aqueous solution of sodium ethyl oxalacetate (0.11 mole) and 7 ml. of acetic acid was added and the mixture heated on the steam-bath for ten minutes. After cooling and allowing to stand for two hours, a yellow solid separated which was filtered off, recrystallized from Butyl Cellosolve and dried *in vacuo*. The yield was 75-85% of a yellow crystalline product, m.p. 210°. Anal. Calcd.: C, 62.05; H, 5.21; N, 12.06. Found: C, 61.93; H, 5.02; N, 12.49. Ethyl  $\alpha$ -(2-Hydroxy-3-quinoxalyl)-propionate.—The recation was corrided out as above with sodium ethyl oxplores.

Ethyl  $\alpha$ -(2-Hydroxy-3-quinoxalyl)-propionate.—The reaction was carried out as above with sodium ethyl oxalpropionate. The product, which was extremely soluble in ethanol and acetone, was obtained in nearly quantitative yields, m.p. 160–162°.

Anal. Caled.: C, 63.40; H, 5.72; N, 11.37. Found: C, 62.95; H, 6.13; N, 11.72.

3-Methyl-2-furo[2,3-b]quinoxalone.—Diphenyl ether (500 ml.) was heated to reflux and ethyl  $\alpha$ -(2-hydroxy-3-quinoxalyl)-propionate (10 g.) added portionwise over a period of one-half hour. After the final addition had boiled out ethanol, the solution was cooled, diluted with ligroin and the solid separating collected, washed with ether and recrystal-lized from Methyl Cellosolve. The yield was 60% of the theoretical, m.p. 310°.

Anal. Caled. C, 65.99; H, 4.02; N, 13.99. Found: C, 65.90; H, 4.01; N, 13.67.

2-Hydroxy-3-ethylquinoxaline Hydrate.—Ethyl  $\alpha$ -(2-hydroxy-3-quinoxalyl)-propionate (12.3 g.) was suspended in a small volume of water containing 3 g. of potassium hydroxide and boiled for one-half hour. Concd. hydrochloric acid was added dropwise until the solution was acidic. Carbon dioxide was evolved and the product separated. After recrystallization from hot water, the yield was 75% of the product, m.p. 198°.

Anal. Calcd.: C, 62.48; H, 6.29; N, 14.58. Found: C, 63.25; H, 6.22; N, 14.84.

2-Hydroxy-3-methylquinoxaline.—This product was obtained from ethyl 2-hydroxy-3-quinoxalylacetate and from 3-methyl-2-furo[2,3-b]quinoxalone by the same procedure as used for the 3-ethyl compound. The product was recrystallized from hot water, yield 72%, m.p. 250°.8

(8) Hinsberg, Ann., 292, 249 (1896), gives the m.p. as 245°.

Anal. Calcd.: C, 67.48; H, 5.03; N, 17.49. Found: C, 67.00; H, 5.06; N, 17.80.

2-Chloro-3-ethylquinoxaline.—2-Hydroxy-3-ethylquinoxaline (0.1 mole) was refluxed in 150 ml. of phosphorus oxychloride. After removing the excess oxychloride, the residue was triturated with ice and water, the solution neutralized with ammonia. A low-melting solid separated and was recrystallized from acetone and water, m.p.  $38-40^{\circ}$ .

Anal. Caled.: C, 62.34; H, 4.70; N, 14.59. Found: C, 62.16; H, 4.52; N, 14.72.

The 2-chloro-3-methyl isomer, m.p.  $79-81^{\circ}$  was prepared similarly.

Anal. Caled.: C, 60.51; H, 3.95; N, 15.68. Found: C, 60.72; H, 4.10; N, 15.12.

2-o-Chloroanilino-3-methylquinoxaline.—Equimolar amounts of 2-chloro-3-methylquinoxaline and o-chloroaniline reacted in slightly acid aqueous suspension at the reflux temperature for three days. The product was recrystallized from water, m.p. 114-115°.

