaction was allowed to run for fifteen hours. A sample of 8.65 mg. of acid 1 in 1 cc. of 0.1 *N* sodium hydroxide was introduced into the reaction vessel and it precipitated immediately. After standing overnight it was completely in solution and had produced 0.80 cc. of nitrogen gas (STP) equivalent to 0.44 mg. or 5.1% of the compound. Calcd. for $C_9H_8O_4N_6$: one NH_2 group 5.34%. Calcd. for $C_7H_5O_3N_5$: 6.76%.

Further Purification of Acid 1.—A. Acid 1 (115 mg.) was dissolved in 15 cc. of 0.1 *N* sodium hydroxide by warming and stirring and then diluted to 50 cc. and made acid to *p*H 2 by the addition of 2 *N* hydrochloric acid. The precipitate was dissolved by making the volume up to 1.5 liters with distilled water and boiling the solution for half an hour. The solution was filtered hot and then boiled down to about 50 cc. where at 60–70° the acid 1 precipitated as a micro crystalline white solid. The solution was at *p*H 2.3. The acid 1 was centrifuged off, washed with a small volume of water and the above process was repeated. On cooling, rosets of micro crystals, some showing the maltese cross under the polarizing microscope, were formed. The solid was collected and the process described above was repeated twice; only micro crystals were obtained. The product was washed thoroughly with 5-cc. portions of water and dried in a vacuum desiccator; yield 78 mg. This product gave analysis 10, Table I.

B. Acid 1 (150 mg.) was stirred at room temperature with 5 cc. of 2 *N* sodium hydroxide. A part readily dissolved and then precipitated as a nicely crystalline sodium salt before solution had been completed. After thorough stirring and cooling the sodium salt was centrifuged off. It was completely dissolved on warming to 60° with 10 cc. of 2 *N* sodium hydroxide but on heating further to 80° a more insoluble "disodium" salt precipitated. This was centrifuged off, dissolved in 10 cc. of hot water, filtered and converted to the free acid by acidification. This was collected, again converted to the crystalline sodium salt and then to the free acid. This acid was heated to 40° with 3.5 cc. of 2 *N* sodium hydroxide, cooled and the sodium salt separated and dissolved in 10 cc. of hot water. The solution was carefully acidified while warm to *p*H 9–10 until a precipitate just started to form. On cooling slowly a brown to orange, somewhat gelatinous solid precipitated before the more yellow crystalline sodium salt and could be separated off with some of the crystalline salt by centrifuging. The soluble salt was converted to the free acid by warming at *p*H 2, separated, washed and dried. This sample, 95 mg., gave analyses 15 and 16 which fit the formula $C_7H_5O_3N_5$. The ultraviolet absorption curves are shown in Fig. 1A.

Acid 1 by Oxidation of Vitamin Bc Methyl Ester with Neutral Permanganate.—Vitamin Bc methyl ester was first hydrolyzed to vitamin Bc by warming 152 mg. of the ester to 60° in 15 cc. of 0.1 *N* sodium hydroxide and allowing the solution to stand at room temperature for twelve hours. A current of carbon dioxide gas was passed through the solution for two hours to neutralize the alkali and stir the solution. With a current of carbon dioxide passing through the solution 10 cc. of a solution of sodium permanganate (0.6 *N*, 3×10^{-4} atom oxygen per cc.) was added dropwise over a period of fifteen minutes. The solution was then heated to 80° in a water-bath and 7.6 cc. of permanganate solution was added dropwise over a period of three and one-half hours. The solution was allowed to cool and stand at room temperature twelve hours. The excess permanganate was removed with dilute sodium sulfite solution and the manganese dioxide was centrifuged off. It was extracted with 15 cc. of 0.05 *N* sodium hydroxide and the sodium hydroxide solution was added to the main solution which was then acidified to *p*H 2–3 with 2 *N* hydrochloric acid. The acid 1 was separated and was coagulated by heating the solution to 70° for one half hour. It was centrifuged off, washed with 5 cc. of water and dried in a vacuum desiccator; yield 68 mg., 45% by weight of the original vitamin Bc. This product, acid

(6) Müller and Tietz, *Ber.*, **74**, 820 (1941).

