This product behaved polarographically as did leucovorin; the bioassay was 0.486 mg./mg. for *Le. citrovorum*.

Leucovorin.—Five grams of neutral barium salt, obtained by chromatographic purification as described above, was dissolved in 400 ml. of distilled water, 0.5 g. of Darco G-60 was added, and the solution clarified. The filtrate at 30° was acidified with formic acid to *p*H 3.5, and the solution was cooled slowly with occasional stirring to 3°. The crystalline material was filtered and washed with water. The filter cake was reprecipitated again as described above. The light cream crystalline material was dried quickly under water-pump vacuum at 30° to give 1.6 g. of leucovorin as the trihydrate.

Anal. Calcd. for $C_{20}H_{23}N_7O_7 \cdot 3H_2O$: C, 45.54; H, 5.54; N, 18.59; CHO, 5.50; H_2O, 10.25. Found: C, 45.2; H, 5.67; N, 18.8; CHO, 5.07; H_2O, 11.15 (by Karl Fischer titration).

Leucovorin trihydrate melted with decomposition at 248–250° (cor.); $[\alpha]^{25}D$ +16.76 (*c* 3.52 on anhydrous basis; sample dissolved in 5% sodium bicarbonate; *p*H 8.36).

By acidification with formic acid of a 0.5% solution of calcium leucovorin at 35°, and slow cooling to 30°, with seeding, leucovorin crystallized as flat plates exhibiting oblique extinction, with a tendency to form twin crystals. There was also a tendency to aggregate into tightly packed spheres. The refractive indices of the flat plates were: n_L 1.80; n_S 1.50; the angle of extinction was 9°.

In many cases when leucovorin was precipitated, two crops were obtained. The first, isolated after cooling 4–12 hours, was insoluble in pyridine and was hydrated to lesser degree (5–8%). The second crop, which appeared when the filtrate from the first crop was cooled further, was soluble in pyridine and was more highly hydrated (9–14%). The latter material was of a slightly higher purity. The difference in pyridine solubility was probably due to the difference in hydration.

Anhydrous Leucovorin.—A sample of crystalline hydrated leucovorin (0.1000 g.) was dried under high vacuum at 100° for two hours. There was no color change, and the weight loss was 11.8%. This gave a bioassay of 1.08 mg./mg.

A second sample of crystalline leucovorin was dried at 155° for four hours at a pressure of 20 μ. The substance turned a bright yellow after heating for a short time. Bioassays on this product indicated 0.401 mg./mg. of leucovorin activity.

A third sample of leucovorin (0.0473 g.) was heated at 200° and 2–3 mm. pressure for five hours. The material became a bright yellow, and the bioassay of 0.038 mg./mg. indicated almost complete loss of activity.

BOUND BROOK, N. J.
PEARL RIVER, N. Y. RECEIVED SEPTEMBER 26, 1951

[CONTRIBUTION FROM THE PHARMACEUTICAL RESEARCH SECTION, CALCO CHEMICAL DIVISION, AMERICAN CYANAMID COMPANY]

Chemistry of Leucovorin

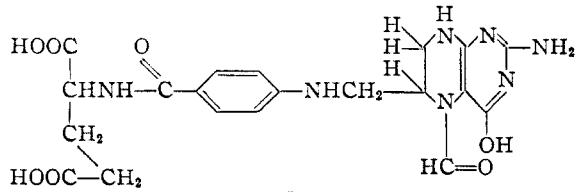
BY DONNA B. COSULICH, BARBARA ROTH, JAMES M. SMITH, JR., MARTIN E. HULTQUIST AND ROBERT P. PARKER

RECEIVED JANUARY 7, 1952

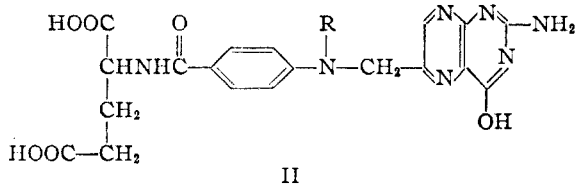
From chemical studies on leucovorin and related formyltetrahydropteridines it is concluded that leucovorin has the structure 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid. Leucovorin nitrate, which is active for *Leuconostoc citrovorum* 8081, is described. Four acid transformation products of leucovorin have been prepared and characterized.