Anal. Caled.: C, 66.78; H, 4.48; N, 15.57. Found: C, 66.70; H, 4.71; N, 15.36.

Tetraethyl  $\alpha, \alpha' - (p$ -Phenylenedinitrilo)-bis-( $\beta$ -methylsuccinate).—p-Phenylenediamine reacted with two equivalents ( $^{2}/_{3}$  mole) of ethyl oxalpropionate on a steam-bath in the same manner as for the  $\sigma$ -phenylenediamine. The product precipitated and was recrystallized from methanol, yield 75%, m.p. 105-106°.

Anal. Caled.: C, 60.48; H, 6.76; N, 5.88. Found: C, 60.94; H, 6.98; N, 6.02.

**3,8-Dicarbethoxy-1,10-dihydroxy-2,9-dimethyl-4,7-phenanthroline**.—The previous bis-anil was heated in boiling diphenyl ether until no more ethanol was evolved. The diphenyl ether was cooled and diluted with ligroin to obtain a solid which was recrystallized from Methyl Cellosolve, yield 88%, m.p. 285-290° dec.

Anal. Caled.: C, 62.48; H, 5.24; N, 7.28. Found: C, 62.48; H, 5.15; N, 7.16.

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## A Proposed Structure for Folinic Acid-SF, a Growth Factor Derived from Pteroylglutamic Acid

## By Albert Pohland, Edwin H. Flynn, Reuben G. Jones and William Shive

The structure 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid is proposed for folinic acid-SF, a synthetic growth factor derived from pteroylglutamic acid.

A new group of factors, the folinic acid group, has recently been reported to be more effective than pteroylglutamic acid in preventing the toxicity of x-methylfolic acid for *Lactobacillus casei*<sup>1</sup> and to be essential for the growth of *Leuconostoc citrovorum*.<sup>2,3</sup> A recent communication has described the preparation from pteroylglutamic acid of a reaction mixture which has the biological activities of folinic acid derived from purified liver extracts.<sup>4</sup> A synthetic factor, folinic acid-SF, has been obtained in crystalline form.<sup>5,6</sup> The synthetic factor is effec-

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(2) H. E. Sauberlich and C. A. Baumann, J. Biol. Chem., 176, 165 (1948).

(3) T. J. Bardos, T. J. Bond, J. Humphries and W. Shive, THIS JOURNAL, **71**, 3852 (1949).

(4) W. Shive, T. J. Bardos, T. J. Bond and L. Rogers, *ibid.*, 72, 2817 (1950).

(5) E. H. Flynn, T. J. Bond, T. J. Bardos and W. Shive, *ibid.*, 73, 1979 (1951).

(6) J. A. Brockman, B. Roth, H. P. Broquist, M. E. Hultquist,

tive in promoting the growth of chicks,<sup>6</sup> and in preventing the toxicity of aminopterin for the mouse.<sup>6</sup> More recently folinic acid-SF has been reported to be an effective antianemic substance for the human.<sup>7</sup> It is the purpose of this paper to propose as a tentative structure, 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid (I), for folinic acid-SF.



The proposed structure is based on evidence ob-

J. M. Smith, M. J. Fahrenbach, D. B. Consulich, R. P. Parker, E. L. R. Stokstad and T. H. Jukes, *ibid.*, **72**, 4326 (1950), have reported a crystalline substance which may be the same as folinic acid.SE

talline substance which may be the same as folinic acid-SF.
(7) T. D. Spies, G. G. Lopez, P. Milanes, R. L. Toca, A. Reboredo and R. E. Stone, Southern Med. J., 43, 1076 (1950).



Fig. 1.— , tetrahydropteroylglutamic acid in 0.1 N sodium hydroxide; -----, formyltetrahydropteroylglutamic acid in 0.1 N sodium hydroxide.



Fig. 3.— -----, formylfolinic acid-SF in 0.1 N sodium hydroxide immediate; —, aged 24 hours; —, aged 7 days.

tained by a study of the method of preparation, comparison of ultraviolet absorption spectra of known model compounds, electrometric titration, and changes caused by treatment with acid and base.