(7) Van Slyke, *J. Biol. Chem.*, **16**, 121 (1913).

1, is identical with the compound obtained by sodium chlorate oxidation of vitamin Bc.

The aqueous solution after removal of the acid 1 was extracted four times with 10-cc. portions of ethyl acetate. The ethyl acetate extract was washed once with 5 cc. of water and evaporated to dryness at 60° with a current of nitrogen to leave 15 mg. of a yellow oil which could not be obtained crystalline. To 5 cc. of the aqueous solution (70 cc.) was added 15 cc. of acetic acid and 2 cc. of methanol containing 200 mg. of freshly prepared xanthydrol. After a few hours a precipitate had formed and was centrifuged off, washed with methanol and dried; yield 1 mg. It was recrystallized from pyridine, m. p. 270–275° dec. It gave no depression in melting point with a known sample of dixanthyl urea, m. p. 270–275°. This corresponds to a yield of 2 mg. of urea from the starting material.

Acid 1 Methyl Ester.—A mixture of 21.3 mg. of acid 1, 25 cc. of anhydrous methanol and 25 cc. of 14% methanolic hydrogen chloride was warmed and shaken for two hours until all the acid 1 had dissolved. The solution was allowed to stand at 25° for twenty-four hours and then refluxed for one hour. After again standing for twelve hours the solution was filtered and then evaporated at 40° under reduced pressure to 3 cc. Acetone (10 cc.) was added and the solid was collected and separated from the acetone solution by centrifuging. It was washed with 3 cc. of acetone, three times with about 5 cc. of water and dried, yield 17 mg. of acid 1 methyl ester. This compound is slightly soluble in aqueous methanol and with difficulty can be obtained crystalline from this solvent. The analyses are shown in Table I, 17–21. It dissolved in dilute aqueous alkali with hydrolysis to the original acid 1, analysis 9.

Acid 1 Hydrochloride.—Acid 1 (35.8 mg.) was stirred on the steam-bath with 12 cc. of 8 *N* hydrochloric acid for one half hour and then centrifuged while hot from a small amount of insoluble solid. The solution was allowed to stand at room temperature for a day and then separated from a small amount of insoluble substance which had formed. The white crystalline acid 1 hydrochloride slowly formed in the solution and after ten days it was collected and separated from the solution. It was dried over phosphorus pentoxide in a vacuum desiccator, yield 30 mg. and then at 56° in high vacuum to constant weight for elementary analyses, 21 and 22, Table I.

Treatment of Acid 1 with Sulfuryl Chloride.—A mixture of 1.2 mg. of acid 1 and 24 cc. of sulfuryl chloride was refluxed in an oil-bath for seven hours and then allowed to stand at room temperature for two days. The undissolved material was separated and dissolved at *p*H 11 by adding 10 cc. of water and sufficient *N* sodium hydroxide to bring the solution to *p*H 11. An ultraviolet absorption curve showed the substance to be acid 1.

Treatment of Acid 1 with Phosphorus Pentachloride–Phosphorus Oxychloride.—A mixture of 10 mg. of acid 1, 75 mg. of phosphorus pentachloride and 0.67 cc. of phosphorus oxychloride was heated for eight hours in a bath at 115°. The phosphorus oxychloride was distilled off under vacuum and the residue decomposed with 1 g. of ice. The aqueous solution was allowed to stand at room temperature for forty-eight hours. After the removal of a small amount of reddish-brown flocculent precipitate the solution was adjusted to *p*H 1.0 which precipitated 6.8 mg. of white micro crystalline product. Its ultraviolet absorption curve proved it to be identical with the starting material.

