turated with water and the crystalline product filtered, 0.081 g., m.p. 103–108°. After recrystallization from alcohol it melted at 114–115°. The material did not depress the melting point of the acetyl derivative prepared from XIV.

Anal. Calcd. for $C_{14}H_{13}N_3O_3$: C, 61.98; H, 4.83, N, 15.49. Found: C, 62.00; H, 4.82; N, 15.38.

Hydantoin-3-DL-benzylacetonitrile (XVII).—The hydantoinamide VIb, 0.2 g., was added to a mixture of 0.064 ml. (1 equiv.) of pyridine and 0.5 ml. of phosphorus oxychloride. After 5 minutes on the steam-bath the solid dissolved and gas was eliminated. The acid chloride was allowed to evaporate, the residue triturated with water and filtered to give 0.177 g. of crystalline material (95%), m.p. 170–173°. Purified from alcohol it melted at 172–173.5°.

Anal. Calcd. for $C_{12}H_{11}N_3O_2$: C, 62.87; H, 4.84; N, 18.33. Found: C, 63.20; H, 4.86; N, 18.30.

When XVII was dissolved in concd. hydrochloric acid and diluted with water after 6 hr., the starting material VIb was obtained, m.p. 209–211°.

Alkaline Rearrangement of XVII.—A suspension of 0.25 g. of XVII in alcohol was treated portionwise with one equivalent (0.52 ml.) of 2.12 *N* sodium hydroxide. Most of the solid had dissolved in 5 minutes with the concomitant separation of a sodium salt. After 10 minutes the crystalline mush was treated with 0.52 ml. of 2.1 *N* hydrochloric acid, resulting in complete solution. An additional 0.52 ml. of hydrochloric acid was added and the solution heated for 10 minutes. On cooling, 0.2 g. of prisms was obtained, m.p. 179–181.5°. The compound did not depress the melting point of 5-benzylhydantoin-3-acetic acid (IXa).

Alkaline Cyclization of XVI.—A suspension of 1.5 g. of XVI in 6 ml. of ethanol was treated with 4 ml. (1.4 equiv.) of 2.1 *N* sodium ethoxide in ethanol. The solid dissolved with simultaneous precipitation of a sodium salt. After 30 minutes the salt was filtered, weight 1.6 g. When an aqueous suspension of the sodium salt, 1.14 g., was treated with excess hydrochloric acid, an oil separated which soon crystallized, 0.68 g., m.p. 178–187°. Purified from ethanol it melted at 187–189° and was identical with VIa in melting point. For further confirmation, a sample was hydrolyzed by alkali at 25° to carbonyl-N-glycine-N′-phenylalanine (VII).

Acknowledgment.—The authors thank Dr. William C. Alford and his associates, all of this Institute, for carrying out the microanalyses, Messrs. Wm. M. Jones and H. K. Miller for measuring the infrared spectra and Mrs. Anne Wright for the ultraviolet spectra.

BETHESDA 14, MARYLAND

[CONTRIBUTION FROM THE NUTRITION AND PHYSIOLOGY SECTION, RESEARCH DIVISION, AMERICAN CYANAMID CO., LEDERLE LABORATORIES]

The Synthesis of a Pteridine Required for the Growth of *Crithidia fasciculata*[1]

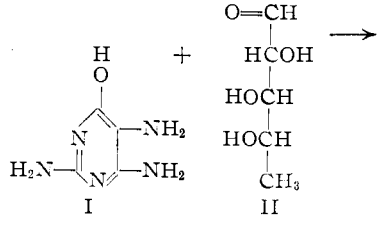
BY E. L. PATTERSON, R. MILSTREY AND E. L. R. STOKSTAD

RECEIVED MAY 10, 1956

The condensation of 5-deoxy-L-arabinose with 2,5,6-triamino-4-hydroxypyrimidine sulfate yielded a mixture from which 2-amino-4-hydroxy-6-[1,2-dihydroxypropyl-(L-*erythro*)]-pteridine was isolated. The biological, chemical and physical properties of this compound were the same as those of biopterin, a pteridine isolated from human urine and required for the growth of *Crithidia fasciculata*.