The optimum conditions for converting pteroyl-

glutamic acid to folinic acid-SF<sup>4,5</sup> involve formylating, reducing catalytically, and either autoclaving the resulting product or treating it with dilute alkali. The formylation and reduction steps may be carried out simultaneously by reducing pteroylglutamic acid in formic acid solution. Omission of any of the steps results in a decreased yield of folinic acid-SF.<sup>5</sup> The reduction product appears to be sensitive to oxygen and should be protected by an inert atmosphere or a reducing medium prior to and during autoclaving. Elemental analyses of folinic acid-SF are in agreement with the formula C20H28N7O7 and the presence of one formyl group also has been demonstrated. It appears from the method of preparation and the analytical data<sup>5,6</sup> that folinic acid-SF is a tetrahydroformylpteroylglutamic acid. Additional support for this belief was obtained from the results of oxidation of folinic acid-SF with potassium chlorate to yield guanidine, oxalic acid and chloranil. Rhizopterin, treated similarly, yields the same products.8 Therefore, the remaining problem is that of establishing the positions of the hydrogens and the formyl group.

There is some question concerning the point of attack in the hydrogenation of pteridines. However, the available evidence indicates that it is the pyrazine ring which undergoes reduction. Pyrazine derivatives are readily reduced to the piperazines by hydrogen and platinum.<sup>9,10</sup> On the other hand the pyrimidine nucleus is more resistant to catalytic hydrogenation.<sup>11</sup> It has been suggested that reduction of pteroylglutamic acid and related pteridines leads to the formation of the 7,8-dihydro compounds by the addi-

tion of one mole of hydrogen.<sup>12</sup> However, there may be exceptions, as in the case of the hydrogenation product of xanthopterin which may not be 7,8-dihydroxanthopterin.<sup>13</sup> Recently Polonovoski and co-workers<sup>14</sup> have shown that the hydrogenation product of 2-ethoxy-4methyl-6,7-diphenylpteridine is probably the 5,6,7,8-tetrahydro compound (II) because the same product is obtained when the known 2ethoxy-4-methyl-6,7-diphenyl-7,8-dihydropteridine is catalytically reduced.



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(12) B. L. O'Dell, J. M. Vandenbelt, E. S. Bloom and J. J. Pfiffner, *ibid.*, **69**, 250 (1947).

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(14) M. Polonovoski, M. Pesson and A. Pinster, Compt. rend., 230, 2005 (1950).

A study of the ultraviolet spectra of tetrahydropteroylglutamic acid and its formyl derivatives gives evidence that these are 5,6,7,8tetrahydro compounds. The spectra (Figs. 1, 2, 3) closely resemble those of model pyrimidines, 16, 17 and the indications are that the ultraviolet absorption of tetrahydropteroylglutamic acid (Fig. 1), the reduction product of formylpteroylglutamic acid (Fig. 1), folinic acid-SF (Fig. 2) and formylated folinic acid-SF (Fig. 3) is due mainly to an intact pyrimidine ring system within these molecules. The absorption curves of pteroylglutamic acid and formylpteroylglutamic acid are given in Fig. 4. Comparison with those of the hydrogenated derivatives illustrates the change brought about by reduction. The ultraviolet absorption spectrum of paminobenzoylglutamic acid (Fig. 5) has a qualitative resemblance to that of the pyrimidines under consideration; however, quantitatively the absorption ( $\epsilon$  15,600 at 273 m $\mu$ , the maximum) is much less than that of folinic acid-SF, and somewhat less than that of dihydropteroylglutamic acid<sup>16</sup> and tetrahydropteroylglutamic acid.