Oxidation of Acid 1 with Sodium Chlorate.—Acid 1 (12 mg.) was heated with a solution of 1.6 cc. of distilled water and 0.3 cc. of concentrated hydrochloric acid to 80° in a 50 cc. centrifuge tube. A stream of nitrogen was bubbled through the solution to cause stirring. The acid 1 would not completely dissolve but as 0.9 cc. of a solution of *M*/8 sodium chlorate was added dropwise over a period of one hour at 80° the acid 1 gradually dissolved. After this time the solution was allowed to cool to 50° over a period of one hour and then centrifuged to remove a small amount of insoluble substance. The solution was made neutral with 6 *N* sodium hydroxide (0.6 cc. approx.) and concentrated with a current of nitrogen to a volume of 1.5 cc. It was again centrifuged to remove an insoluble substance and to the clarified solution was added 1.2 cc. of saturated aqueous picric acid solution. A precipitate of guanidine picrate immediately began to form and after a time was filtered off and recrystallized from a small volume of water, yield 5 mg. The guanidine picrate melted at 324° with decomposition and gave no melting point depression when mixed with a known sample of guanidine picrate. *Anal.* Calcd. for $C_7H_8N_6O_7$: C, 29.17; H, 2.80; N, 29.17. Found: C, 29.52; H, 2.96; N, 29.24.

Oxidation of Acid 1 with Alkaline Permanganate.—**A.** A solution of 11.6 mg. of acid 1 in 2 cc. of 0.1 *N* sodium hydroxide was oxidized at 90–95° with 0.4 cc. of sodium permanganate solution (0.6 *N*, 3×10^{-4} atoms O per cc.) over a period of four hours. After cooling the solution the excess permanganate was removed with sodium sulfite solution (0.26 cc. permanganate was used). The manganese dioxide was centrifuged off and extracted with 0.5 cc. of 0.1 *N* sodium hydroxide. The extract was combined with the main solution which was acidified with *N* hydrochloric acid to *p*H 2 and heated on the steam-bath for a short time to coagulate the solid. The solid was centrifuged off, washed with 0.5-cc. portions of water until it started to become colloidal and then dried. Ultraviolet absorption curves showed the compound to be unchanged acid 1, yield 9 mg., 78% recovery; analysis 8, Table I.

B. A solution of 8.8 mg. of acid 1 in 2 cc. of *N* sodium hydroxide was oxidized at 90–92° with 0.6 cc. of 0.6 *N* sodium permanganate solution added about 0.1 cc. an hour over a period of five hours. Removal of the excess permanganate with sodium sulfite solution indicated 0.4 cc. had been used. The manganese dioxide was centrifuged off and the solution was acidified to *p*H 2 and warmed on the steam-bath. The insoluble product was separated, washed and dried in a vacuum desiccator over phosphorus pentoxide. The ultraviolet absorption curves were identical with those of the starting material, yield 3.9 mg., 40%.

2-Amino-4-hydroxy-6-carboxy-7-methylpteridine (IV).—To a solution of 1 g. of 2,4,5-triamino-6-hydroxypyrimidine[8] in 10 cc. of 1 *N* hydrochloric acid and 40 cc. of water was added a solution of 1 g. of ethyl α,β-diketobutyrate[9] dissolved in 10 cc. of ethanol. The solution was heated on the steam-bath for one half hour, cooled and the solid which had formed was filtered off. It was dissolved in 25 cc. of 1 *N* sodium hydroxide with warming and precipitated while hot with 35 cc. of 1 *N* hydrochloric acid. The product was centrifuged off, washed thoroughly with water and dried; yield 1.2–1.5 g. The compound can be further purified by dissolving it in alkali and reprecipitating with excess acid if desired, or by preparation of a crystalline hydrochloride from 8 *N* hydrochloric acid and regeneration of the compound with alkali. This 2-amino-4-hydroxy-6-carboxy-7-methylpteridine is a pale yellow solid having a characteristic ultraviolet absorption curve shown in Fig. 1B. The product was dried at 145° in high vacuum to constant weight, loss 4.4%.