The synthesis of leucovorin (I), a pteroylglutamic acid derivative with biological properties similar to the citrovorum factor (CF)[1] of Sauberlich and Baumann, was reported in earlier communications from these laboratories.[2,3] On the basis of chemical studies on the pure crystalline synthetic substance, its transformation products, and related model compounds, the structure N-(4-[(2-amino-4-hydroxy-5-formyl-5,6,7,8-tetrahydropyrimido(4,5,b)-pyrazinyl-6)-methyl]-aminobenzoyl)-glutamic acid (I) is considered to be the most probable for leucovorin.[4] The purpose of the present paper is to present new chemical evidence for this structure.

The essential steps in the synthesis[3] of leucovorin are: reduction of pteroylglutamic acid (PGA)[5] (II, R = H) or 10-formylpteroylglutamic acid[6] (II, R = CHO) in formic acid solution at 0° to 30° over a platinum catalyst, whereby two moles of hydrogen are absorbed; neutralization with aqueous sodium bicarbonate solution; adjustment with sodium hydroxide to *p*H 10 to 12, followed by heating; and subsequent isolation of the active material.



I



II

R = H, pteroylglutamic acid (PGA)

R = CHO, 10-formyl PGA

The formic acid solution of the crude reduction product had a very low activity for *Le. citrovorum*, but following the step of heating in alkaline solution the activity was maximum. The empirical formula of the crystalline factor was $C_{20}H_{27}N_7O_7$, and the presence of one formyl group was demonstrated. From this it was concluded that leucovorin was a formyltetrahydropteroylglutamic acid,[3] and for elucidation of the structure it was necessary to determine the location of the four hydrogen atoms and the formyl group. The fact that the formyl group was not lost by heating at *p*H 10 to 12 indicated strongly that it did not exist as a simple 10-formyl

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

(2) J. A. Brockman, Jr., *et al.*, THIS JOURNAL, **72**, 4325 (1950).

(3) B. Roth, *et al.*, *ibid.*, **74**, 3247 (1952).

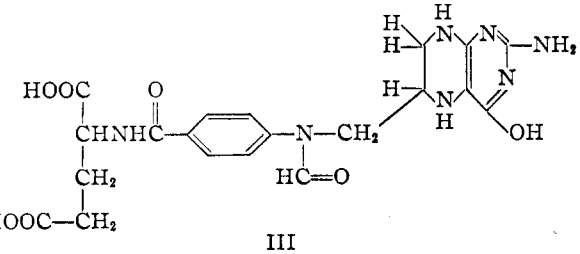
(4) The same conclusion has been reached by W. Shive and coworkers for the structure of "folinic acid-SF," based upon different approaches, as reported in papers before the 119th Meeting of the American Chemical Society, Boston, Massachusetts, April, 1951.

(5) R. B. Angier, *et al.*, *Science*, **103**, 667 (1946).

(6) M. Gordon, *et al.*, THIS JOURNAL, **70**, 878 (1948).

substituent, since in the case of 10-formyl PGA the formyl group is readily hydrolyzed under these conditions.

Dihydro- and tetrahydropteridines have been described[7] in which the pyrazine ring is reduced. Both dihydro- and tetrahydropteroylglutamic acid[8] have been reported, the latter being prepared by catalytic reduction of PGA in acetic acid; and presumably the pyrazine ring was reduced in both cases. The oxidation of reduced pteridines to the aromatic forms is well known.[7,8] As a means of studying the stage of reduction of pteridines, the potential at the dropping mercury electrode in the polarograph was utilized, and it was shown by Allen and co-workers,[9] of these Laboratories, that it was possible to distinguish between PGA, 10-formyl PGA, and dihydro- and tetrahydro-PGA by means of the potential at *p*H 9. In the case of PGA (and pteridines in general) the polarograph gave three inflection points in the curve corresponding to the tetrahydro, dihydro and aromatized forms; the height of the wave between inflection points was a measure of the concentration of a particular reduction state. By means of the data thus obtained it was demonstrated that the crude product formed by the reduction of PGA or 10-formyl PGA under the conditions used in the synthesis[3] was a tetrahydropteridine derivative because it produced the typical tetrahydropteridine wave in the polarograph at *p*H 9. Both the pyrimidine ring and *p*-aminobenzoylglutamic acid were found to be nonreducible polarographically under similar conditions. It was logical therefore to locate the four added hydrogen atoms in the crude reduction product of 10-formyl PGA in positions 5, 6, 7 and 8 of the pyrazine ring, and to assign to this product the structure 10-formyl-5,6,7,8-tetrahydro PGA (III). The possibility of tautomeric ring structures was also considered.