Additional support for structure I for folinic acid-SF is provided by electrometric titration data. Folinic acid-SF has three titratable groups. They are all acidic and have  $pK'_a$  values of 3.1, 4.8 and 10.4. Pteroylglutamic acid has two titratable, acidic groups in its solubility range of pH 5 to 10.5, with  $pK'_a$  values of 5.0 and 8.2. In both compounds the  $pK'_{a}$  values at or below 5 may be assigned to the glutamic acid portion of the molecules, and in pteroylglutamic acid the  $pK'_a$  value 8.2 may be assigned to the phenolic hydroxyl at position 4. The only logical acidic group with  $pK'_a$ 10.4 in folinic acid-SF is also a phenolic hydroxyl at position 4. Two other model compounds were titrated for comparison with folinic acid-SF. These were 2,5,6-triamino-4-hydroxypyrimidine and its formylated derivative, 2,6-diamino-5-formylamino-4-hydroxypyrimidine. The former had three titratable groups with  $pK'_a$  values 2.0, 5.1 and 10.1 and the latter had two with  $pK'_a$  values of 2.5 and 9.9. The  $pK'_{a}$  values, 10.1 and 9.9, of the phenolic hydroxyl groups in these pyrimidine models are almost identical with the 10.4 value of folinic acid-SF and it may be inferred from this that the pyrimidine ring system in folinic acid-SF is completely aromatic.

Although formylpteroylglutamic acid<sup>18</sup> has not been completely characterized, there seems to be little doubt that the formyl group is attached in the

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(16) L. F. Cavalieri, A. Bendich, J. F. Tinker and G. B. Brown, *ibid.*, 70, 3875 (1948).

(17) L. F. Cavalieri and A. Bendich, ibid., 72, 2587 (1950).

(18) M. Gordon, J. M. Ravel, R. E. Eakin and W. Shive, *ibid.*, 70, 878 (1948).



Fig. 4.— ——, pteroylglutamic acid in 0.1 N sodium hydroxide; ----, formylpteroylglutamic acid in 0.1 N sodium hydroxide; —, formylpteroylglutamic acid aged 24 hours in 0.1 N sodium hydroxide.



Fig. 5.— , *p*-aminobenzoylglutamic acid in 0.1 N sodium hydroxide; ---, 2,4,5-triamino-6-hydroxypyrimidine in 0.1 N sodium hydroxide immediate.

ten position. This conclusion is based on analogy with the known structure of rhizopterin (10-formyl-pteroic acid).<sup>8</sup> The hydrogenation of 10-formylpteroylglutamic acid (V) in aqueous solution or in formic acid solution gives a crude product which we have pictured as 10-formy1-5,6,7,8-tetrahydropteroylglutamic acid (VII). This product has essen-tially no activity for L. citrovorum. However, when allowed to stand in alkaline solution or heated, biological activity (folinic acid-SF) is acquired. The progressive changes in ultraviolet spectrum of the product aged in 0.1 N sodium hydroxide are shown in Fig. 6. After 24 hours the spectrum closely resembled that of folinic acid-SF, the single maximum shifting from 253 to  $281 \text{ m}\mu$ . At the same time the L. citrovorum activity increased tenfold. The paper chromatograph and agar plate bioautograph using L. citrovorum showed the acquired activity to be that of folinic acid-SF. This formation of folinic acid-SF is most probably due to an intramolecular rearrangement, 10-formy1-5,6,7,-8-tetrahydropteroylglutamic acid being isomeric with folinic acid-SF. We suggest the most likely rearrangement is a migration of the formyl group, structural considerations favoring migration to position five. The various transformations which ap-



Fig. 6.— ----, formyltetrahydropteroylglutamic acid in 0.1 N sodium hydroxide immediate; ——, aged 5 hours: ——, aged 24 hours.

pear to be in best accord with the experimental facts are indicated in the accompanying flow sheet.