In a previous communication[2] it was reported that a factor named biopterin required for the growth of *Crithidia fasciculata* had been isolated from human urine and characterized as 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine. The optical configuration was not established. The details of the isolation and characterization are described in the accompanying paper.[3] In this paper the synthesis and some of the properties of 2-amino-4-hydroxy-6-[1,2-dihydroxypropyl-(L-*erythro*)]-pteridine are presented.

The usual method for the preparation of polyhydroxyalkyl pteridines of this type consists of the condensation of 2,5,6-triamino-4-hydroxypyrimidine with a sugar or sugar derivative[4–7] as shown. The yield of pteridine was not high, and two isomers having the alkyl side chain in the 6- and 7-positions were obtained starting with either an aldose, ketose or osone.[6] Hydrazine had some directive effect with the hexoses favoring the 6-isomer,[5] but little effect with pentoses.[5]



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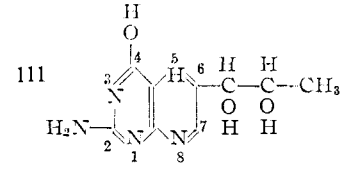

III

For the synthesis of the pteridine having the structure proposed for biopterin[2] by this method, the starting sugar should be a 5-deoxypentose having the proper optical configuration about carbon atoms three and four. Forrest and Mitchell[7] treated rhamnotetrose, prepared in solution, with 2,5,6-triamino-4-hydroxypyrimidine in the presence of hydrazine, and from the reaction mixture they obtained a product which was a mixture of 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine and 2-amino-4-hydroxy-7-(1,2-dihydroxypropyl)-pteridine. By ultraviolet absorption and paper chromatography the synthetic preparation

(1) Presented in part at the 128th Meeting of the American Chemical Society, Minneapolis, Minn., September 11–16, 1955, Abstracts p. 65C.

(2) E. L. Patterson, H. P. Broquist, A. M. Albrecht, M. H. von Saltza and E. L. R. Stokstad, THIS JOURNAL, **77**, 3167 (1955).

(3) E. L. Patterson, M. von Saltza and E. L. R. Stokstad, *ibid.*, **78**, 5781 (1956).

(4) P. Karrer, R. Schwyzer, B. Erden and A. Siegwart, *Helv. Chim. Acta*, **30**, 1031 (1947).

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was indistinguishable from one of the pteridines isolated from Drosophila and assigned the structure 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine. The optical configuration of neither the natural nor synthetic materials was reported.

Since the 5-deoxypentoses are not readily available some information first was sought regarding the optical configuration of biopterin by studying the condensation products of I with the pentoses having the proper configuration to give the four optical isomers of 2-amino-4-hydroxy-6-(1,2,3-trihydroxypropyl)-pteridine. D- and L-arabinose and D-and L-xylose were condensed with I by two slightly different procedures, and the crude reaction products were tested for their activity in supporting the growth of *C. fasciculata*. The condensation product of L-arabinose with I was about 10% as active as biopterin from urine and 100 to 1000 times more active than the other three products. These three products had activity in the same range as certain other 2-amino-4-hydroxy-6-alkylpteridines.[8] It was since found that pure 2-amino-4-hydroxy-6-[1,2,3-trihydroxypropyl-(L-*erythro*)]-pteridine was equally as active as biopterin in supporting the growth of *C. fasciculata*. On the basis of these results it was tentatively concluded that the dihydroxypropyl side chain of biopterin was the L-*erythro* configuration and that either 5-deoxy-L-arabinose or 5-deoxy-L-ribose was the proper sugar to condense with 2,5,6-triamino-4-hydroxypyrimidine to prepare biopterin by the above reaction. 5-Deoxy-L-arabinose was selected and synthesized for this purpose.

Methyl 5-deoxy-L-arabinofuranoside was prepared in 5% over-all yield starting with L-arabinose following the procedures of Levene and Compton,[9] and two other methods were tried with no improvement in yield. In one L-arabinose was converted to methyl 5-deoxy-L-arabinofuranoside by reduction of the primary alcohol through the 5-tosyl and 5-deoxy-5-iodo derivatives according to the procedures of Haskins, Hann and Hudson[10] for preparing D-rhamnose from D-mannose. None of the intermediates were crystalline. However, the final product, methyl 5-deoxy-L-arabinofuranoside had the expected properties,[9,11] b.p. 96–100° at 0.2 mm., m.p. 87.5–88°, $[\alpha]^{25}D$ −135° (methanol, *c* 2.2).