III

Presumably a new asymmetric center has been formed at the 6-carbon atom of the tetrahydropteridines I and III, and the existence of two diastereoisomers of I, having different biological activity, has been considered.

When the crude reduction product (III) was subsequently heated in alkaline solution and analytically pure leucovorin had been isolated, it was found to give no wave in the polarograph at *p*H 9. It was also found to be stable to air oxidation in neutral or weakly alkaline solution. Thus, the tetrahydropyrazine ring had become stabilized and the molecule was difficult to oxidize or reduce at *p*H 9. By contrast the crude 10-formyltetrahydro-PGA was quite sensitive to oxidation both in the polarograph and by atmospheric oxygen. It was thus shown that the increased stability of leucovorin to oxidation was the result of the treatment in alkaline solution, and that this increased stability paralleled the previously observed increase in biological activity. During the step of bicarbonate neutralization in the synthesis[3] it was probable that the crude 10-formyltetrahydro PGA (III) was protected from air oxidation by the large amounts of carbon dioxide evolved. This also suggested that the function of ascorbic acid in the syntheses of the citrovorum factor reported by other investigators was that of an antioxidant.[10]

Among the outstanding chemical properties of naturally occurring citrovorum factor are its stability in alkaline solution, as evidenced by no loss in activity for *Le. citrovorum*,[11] and its instability in acid solution, accompanied by loss of activity for *Le. citrovorum*.[10b,12] When subjected to acid conditions natural factor retained growth-promoting activity for *Streptococcus faecalis* R. and thus closely resembled PGA. Leucovorin was similarly stable in alkaline solution, and it was inactivated for *Le. citrovorum* by acid treatment but retained activity for *S. faecalis* R.[3] While leucovorin gave neither oxidation nor reduction waves in the polarograph at *p*H 9, after being subjected to treatment with mineral acid at room temperature for a short time it was then found to give the characteristic tetrahydropteridine wave in the polarograph at *p*H 9 under anaerobic conditions. The polarographic data together with the previously observed absorption of two moles of hydrogen during synthesis[3] established that leucovorin was a tetrahydro-PGA derivative in which the pyrazine ring of the pyrimido(4,5-b)pyrazine nucleus was reduced and stabilized. A study of the product or products from acid treatment of leucovorin appeared to be an important approach to the problem of its structure. The above changes in biological activity and polarographic behavior strongly suggested that a base-catalyzed isomerization had been accomplished during the full synthesis of leucovorin, and that subsequent acid treatment reversed the process in some manner. Possible explanations included the formation of a new ring linking the N^{10}-position by a one-carbon bridge to positions 5, 7 or 8 in the tetrahydropyrazine ring, or a shift of the N^{10}-formyl group to the pyrazine ring, or the introduction of an additional formyl group (or groups) in the tetrahydro pyrazine ring with subsequent removal of this group, or the N^{10}-formyl group, by hydrolysis in the alkaline treatment.

The oxidation with alkaline permanganate of 2-

(7) J. H. Boothe, *et al.*, *ibid.*, **70**, 27 (1948); M. Polanovski, *et al.*, *Compt. rend.*, **230**, 2205 (1950); M. Pesson, *Bull. soc. chim.*, **15**, 963 (1948); W. R. Boon, *et al.*, *J. Chem. Soc.*, 96 (1951).

(8) B. L. O'Dell, *et al.*, THIS JOURNAL, **69**, 250 (1947).

(9) W. Allen, *et al.*, *ibid.*, **74**, 3264 (1952).

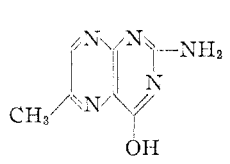
(10) (a) W. Shive, *et al.*, *ibid.*, **72**, 2818 (1950); (b) C. A. Nichol and A. D. Welch, *Proc. Soc. Exp. Biol. Med.*, **74**, 72 (1950); E. H. Flynn, *et al.*, THIS JOURNAL, **73**, 1979 (1951).