Analogous acyl migrations are known in certain tetrahydroquinoline and tetrahydroisoquinoline compounds, where the participating nitrogen atoms are on adjacent carbon atoms, *i.e.*, the catalytic hydrogenation of 1-benzoyl-2-cyano-1,2-dihydroquinoline (VIII) yields 2-benzoylaminoinethyl-1,2,3,4-tetrahydroquinoline (IX).<sup>19</sup>



Migration of acyl radicals between an amino group and an adjacent hydroxyl group also is well-known.<sup>20</sup> A general treatment of this subject has appeared recently.<sup>21</sup>

The much higher intensity of absorption of folinic acid-SF at 282 m $\mu$  than that of tetrahydropteroylglutamic acid (Figures 2 and 1) again suggests the presence of a formyl group in folinic acid-SF in position 5, as indicated in structure I. Cavalieri and Bendich<sup>17</sup>

in structure I. Cavalieri and Bendich<sup>17</sup> have pointed out that the introduction of a formyl group on the 5-amino group of their tetrasubstituted pyrimidines increased the intensity of absorption by about 50%. A comparison of the ultraviolet spectra of two model compounds prepared in the present study clearly demonstrates the effect of formylation of the amino group in position 5 of a substituted pteridine. These model compounds are 2-amino-4-hydroxy-6,7-dimethyl-5,6,-7,8-tetrahydropteridine (X) and the corresponding 5-formyl compound (XI).<sup>22</sup> Figure 7 shows that the



presence of the formyl group in compound XI has more than doubled the molecular extinction. It is noteworthy that the position of the maximum corresponds closely to that of folinic acid-SF. Electrometric titration of compound X shows two titratable groups having  $pK'_a$  values of 5.6 and 10.4. The formylated compound (XI) has only one titratable group with  $pK'_a$  10.0 indicating that formylation has covered the basic group with  $pK'_a$  5.6. Folinic acid-SF has no basic titratable group in the pH range 2 to 10.5. This may be taken as further evidence that the amino group at position five is formylated as shown in structure I.

Folinic acid-SF may be prepared from pteroylglutamic acid by another reaction sequence as indicated in the above flow sheet. Pteroylglutamic acid was hydrogenated in formic acid solution and then acetic anhydride was added to effect formylation. This, presumably, yielded compound VI

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(1923); A. P. Phillips and R. Baltzly, THIS JOURNAL, 69, 200 (1947).
(21) S. Winstein and R. Boschan, *ibid.*, 72, 4669 (1950).

(22) The formyl group is assigned to position 5 in compound XI because 2,5,6-triamino-4-hydroxypyrimidine is preferentially acylated on the amino group in position 5 [see W. Wilson, J. Chem. Soc., 1157 (1948)]. See also reference 13.



Fig. 7.— - - - , 2-amino-4-hydroxy-5-formyl-6,7-dimethyl-5,6,7,8-tetrahydropteridine in 0.1 N sodium hydroxide; —, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine in 0.1 N sodium hydroxide immediate.

having formyl groups at positions 5 and 10. The product (VI) had essentially no activity for L. citrovorum. However, when VI was dissolved in dilute alkali and the solution allowed to stand at room temperature L. citrovorum activity slowly appeared. The low activity initially present was increased fortyfold after the solution had stood for thirteen days. A concomitant change in the ultraviolet absorption spectrum of the solution occurred, with the maximum shifting from 266 to 278 m $\mu$  after eight days (Fig. 8). This new spectrum closely resembles that of folinic acid-SF (Fig. 2) and in this experiment folinic acid-SF apparently has been formed by hydrolysis of the 10-formyl group of compound сн. СШ. VI. A product which was apparently iden-E tical with VI was prepared also from pure folinic acid-SF by formylation with formic acid and acetic anhydride. This product had an activity equivalent to 0.5% of pure folinic acid-SF by weight. Its ultraviolet spectrum very closely resembled that of VI (compare Figs. 3 and 8) and it exhibited the same behavior as VI when aged in alkaline solution, i.e., the L. citrovorum activity increased fiftyfold and the ultraviolet absorption spectrum changed back to that characteristic of folinic acid-SF (see Fig. 2). davs.