By the other method L-rhamnose was degraded to 5-deoxy-L-arabinose through the acetylated disulfone by the method of MacDonald and Fischer.[12] The 5-deoxy-L-arabinose thus obtained was a sirup and was used as such.

L-rhamnose —(conc'd. HCl; ethyl mercaptan; 25°, 1 hour)→ L-rhamnose diethylmercaptal —(acetic anhyd., pyridine)→ L-rhamnose diethyl mercaptaltetraacetate —(perphthalic acid; ether)→ L-arabo-3,4,5-triacetoxy-1,1-bis-(ethanesulfonyl)-hexene-1 —(hydrazine)→ 5-deoxy-L-arabinose

5-Deoxy-L-arabinose was condensed with 2,5,6-triamino-4-hydroxypyrimidine sulfate by the procedure of Forrest and Walker[6] for the preparation of 2-amino-4-hydroxy-6-tetrahydroxybutyl-(D-arabino)-pteridine. The crude reaction products from different runs were 2 to 10% as active as biopterin from urine in the regular bioassay. The purification proved to be difficult due to the similarity in properties of the constituents in the reaction mixtures and their general low solubility. Using procedures of adsorption and ion exchange chromatography and the growth of *C. fasciculata* as an assay, about 100 mg. of highly purified 2-amino-4-hydroxy-6-[1,2-dihydroxypropyl-(L-*erythro*)]-pteridine was isolated.

The material was equal in activity to biopterin from urine in supporting the growth of *C. fasciculata*. The alkaline permanganate oxidation product had the characteristic ultraviolet absorption of 2-amino-4-hydroxy-6-carboxypteridine indicating it was the desired 6-isomer. When titrated with periodate, 1.4 and 4.7 oxidation equivalents per mole of pteridine were consumed at *p*H 2 and 8.5, respectively (Fig. 1). At the alkaline *p*H two equivalents

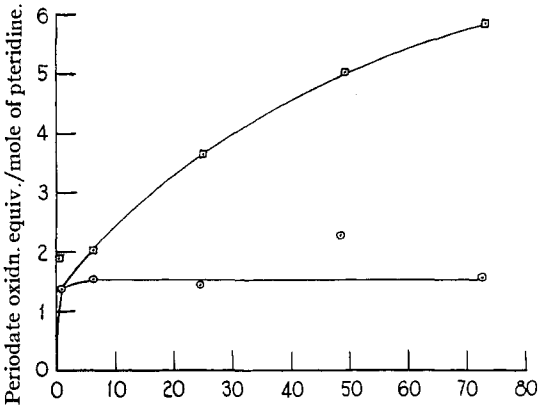

Fig. 1.—The periodate oxidation of 2-amino-4-hydroxy-6-[1,2-dihydroxypropyl-(L-*erythro*)]-pteridine: ⊡, *p*H 8.5; ⊙, *p*H 2. The experimental conditions were those previously described.[3]

were consumed rapidly and two quite slowly. This was also the case with biopterin isolated from urine, and it was shown there that the second two equivalents were consumed in converting 2-amino-4-hydroxy-6-formylpteridine to 2-amino-4-hydroxy-6-carboxypteridine.[3] The infrared and ultraviolet absorption spectra of the synthetic material and biopterin are compared in Figs. 2 and 3. From these and other similar properties of the synthetic and natural materials it was concluded that they were identical and that biopterin is 2-amino-4-hydroxy-6-[1,2-dihydroxypropyl-(L-*erythro*)]-pteridine.

Experimental

L-Rhamnose Diethylmercaptaltetraacetate.—L-Rhamnose diethylmercaptal was prepared by the method of Fischer[13]

(8) H. P. Broquist and A. M. Albrecht, *Proc. Soc. Expll. Biol. Med.*, **89**, 178 (1955).
(9) P. A. Levene and J. Compton, *J. Biol. Chem.*, **116**, 189 (1936).
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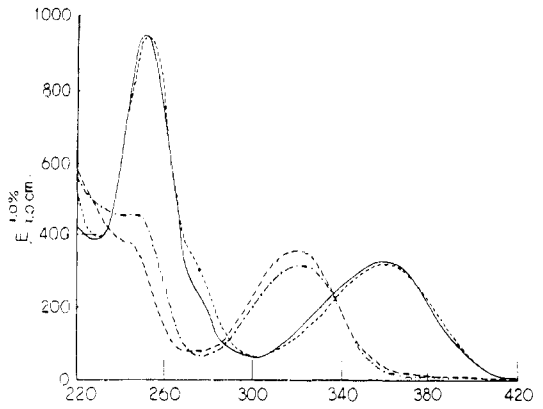