(11) C. N. Lyman and J. M. Prescott, *J. Biol. Chem.*, **178**, 523 (1949); K. A. Lees and W. B. Emery, *Biochem. J.*, **45**, Proc., ii (1949); H. P. Broquist, *et al.*, *Proc. Soc. Exp. Biol. Med.*, **71**, 549 (1949).

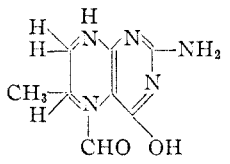
(12) T. J. Bardos, *et al.*, THIS JOURNAL, **71**, 3852 (1949); T. H. Jukes, H. P. Broquist and E. L. R. Stokstad, *Arch. Biochem.*, **26**, 157 (1950); H. P. Broquist, E. L. R. Stokstad and T. H. Jukes, *J. Biol. Chem.*, **185**, 404 (1950); J. C. Keresztesy and M. Silverman, *ibid.*, **183**, 473 (1950); M. Silverman and J. C. Keresztesy, THIS JOURNAL, **73**, 1897 (1951).

amino-4-hydroxy-6- or 7-alkylpteridines, including pteroylglutamic acid and its analogs, yields the corresponding 6- or 7-carboxypteridines.[13] When treated directly with alkaline permanganate leucovorin yielded no recognizable product, due perhaps to a deep-seated disruption of the molecule. However, when it was first treated with mineral acid and then oxidized with alkaline permanganate, 2-amino-4-hydroxypteridine-6-carboxylic acid was obtained in 68% yield. No 2-amino-4-hydroxypteridine-6,7-dicarboxylic acid was found in either experiment, which make it unlikely that a new carbon–carbon linkage had been formed in the 7-position of the tetrahydropteridine ring. There remained then the possibilities of a shift of the formyl group, or a new ring formation involving the N^5- or N^8-position. Because of its proximity to the N^{10}-formyl group the N^5-position was considered the site most probably implicated.

As a model compound closely related to leucovorin, 2-amino-4-hydroxy-6-methylpteridine (IV)[13] was investigated. It was readily reduced in formic



IV



V

acid solution over platinum, with absorption of two moles of hydrogen, to form a tetrahydropteridine which gave the characteristic tetrahydropteridine wave in the polarograph at *p*H 9. To effect formylation of this reduction product it was found to be necessary to carry out the reaction in 98–100% formic acid and subsequently to treat the reduced solution with acetic anhydride at room temperature. Under these conditions a monoformyl derivative, subsequently shown to be 2-amino-4-hydroxy-5-formyl-6-methyl-5,6,7,8-tetrahydropteridine (V) was obtained which had many of the same chemical properties previously observed for leucovorin. The formyl group was not removed by heating for one hour in 0.1 *N* sodium hydroxide solution at 95°. The compound showed no wave in the polarograph at *p*H 9, but after it had been treated with mineral acid the characteristic tetrahydropteridine wave was found. It did not react with nitrous acid in the cold but after it was subjected to acid hydrolysis, which removed the formyl group, it then absorbed one mole of nitrous acid. 2-Amino-4-hydroxy-6-methylpteridine did not react with nitrous acid under these conditions,[14] indicating that the 2-amino group is not involved. Thus, the introduction of one formyl group in the N^5- or N^8-position stabilized the tetrahydropteridine ring notably, and the remaining secondary nitrogen in the tetrahydropyrazine ring was inert to formylation or to reaction with nitrous acid.

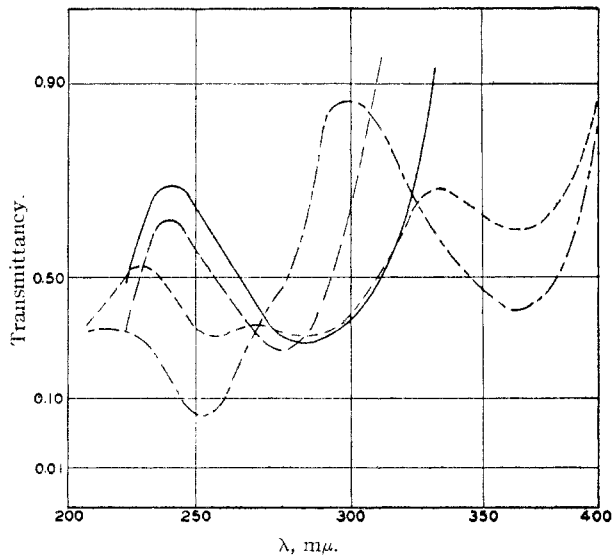


Fig. 1.—Ultraviolet absorption spectra, in 0.1 *N* sodium hydroxide (10 mg./l.): ———, leucovorin; — — —, 2-amino-4-hydroxy-5-formyl-6-methyltetrahydropteridine; - - - - -, pteroylglutamic acid; — · —, 2-amino-4-hydroxy-6-methylpteridine.