It is of interest to note that folinic acid-SF is surprisingly stable in basic solution. A solution in 0.1 N sodium hydroxide was heated at 90°, and after six hours there was no appreciable loss of activity. After 22 hours about 35% of the original activity still remained. On the other hand formylpteroylglutamic acid, V, appears to be readily susceptible to basic hydrolysis. This was evidenced by the fact that the ultraviolet spectrum of a solution of V in 0.1 N sodium hydroxide had completely changed after twenty-four hours at room temperature, to a spectrum identical with that of pteroylglutamic acid (see Fig. 4). Thus the ease with which the formyl group at position 10 is hydrolyzed provides additional evidence that in folinic acid-SF the formyl group is not on position 10 and is probably, as indicated, on position 5.

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## Experimental

**Physical and Analytical Measurements.**—Assay values in the Experimental section are based on the activity of pure folinic acid-SF which gives a half-maximal response in the *L. citrovorum* assay<sup>5</sup> at a concentration of  $1.8 \times 10^{-5} \gamma$  per ml. of assay medium. Ultraviolet absorption measurements were obtained with a model DU Beckman spectrophotometer. Where changes in absorption with varying periods of time are given in the legends of the figures, the time indicated is that when the readings were begun. Potentiometric titration of folinic acid-SF

was carried out in the usual manner and showed the presence of three acidic groups with pK'a's of 3.1, 4.8 and  $10.4 \pm 0.2$ . Analytical data were obtained on folinic acid-SF which had been purified as reported previously<sup>5</sup>;  $[\alpha]^{25}D + 10.5^{\circ}$  (c, 8.3 in H<sub>2</sub>O at pH 8.5).

Anal. Calcd. for  $C_{20}H_{23}N_7O_7$ : C, 50.73; H, 4.90; N, 20.71; -CHO, 6.1. Found: C, 50.64, 50.99; H, 5.01, 5.09; N, 20.97, 18.55; -CHO, 5.50.

Alkali Stability of Folinic Acid-SF.—Folinic acid-SF was dissolved in 0.1 N sodium hydroxide solution at 5 mg. per



Fig. 8.— ----, diformyltetrahydropteroylglutamic acid in 0.1 N sodium hydroxide immediate; —, aged 24 hours; —, aged 8

ml. and the solution was heated on the steam-bath. Aliquots were withdrawn for ultraviolet measurements and for microbiological assay at appropriate intervals. After six hours, 70% of the initial activity for *L. citrovorum* was still present. After 22 hours, 35% of the activity remained.

The above procedure was also carried out using 0.5 N sodium hydroxide, except for assay. Qualitatively the ultraviolet absorption changed only slightly in two hours, the major difference being a drop in  $E_{1 \text{ cm.}}^{1\%}$  from 560 to 450 at the maximum wave length and a shift of the maximum from 282 to 280 m $\mu$ . After 18 hours the  $E_{1 \text{ cm.}}^{1\%}$  value was 395 at the maximum wave length of 275 m $\mu$ .

**Reduction and Alkali Treatment of Formylpteroylglutamic Acid.**—Formylpteroylglutamic acid,<sup>18</sup> 940 mg. (0.002 mole), was reduced in 50 ml. of 98–100% formic acid using 100 mg. of platinum oxide catalyst. After 45 minutes the reduction had ceased, approximately 0.0022 mole of hydrogen having been consumed. The reduction was continued at 40° for 30 minutes and then 12 hours at room temperature. The total hydrogen consumed in the reduction was 0.0037 mole. The reaction mixture was filtered and the filtrate dried in the frozen state. Assay with L. citrovorum showed the fluffy yellow product to have an activity equivalent to 2.2% folinic acid-SF by weight. Changes in the ultraviolet absorption spectrum of this product in 0.1 N sodium hydroxide are shown in Fig. 6. A solution containing 1 mg. per ml. in 0.1 N sodium hydroxide was allowed to stand 24 hours at room temperature and was then adjusted to pH 6.5 with hydrochloric acid. The assay with L. citrovorum showed an activity equivalent to 18% folinic acid-SF by weight. The activity was shown to be folinic acid (9:1) saturated with water. The strips were bioautographed on agar plates seeded with L. citrovorum. The active material in the solution and an authentic sample of folinic acid-SF exhibited the same mobility,  $R_t 0.396$ .