Fig. 2.—The ultraviolet absorption of 2-amino-4-hydroxy-6-[1,2-dihydroxypropyl-(L-*erythro*)]-pteridine: ———, 0.1 *N* NaOH; - - - -, 0.1 *N* HCl; and 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine from urine: ·····, 0.1 *N* NaOH; —·—·—·, 0.1 *N* HCl.

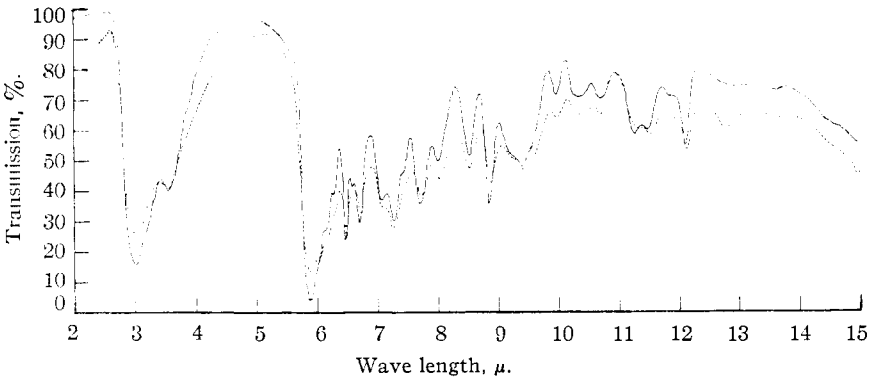

Fig. 3.—The infrared absorption of 2-amino-4-hydroxy-6-[1,2-dihydroxypropyl-(L-*erythro*)]-pteridine ····· and 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine from urine ———

in 64% yield, m.p. 136–137°, $[\alpha]^{25}D$ −12.4 (methanol, *c* 2.18).

Anal. Calcd. for $C_{10}H_{22}O_4S_2$: C, 44.5; H, 8.14; S, 23.7. Found: C, 44.5; H, 8.27; S, 23.9.

Eighty-seven grams (0.32 mole) of L-rhamnose diethylmercaptal was dissolved in 270 ml. of dry pyridine and cooled to −10°, and 390 ml. (3.8 moles) of acetic anhydride was added slowly. After standing for two hours at −10° and an additional two hours at room temperature, the solution was poured into 20 l. of ice-water. The crystalline mass was collected by filtration and dissolved in 100 ml. of methanol by warming. Water was added to the warm solution until a slight turbidity persisted. After standing 18 hours at 4° the product was filtered off and recrystallized from methanol–water; yield 126 g. or 90%, m.p. 60–62°, $[\alpha]^{26}D$ −42.1 (methanol, *c* 2.1), soluble in alcohol–ether; insoluble in benzene–water.

Anal. Calcd. for $C_{18}H_{30}O_8S_2$: C, 49.3; H, 6.83; S, 14.6. Found: C, 49.2; H, 6.67; S, 15.0.

L-Arabo-3,4,5-triacetoxy-1,1-bis-(ethanesulfonyl)-hexene-1.—Twenty-five grams (0.057 mole) of L-rhamnose diethylmercaptaltetraacetate was dissolved in 200 ml. of dry ether and cooled to −5°. A 20% excess calculated on 8 oxidation equivalents per mole of sugar derivative of perphthalic acid (prepared according to H. Bohme, *Org. Syntheses*, **20**, 70 (1940)), in about 1 liter of dry ether was added slowly during one hour. After standing one hour at 0° the solution was left 18 hours at room temperature. The ether was evaporated under reduced pressure, and the residue was extracted with five 50-ml portions of chloroform. The chloroform extract was washed with saturated aqueous sodium bicarbonate then water and dried over sodium sulfate. The chloroform was evaporated under reduced pressure, and the thick light brown residue from the composite of five equal batches oxidized as above was dissolved in one volume of *n*-butyl alcohol. After storing 7 days at 4° the crystals were filtered off and washed with a little cold *n*-butyl alcohol; yield 60 g. or 41%. Reoxidation of the sirup from the crystallization with perphthalic acid and subsequent working up as above did not produce a second crop of crystals. Recrystallized from chloroform:petroleum ether 60–80° 1:1, methyl isobutyl ketone and then *n*-butyl alcohol for analysis, m.p. 101–102°, $[\alpha]^{26}D$ −36.9 (methanol, *c* 2.1); soluble in alcohol, ether–benzene, chloroform; insoluble in water.