The ultraviolet absorption spectra for PGA, leucovorin, 2-amino-4-hydroxy-6-methylpteridine and 2-amino-4-hydroxy-5-formyl-6-methyl-5,6,7,8-tetrahydropteridine were compared (Fig. 1), and additional evidence for the 5-formyltetrahydropteridine structure in leucovorin and in the reduced and formylated pteridine (V) was thus obtained. The absorption at 255–260 and 365 mμ in the pteridines was lost in the two reduced and formylated substances, while strong absorption in the region 277–282 mμ was present.

Since leucovorin was prepared without the aid of acetic anhydride, the results obtained in the formylation of the simple tetrahydro-6-methylpteridine also indicated that the crude intermediate in the leucovorin preparation was indeed 10-formyltetrahydro PGA.

Investigation of the properties of 2,4-diamino-5-formamidopyrimidine[15] revealed some close similarities with the properties of leucovorin and those of the monoformyl derivative of 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine. The 5-formamidopyrimidine was found to be stable to hydrolysis upon heating in 0.1 *N* sodium hydroxide solution for one hour. It did not react with nitrous acid in the cold, but after the formyl group was hydrolyzed with mineral acid at room temperature reaction with one mole of nitrous acid occurred. These properties showed that the amino groups in the 2- and 4-positions were inert to formylation or nitrosation, and suggested that the N^8-position in the tetrahydropteridine nucleus was unreactive to either forylation or reaction with nitrous acid, therefore assignment of the formyl group to the N^5 position in leucovorin and the formylated tetrahydro-6-methylpteridine was logical.

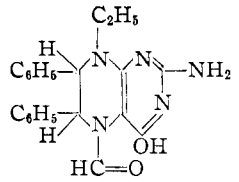
The assignment of the formyl group to the N^5- rather than the N^8-position resulted from the preparation of 2-amino-4-hydroxy-5-formyl-6,7-diphenyl-8-ethyl-5,6,7,8-tetrahydropteridine (VI) by the reduction in formic acid and formylation, in the presence of acetic anhydride, of 2-amino-4-hydroxy-6,7-diphenyl-8-ethyl-7,8-dihydropteridine

(13) I. H. Mowat, *et al.*, THIS JOURNAL, **70**, 14 (1948).

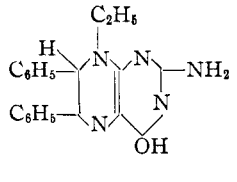
(14) D. B. Cosulich and J. M. Smith, Jr., *ibid.*, **71**, 3574 (1949).

(15) W. Traube and H. W. Dudley, *Ber.*, **46**, 3845 (1913); see also L. F. Cavalieri and A. Bendich, THIS JOURNAL, **72**, 2587 (1950).

(VII).[16] The preparation proceeded smoothly and in a manner identical with that of the 6-methyl-



VI



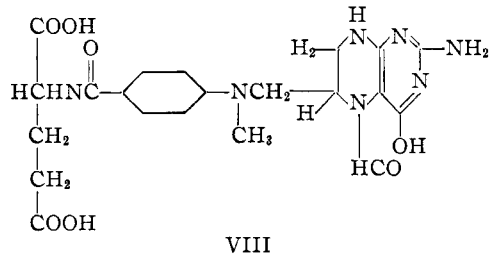
VII

pteridine model (V). The formyl group of VI was not removed by heating in 0.1 *N* sodium hydroxide at 95° for one hour, nor did VI react with nitrous acid until it had been treated with mineral acid at *p*H 2, whereupon about one mole of nitrous acid was absorbed. It should be noted that titration with sodium nitrite of the acid-hydrolyzed formyltetrahydropteridines did not result in sharp end-points. In the case of the compound in question, the original dihydropteridine (VII) was isolated after reaction with nitrous acid. This gave a clear indication that the nature of the nitrite reaction on tetrahydropteridines was one of oxidation rather than nitrosation. In general, the polarographic behavior of VI was similar to leucovorin.