Anal. Calcd. for $C_8H_7O_3N_5$: C, 43.44; H, 3.19; N, 31.67. Found: C, 43.30; H, 3.29; N, 31.68.

The compound on heating in a steam-bath with 8 *N* hydrochloric acid dissolves and on subsequent cooling precipitates as a nicely crystalline hydrochloride. The product was dried at 56° in high vacuum to constant weight.

Anal. Calcd. for $C_8H_7O_3N_5 \cdot HCl \cdot H_2O$: C, 34.85; H, 3.66; N, 25.41; Cl, 12.86. Found: C, 35.00; H, 3.58; N, 24.5; Cl, 12.93.

2-Amino-4-hydroxy-6-carboxy-7-methylpteridine Methyl Ester.—A solution of 50 mg. of 2-amino-4-hydroxy-6-carboxy-7-methylpteridine in 40 cc. of 10% methanolic hydrogen chloride was allowed to stand for twenty-four hours and then refluxed for five hours. The solution was evaporated to dryness under reduced pressure

(8) Traube and Dudley, *Ber.*, **46**, 3843 (1913).

(9) Dennis, *Am. Chem. J.*, **38**, 357 (1907).

and 10 cc. of methanol was added to the residue. The solution was again evaporated to dryness and the residue stirred up with 15 cc. of water and centrifuged. The solid was separated from the water, washed thoroughly with distilled water, separated and dried; yield 45 mg. It was further dried at 145° in high vacuum to constant weight for analysis.

Anal. Calcd. for $C_9H_9O_2N_5$: C, 45.96; H, 3.86; N, 29.78. Found: C, 45.47; H, 4.03; N, 30.12.

Oxidation of 2-Amino-4-hydroxy-6-carboxy-7-methylpteridine (IV).—2-Amino-4-hydroxy-6-carboxy-7-methylpteridine (IV) (500 mg., 0.002 mole) was dissolved in 50 cc. of 1 *N* sodium hydroxide and an aqueous solution containing 0.7 g. of potassium permanganate was added slowly (ninety minutes) with stirring while the solution was maintained at reflux temperature. The excess permanganate was reduced with alcohol and the manganese dioxide was removed and washed. Upon acidification of the solution a precipitate was formed. It was collected, dissolved in 0.1 *N* sodium hydroxide, treated with 50 mg. of norite and added slowly to an excess of hot 0.5 *N* hydrochloric acid. Upon cooling 462 mg. of product precipitated. It was esterified by dissolving 100 mg. of the dried product in 50 cc. of methanol containing 2 g. of hydrogen chloride and the solution allowed to stand at room temperature for forty-eight hours. The solvent was removed under reduced pressure and the residue taken up in water. The solution was adjusted to *p*H 3.0, the precipitate collected and crystallized from 50% methanol. Recrystallization from methanol gave 45 mg. of crystalline product (rod-shaped crystals). The analyses show this compound to be 2-amino-4-hydroxy-6,7-dicarboxypteridine dimethyl ester. It was dried at 145° in high vacuum to constant weight, volatile loss 3.5%.

Anal. Calcd. for $C_{10}H_9O_6N_5$: C, 43.01; H, 3.22; N, 25.08. Found: C, 43.07; H, 3.25; N, 25.04.

The Non-Pterine Part

3,5-Dichloro-4-aminobenzoylglutamic Acid from Vitamin Bc.—The chlorate oxidation mixture from 500 mg. of vitamin Bc, after removal of acid 1, was acidified further with 1 cc. of concentrated hydrochloric acid and extracted four times with ethyl acetate, 30, 25, 20 and 20 cc., respectively. The combined ethyl acetate solution was washed twice with 5 cc. of water, dried over a small amount of anhydrous magnesium carbonate, and evaporated to dryness with a stream of nitrogen at 60°. The residue was a red colored semi-solid weighing 230 mg. after drying over phosphorus pentoxide in a vacuum desiccator, yield 46% from the starting material. This material was thoroughly stirred up with 5 cc. of ethyl acetate and the insoluble white solid was separated by centrifuging from the red ethyl acetate solution. The white solid was purified by repeated crystallizations from ethyl acetate to give 75 mg. of 3,5-dichloro-4-aminobenzoylglutamic acid, yield 15% from the original vitamin Bc. This compound is a white crystalline solid softening at 165° and melting at 175–178° on the hot stage microscope. It has the ultraviolet absorption shown in Fig. 2A. For analysis the compound was dried at 100° for three hours in high vacuum.