Anal. Calcd. for $C_{16}H_{26}O_{10}S_2$: C, 43.5; H, 5.88; S, 14.5. Found: C, 44.2; H, 6.00; S, 14.6.

5-Deoxy-L-arabinose.—Sixty grams (0.135 mole) of L-arabo-3,4,5-triacetoxy-1,1-bis-(ethanesulfonyl)-hexene-1 was dissolved in 1000 ml. of methanol, and 50 ml. of hydrazine hydrate was added. After standing for 20 hours at room temperature the solvent was evaporated under reduced pressure. The residue in 500 ml. of water was extracted with four 100-ml. portions of chloroform. Three hundred fifty ml. of benzaldehyde and 5 g. of benzoic acid were added to the aqueous solution which was then refluxed for 2.5 hours. The cooled solution was extracted with three 100-ml. portions of chloroform and once with 100 ml. of ether. The aqueous solution was then passed through a cation exchanger (IR-120) and then an anion exchanger (IR-400). The effluent which was collected on the basis of sugar content using Shaffer–Somogyi reagent was reduced to a thick sirup weighing 5.7 g. which was presumed to be 5-deoxy-L-arabinose; yield 35%, over-all yield from L-rhamnose 7%.

2-Amino-4-hydroxy-6-[1,2-dihydroxypropyl)-(L-*erythro*)]-pteridine.—Six grams (0.0405 mole) of methyl 5-deoxy-L-arabinofuranoside was warmed on the steam-bath for 30 minutes in 0.01 *N* HCl; 6.6 g. (0.08 mole) of sodium acetate, 4.8 g. (0.077 mole) of boric acid and 11 g. (0.043 mole) of powdered 2,5,6-triamino-4-hydroxypyrimidine sulfate as a slurry in 35 ml. of water were added in that order. The system was swept with nitrogen through a bubbler extending to the bottom of the solution. Two ml. (0.04 mole) hydrazine hydrate was added. The mixture was heated on the steam-bath while being agitated with a stream of nitrogen. A brownish-yellow precipitate began to form in 15 minutes. After heating for two hours the reaction mixture was cooled in ice for two hours and filtered. The wet cake, 7 g. of dry solid, was suspended in 800 ml. of water saturated with calcium oxide and filtered. The filtrate was heated to 50°, and a 5% solution of zinc acetate was slowly added with good stirring until the *p*H had dropped to 9. The dark brown precipitate was filtered off and discarded. The material in the filtrate was further purified by chromatographic adsorption on Magnesol:Celite 545 1:4, developed with water and adsorption on Dowex-50 using 2 *N* hydrochloric acid as the developer. The desired fractions from each column were selected using the growth of *C. fasciculata* as an assay. The final product was crystallized from hot water

three times; yield 40 to 55 mg. on different runs, dried 16 hours at 115°, $[\alpha]^{26}D$ −52° (0.1 *N* HCl, *c*, .54); solubility: in mg./ml. water, 90°, 3; 4°, 0.5; 1 *N* HCl and 1 *N* NaOH, more than 25 at 25°; ether, acetone and alcohol less than 0.1; biological activity, 0.05 millimicrogram per ml. required for one-half maximum growth of *C. fasciculata* in 144 hours.

Anal. Calcd. for $C_9H_{11}N_5O_3$: C, 45.6; H, 4.64; N, 29.5. Found: C, 46.0; H, 4.85; N, 28.4; C, 45.9; H, 4.83; N, 30.3.

Acknowledgment.—We wish to thank Dr. R. C. Gore, Stamford Laboratories, American Cyanamid Co., for the infrared analysis and Mr. L. Brancone and staff for the microanalytical determinations.