Formylpteroylglutamic acid, 471 mg. (0.001 mole), was dissolved in 100 ml. of water by adding dilute sodium hydroxide to pH7. The solution was reduced using 100 mg. of platinum oxide catalyst. The reduction was complete in one hour, 0.0025 mole of hydrogen having been consumed. An aliquot of this solution was diluted with 0.1 N sodium hydroxide and the changes in the ultraviolet absorption spectrum followed. The same changes were observed as those shown by the product from the reduction in formic acid (Fig. 6).

Reduction of Pteroylglutamic Acid Followed by Formylation.—Pteroylglutamic acid, 886 mg. (0.002 mole), was reduced in 50 ml. of 98-100% formic acid using 100 mg. of platinum oxide catalyst. The reduction was complete in one hour, 0.0045 mole of hydrogen having been consumed. Ten ml. of acetic anhydride was added and the reaction mixture allowed to stand at room temperature for one hour. The catalyst was collected on a filter and the filtrate dried in the frozen state to yield a light brown solid. Assay with L. citrovorum showed the product to have an activity equivalent to 0.5% folinic acid-SF by weight. Changes in the ultraviolet absorption spectrum of this product in 0.1 N sodium hydroxide are shown in Fig. 8. After 24 hours at room temperature in 0.1 N sodium hydroxide, assay with L. citrovorum showed the product to have activity equivalent to 4.1 per cent. folinic acid-SF by weight.

In a similar experiment the activity increased steadily from an initial value of 0.2% to 20% by weight of folinic acid-SF on aging at room temperature in 0.1 N sodium hydroxide for thirteen days. At steam-bath temperatures  $(90-95^{\circ})$  an increase in folinic acid-SF activity from an initial value of 0.2% to a value of 17% was obtained in five hours.

2-Amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine.—2-Amino-6,7-dimethyl-4-hydroxypteridine,<sup>23</sup> 3.30 g. (0.0173 mole) was reduced in 100 ml. of 5 N hydrochloric acid using 200 mg. of platinum oxide catalyst. The reduction was complete in three hours, 0.031 mole of hydrogen having been consumed. The catalyst was collected on a filter and the filtrate concentrated *in vacuo*. The oily residue was crystallized from alcohol-water-acetone solution (1:1:3) in the form of colorless rosettes, weight 3.4 g. For analysis a sample was recrystallized twice from the same solvent mixture and dried at 120° *in vacuo*.

Anal. Calcd. for  $C_8H_{13}N_5O$ ·HCl: C, 41.47; H, 6.09; N, 30.23; Cl, 15.30. Found: C, 41.47; H, 6.00; N, 29.98; Cl, 14.90.

2-Amino-6,7-dimethyl-5-formyl-4-hydroxy-5,6,7,8-tetrahydropteridine.—2-Amino-6,7-dimethyl-4-hydroxypteridine, 3.30 g. (0.0173 mole), was reduced in 100 ml. of 98-100% formic acid using 200 mg. of platinum oxide catalyst. The reduction was complete in three hours, 0.031 mole of hydrogen having been consumed. Ten ml. of acetic an-

(23) C. K. Cain, M. F. Mallette and E. C. Taylor, Jr., THIS JOURNAL, 68, 1998 (1946).

hydride was added and the reaction mixture allowed to stand at room temperature overnight. The catalyst was collected on a filter and the filtrate concentrated *in vacuo* to dryness. The oily residue was dissolved in 200 ml. of water and sodium carbonate added to pH4.5. A pale yellow product slowly separated, weight 3.50 g. For analysis a sample was recrystallized three times from water in the form of pale yellow prisms and dried *in vacuo* at 120° for two hours. The product melted at 215° (dec.).