Anal. Calcd. for $C_{12}H_{12}N_2Cl_2O_5$: C, 43.00; H, 3.61; N, 8.36; Cl, 21.16; mol. wt., 335.15. Found: C, 43.15, 43.69; H, 3.52, 3.40; N, 8.65, 8.62; Cl, 20.75, 20.81; mol. wt. (in camphor), 351.

This compound gave no depression in melting point when mixed with a synthetic sample of 3,5-dichloro-4-aminobenzoylglutamic acid. It has a low negative rotation $[\alpha]^{27}D$ −3° which could not be determined too accurately.

Methyl 3,5-Dichloro-4-aminobenzoylglutamate.—A. 3,5-Dichloro-4-aminobenzoylglutamic acid (50 mg.), isolated as above, was dissolved in 10 cc. of anhydrous methanol and 2 cc. of 20% methanolic hydrogen chloride was added. The solution was allowed to stand at room temperature for twelve hours, refluxed for one half hour and again allowed to stand for twelve hours. It was evaporated to dryness at 50° under reduced pressure to leave a light brown resin. This residue was dissolved in 10 cc. of ether and the ether solution was washed with 2 cc. of 10% sodium bicarbonate, 2 cc. of water and then evaporated. The residue was dissolved in 3 cc. of methanol and 1 cc. of water and separated from a small amount of insoluble oil. The solution was concentrated and cooled, the crystals which formed were separated and recrystallized from dilute methanol to yield 28 mg. of methyl ester, m. p. 117–118° on the hot stage microscope.

B. The same ester can be obtained in quantitative yield by treating the acid with excess diazomethane and crystallizing the product from dilute methanol. For analysis the ester was dried at 80° for two hours in high vacuum.

Anal. Calcd. for $C_{14}H_{16}N_2Cl_2O_5$: C, 46.30; H, 4.44; N, 7.72; Cl, 19.52; mol. wt., 363.2; OMe, 17.08. Found: C, 46.50, 45.83; H, 4.27, 4.25; N, 8.05; Cl, 19.65; mol. wt. (in camphor), 379; OMe, 16.51.

3,5-Dichloro-4-aminobenzoic Acid from Vitamin Bc.—3,5-Dichloro-4-aminobenzoylglutamic acid (78 mg.), isolated as above, was placed in a small molecular still and heated at atmospheric pressure in an oil-bath to 215–240° for five minutes. During this time a white solid distilled onto the cold finger condenser. The still was cooled and then placed under a vacuum of 10^{-3} mm. and again heated at 160–230° for twenty minutes. The white solid on the condenser was removed with methanol and redistilled in the molecular still at 10^{-3} mm. at 100–180°. The white solid on the condenser (40 mg.) was dissolved in 5 cc. of dilute methanol, treated with a small amount of norite and crystallized by cooling and concentrating the solution. It was recrystallized from dilute methanol to give 19 mg. of 3,5-dichloro-4-aminobenzoic acid. For analysis the compound was dried at 25° in high vacuum for three hours.

Anal. Calcd. for $C_7H_5O_2NCl_2$: C, 40.81; H, 2.45; N, 6.80; Cl, 34.42; mol. wt., 206.0. Found: C, 41.05; H, 2.82; N, 7.35, 7.28; Cl, 33.77; mol. wt., 207.