PEARL RIVER, NEW YORK

[CONTRIBUTION FROM THE NUTRITION AND PHYSIOLOGY SECTION, RESEARCH DIVISION, AMERICAN CYANAMID CO., LEDERLE LABORATORIES]

The Isolation and Characterization of a Pteridine Required for the Growth of *Crithidia fasciculata*[1]

BY E. L. PATTERSON, M. H. VON SALTZA AND E. L. R. STOKSTAD

RECEIVED MAY 10, 1956

Procedures are described for the isolation from human urine of a compound named biopterin required for the growth of the protozoön *Crithidia fasciculata*. These involve adsorption on charcoal, counter-current solvent distribution and partition chromatography. Pure biopterin crystallized from water in pale yellow spheres. Biopterin was tentatively characterized as a 2-amino-4-hydroxyalkylpteridine from its ultraviolet absorption spectra. Alkyl substituents of $C_3H_7O_2$ were indicated from the elemental analyses, and 1.7 periodate oxidation equivalents per mole were consumed at *p*H 2. The pteridine fragment was 2-amino-4-hydroxy-6-formylpteridine identified by ultraviolet absorption. No formaldehyde, formic acid or ammonia were in the reaction mixtures. The presence of a methyl group in biopterin was established by infrared absorption. From these data it was concluded that biopterin is 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine.

Previous observations indicated that the protozoön *Crithidia fasciculata* required an unknown factor for growth which was present in a number of natural materials.[2] A preliminary report was made on the isolation of this factor which was named biopterin and was identified as 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine.[3] Forrest and Mitchell[4] have since reported the isolation of 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine from Drosophila. It was identified from degradation studies and by comparison with a synthetic specimen. However, the optical configuration was not determined. Some of the properties of one of the pteridines isolated from Drosophila by Viscontini, *et al.*,[5] are similar to those of biopterin, but these authors have not yet established beyond doubt the structure of their compound.[6,7] This paper describes the isolation and characterization of biopterin in detail.

Isolation.—For the isolation of biopterin, human adult male urine was chosen as a starting material because of its availability and relatively constant potency. The bioassay previously described[2] was used to develop the isolation procedures. Batches of 200 liters of normal adult male urine were purified by the procedures outlined in Table I.

Adsorption on charcoal was used as a first step to separate biopterin from the large volumes of urine.

TABLE I

THE SCHEME FOR THE ISOLATION OF BIOPTERIN FROM HUMAN ADULT MALE URINE

Procedure	Bioactivity, mg. Biopterin	Solids, g.
1. Five 200-liter batches of urine	314	11,850
2. 1 kilo charcoal/100 liters urine. Eluate, NH_4OH:ethanol:water, 10:50:50	113	1,715
3. Counter-current solvent distribution *n*-butyl alc./water, *p*H 5; 40 tubes, 40 transfers, one liter per phase	115	360
4. Partition chromatography *n*-butyl alc./0.05 *M* phosphate, *p*H 4.5	80	
5. Partition chromatography on composite of 5 batches		
(a) *n*-butyl alc./0.01 *M* phosphate, *p*H 8.2	46	
(b) *n*-butyl alc./0.01 *M* sulfate, *p*H 2.3	39	
(c) *n*-butyl alc.:ethyl acetate 15:85/0.01 *M* sulfate, *p*H 2.2	64	1.5
(d) *n*-butyl alc.:ethyl acetate 25:75/0.01 *M* phosphate, *p*H 5	26	
6. Chromatographic adsorption on Magnesol[8] and developed with 0.05 *N* aqueous ammonia; fractions collected by fluorescence and evaporated to dryness		
7. Residue extracted with 5 ml. 0.5 *M* HCl; soln. was adjusted to *p*H 6, and the ppt. crystallized from 12 and 7 ml. hot water; washed twice with alcohol, ether and dried at 115° over P_2O_5 for 16 hr.; weight 13 mg.		

(1) Presented in part at the 128th Meeting of the American Chemical Society, Minneapolis, Minn., September 11–16, 1955, Abstracts p. 65C.

(2) H. P. Broquist and A. M. Albrecht, *Proc. Soc. Expll. Biol. Med.*, **89**, 178 (1955).

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(7) M. Viscontini, E. Loeser, P. Karrer and E. Hadorn, *ibid.*, **38**, 2034 (1955).

(8) Westvaco Chemical Division, South Charleston, West Virginia.