Anal. Calcd. for  $C_9H_{13}N_8O_2$ : C, 48.43; H, 5.87; N, 31.38; CHO, 13.00. Found: C, 48.58; H, 6.00; N, 31.35; CHO, 13.00.

Tetrahydropteroylglutamic Acid.—Following the procedure of O'Dell, *et al.*,<sup>12</sup> pteroylglutamic acid was hydrogenated in acetic acid using platinum oxide catalyst. The solution was filtered and the filtrate was dried in the frozen state.

Formylfolinic Acid-SF.—Folinic acid-SF (20 mg.) was dissolved in 0.5 ml. of formic acid (98–100%). Acetic anhydride (0.05 ml.) was added and the solution allowed to stand for two hours. Excess reagents were removed *in vacuo* and the sirupy residue was triturated with ether. The amorphous light yellow powder was removed by centrifuging and washed three times with ether and dried *in vacuo* at 120° for two hours.

Anal. Calcd. for  $C_{21}H_{23}N_7O_8$ : C, 50.29; H, 4.62; -2CHO, 11.6; N, 19.56. Found: C, 50.72; H, 5.05; -CHO, 12.4; N, 19.09.

In the L. citrovorum assay for folinic acid activity, the product had an activity equivalent to 0.5% folinic acid-SI<sup> $\circ$ </sup> by weight. After aging for eight days at room temperature in 0.1 N sodium hydroxide, its activity was equivalent to 25% folinic acid-SF by weight.

In 0.1 W solutin hydroxide, its activity was equivalent to 25% folinic acid-SF by weight. **Reaction of Folinic Acid-SF with Bisulfite**.—Folinic acid-SF, 200 mg., was dissolved in 85 ml. of distilled water by adding 2.0 N sodium hydroxide to pH 8.8. To this solution was added 2.08 g. of anhydrous sodium sulfite and 4.1 ml. of glacial acetic acid. The pH was 4.2. The solution was heated on the steam-bath for 15 hours, then assayed for L. citrovorum activity. About 40% of the initial activity was retained. The reaction mixture remained nearly colorless. The ultraviolet absorption showed no marked change in shape or position of the maximum absorption. Extinction at the maximum was decreased about 20%.

Pteroylglutamic acid, treated similarly, reacted in the manner described by Waller, *et al.*<sup>16</sup> The reaction mixture developed a dark color during the heating period. Isolation of the reaction product, 2-amino-4-hydroxypteridine-6-carboxaldehyde, was carried out as described by Waller, *et al.*<sup>16</sup>

Oxidation of Folinic Acid-SF.<sup>8</sup>—Folinic acid-SF, 400 mg., was dissolved in 25.6 ml. of distilled water by adding 9.6 ml. of concentrated hydrochloric acid. The solution was divided into four equal parts and each portion oxidized by heating it on the steam-bath and adding 1.2 ml. of 0.5 M potassium chlorate in 0.1-ml. portions to the hot solution. The solutions were then cooled to room temperature and combined. The crystalline precipitate was removed by filtration and air-dried, giving 30 mg. of product shown to be chloranil by comparison with an authentic sample.

The filtrate was concentrated to dryness in vacuo and the residue extracted with three 3-ml. portions of cold water. The water insoluble fraction, 50 mg., was refluxed with 2.5 N hydrochloric acid for five hours, then concentrated to dryness under reduced pressure. The residue was dissolved in 5 ml. of water and an excess (8 ml.) of a saturated aqueous solution of pieric acid was added. The crystalline precipitate which formed, 35 mg., was recrystallized from hot water. The compound was identical with known guanidine pierate on the basis of X-ray diffraction pattern, melting point and nitrogen content.

The filtrate remaining after removal of guanidine picrate was extracted with ether, then treated with calcium chloride to give calcium oxalate, identified by comparison with an authentic sample.

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