This compound sublimed completely from the hot stage microscope at 250° without melting and melted in a capillary tube at 290–293°. It gave no depression in melting point with a known sample of 3,5-dichloro-4-aminobenzoic acid prepared according to the procedure of Müller and Tietz.[6] The ultraviolet absorption curves (Fig. 2C) are identical with those of the known compound.

Pyrrolidonecarboxylic Acid.—The aqueous methanol mother liquors from crystallization of 3,5-dichloro-4-aminobenzoic acid above were evaporated to a small volume (0.25 cc.) and centrifuged to remove a small amount of insoluble product. The solution was evaporated to dryness to leave 19 mg. of a clear colorless oil which soon set to a solid. It was crystallized by dissolving it in a large volume of acetone and concentrating the solution. The product was combined with a second similar batch of 30 mg. and recrystallized twice from acetone to yield 18.4 mg., m. p. 181–183°. For analysis it was dried at 25° in high vacuum for two hours.

Anal. Calcd. for $C_5H_7O_3N$: C, 46.5; H, 5.47; N, 10.85. Found: C, 46.43; H, 5.53; N, 10.77.

This compound showed no ultraviolet absorption. It gave no depression in melting point when mixed with a known sample of *dl*-pyrrolidone-carboxylic acid, m. p. 181–183°, prepared by heating *l*(+)glutamic acid at 200–220°.

3,5-Dibromo-4-aminobenzoylglutamic Acid from Vitamin Bc.—In a 50-cc. centrifuge tube fitted with an inlet tube for nitrogen extending to the bottom, an outlet tube and a buret was placed 100 mg. of vitamin Bc, 18 cc. of water and 5.6 cc. of 49% hydrobromic acid. With a stream of nitrogen passing through the solution it was heated in water-bath to 80° and 3 cc. of *M*/8 sodium bromate was added dropwise over a period of one half hour. The solution was kept at 80° for forty minutes and then allowed to cool to room temperature over a period of one hour. The solution had crystals present and after standing for twelve hours they were separated. An additional quantity could be obtained by extracting the aqueous solution with ethyl acetate and evaporating the ethyl

acetate solution. The product was purified by recrystallization from ethyl acetate, yield 45 mg., m. p. 206–208° on the hot stage microscope. 3,5-Dibromo-4-aminobenzoylglutamic acid is a white solid soluble in organic solvents and having a typical ultraviolet absorption spectrum, Fig. 2B. For analysis the compound was dried at 100° in high vacuum for two hours, loss 0.83%.

Anal. Calcd. for $C_{12}H_{12}O_5N_2Br_2$: C, 33.98; H, 2.89; N, 6.61; Br, 37.69. Found: C, 34.34, 34.34; H, 2.98, 3.02; N, 7.17, 6.95; Br, 37.44.

Preparation of 3,5-Dichloro-4-aminobenzoylglutamic Acid.—4-Aminobenzoyl-*l*(+)-glutamic acid[8] (300 mg.) was dissolved in 107 cc. of water and 20 cc. of concentrated hydrochloric acid and heated in a water-bath to 80° with a stream of nitrogen passing through the solution. A solution of 6 cc. of *M*/8 sodium chlorate was added dropwise in fifteen minutes, kept at 80° for one hour and then cooled and extracted once with 50 cc. of ethyl acetate. Evaporation of the ethyl acetate gave a dark red oil containing some white solid. It was stirred and cooled with 3 cc. of ethyl acetate and the solid was separated. It was recrystallized from ethyl acetate six times and obtained as white feathery needles, yield 56 mg., 19%. This compound does not melt sharply but softens at about 165° and melts at 177–179° in a capillary tube. On the hot stage microscope it decomposes at 200° to give 3,5-dichloro-4-aminobenzoic acid as does the compound from the vitamin. It gives no depression when mixed with the product from oxidation of vitamin Bc.

Anal. Calcd. for $C_{12}H_{12}O_5N_2Cl_2$: C, 43.00; H, 3.61; N, 8.36; Cl, 21.16. Found: C, 43.16; H, 3.71; N, 8.59; Cl, 20.80.

The ultraviolet absorption curves are identical with those of the compound from the vitamin (Fig. 2A). It shows a low negative rotation $[\alpha]^{27}D$ −3° which could not be determined too accurately.

Summary

Vitamin Bc (pteroylglutamic acid) has been split by oxidation into two parts, a pterine which was found to have the formula $C_7H_5O_3N_5$ and a non-pterine fragment $C_{12}H_{12}O_5N_2Cl_2$. The analytical results on the vitamin itself indicated a molecular formula of $C_{19}H_{19}O_6N_7$ or $C_{21}H_{20}O_6N_8$. The formulas of the oxidation fragments eliminated the latter.

The pterine part yielded guanidine on oxidation.

The non-pterine part on heating decomposed into *dl*-pyrrolidone-carboxylic acid and a compound $C_7H_5O_2NCl_2$ identical with 3,5-dichloro-4-aminobenzoic acid.

The non-pterine part is identical with 3,5-dichloro-4-aminobenzoylglutamic acid.

DETROIT, MICHIGAN RECEIVED[10] FEBRUARY 7, 1947

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF GEORGE A. BREON AND CO.]

Analgesics. II.[1] The Grignard Reaction with Schiff Bases[2]

BY ROBERT BRUCE MOFFETT AND WILLARD M. HOEHN

It is well-known that Grignard reagents will add to aldimines or Schiff bases to give amines in which the hydrocarbon residue of the Grignard reagent is attached to the carbon atom. This reaction, however, has seldom been used as a preparative method. In our work a series of substituted 1,2-diphenylethylamines was desired for testing as analgesics. To prepare these we used the reaction of benzylmagnesium chloride with Schiff bases prepared from substituted benzaldehydes and primary amines.

$$ArCHO + RNH_2 \longrightarrow$$

$$ArCH{=}NR \xrightarrow{C_6H_5CH_2MgCl} ArCH(NHR){-}CH_2{-}C_6H_5$$

The Schiff bases (Table I) were made by allowing the aldehyde and primary amine to react in benzene and then refluxing the benzene with a trap to remove the water. They were usually purified by distillation, followed by recrystallization in cases where they were solids.

A solution of the Schiff base was added to an excess of benzylmagnesium chloride and the addition complex was decomposed with ice and hydrochloric acid. In many cases the hydrochloride of the desired amine was relatively insoluble in the aqueous solution and could be separated in crystalline form. In several cases the hydrochloride was an oil which could be crystallized from non-aqueous solvents after thorough drying. In cases where the hydrochloride remained in the aqueous solution it was necessary to separate the ether layer, make the aqueous solution basic and extract the free amine with ether from the suspension of magnesium hydroxide. In these cases the amine was usually distilled and converted to the hydrochloride in a non-aqueous solvent by hydrogen chloride gas.

Preliminary pharmacological testing in this Laboratory by Mr. Benjamin Vaughan, Jr., by the method of Ercoli and Lewis[3] indicates that of these compounds, the hydrochlorides of the following amines have weak analgesic activity as compared to morphine.

N-Methyl-1,2-diphenylethylamine
N-Methyl-1-(*m*-hydroxyphenyl)-2-phenylethylamine
N-Methyl-1-(*p*-hydroxyphenyl)-2-phenylethylamine
N-Methyl-1-(*m*-ethoxyphenyl)-2-phenylethylamine
N-Benzyl-1-(*p*-hydroxyphenyl)-2-phenylethylamine

Experimental

The Schiff bases which have not previously been reported are listed with their physical constants in Table I. These were prepared by two general methods. Method

(1) The first paper of this series is: Goodson, Weigand and Splitter, THIS JOURNAL, **68**, 2174 (1946).

(2) Presented before the Division of Organic Chemistry at the Chicago meeting of the American Chemical Society, September, 1946.

(3) Ercoli and Lewis, *J. Pharmacol.*, **84**, 301–317 (1945).