The preparation and properties of this compound also eliminated the possibility of an N^5,N^8-linked ring system, which previously could not have been entirely disregarded.

Because it was considered possible that in leucovorin the formyl group might be involved in the formation of a new ring linking the N^5- and N^{10}-positions, it was of interest to determine whether the N^{10}-position retained the same type of reactivity as found in PGA, *i.e.*, whether it reacted as a secondary aromatic amino nitrogen. To this end the reaction of nitrous acid upon leucovorin and some related structures was investigated. 10-Nitrosopteroylglutamic acid resulted when PGA in cold mineral acid was treated with nitrous acid.[14] Under similar conditions, 10-formyl PGA (II, R = CHO) did not react with nitrous acid, and this may be taken as positive evidence for the location of the formyl group in the 10-position in this compound. When leucovorin suspended in cold dilute hydrochloric acid was immediately and rapidly titrated with nitrous acid, one mole was readily absorbed, and by neutralization of the resulting solution a mononitroso compound precipitated. Upon isolation and purification it was proved by analysis to have the empirical formula $C_{20}H_{22}N_8O_8 \cdot 2H_2O$. Under these conditions it appeared that the N^{10}-position was capable of reaction as a secondary amine. However, if leucovorin was allowed to dissolve in the aqueous mineral acid and stand for a short time at room temperature, there was no reaction with nitrous acid in the cold. This indicated that both the N^5- and N^{10}-positions were blocked, and this was in fact subsequently proved by the isolation of an imidazolinium salt after treatment of leucovorin with hydrochloric acid under similar conditions. The significance of the lack of reactivity will be discussed below in more detail.

As additional evidence for the structure 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid for leucovorin, 10-methylpteroylglutamic acid[17] was hydrogenated in 98–100% formic acid and then the solution of the tetrahydro compound was treated with acetic anhydride to effect formylation. The 10-methyl analog (VIII) of leucovorin was obtained, which contained the same type of alkali-stable, acid-labile formyl group, and had the same behavior in the polarograph. VIII did not react with



VIII

nitrous acid when titrated immediately, as was expected since the N^{10}-position was blocked. However, after standing a short while in mineral acid, the solution readily absorbed nitrous acid. This further indicated that a new linkage in acid-treated leucovorin existed between the N^5- and N^{10}-positions. VIII, when treated with 0.1 *N* hydrochloric acid, did not exhibit a maximum at 355 mμ in the ultraviolet absorption spectra (Fig. 2). The appearance of such a maximum in acid-treated leucovorin, therefore, is probably due to the formation of the N^5–N^{10}-imidazolinium ring.

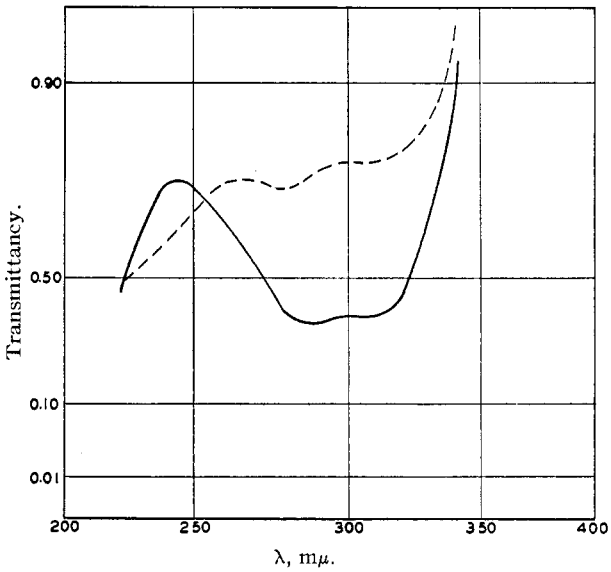


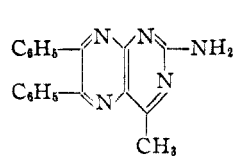
Fig. 2.—Ultraviolet absorption spectra of 5-formyl-10-methyltetrahydropteroylglutamic acid (10 mg./l.): ———, 0.1 *N* sodium hydroxide; - - - - - -, 0.1 *N* hydrochloric acid.

The unusual stability of the formyl group of the 5-formyltetrahydropteridines under alkaline conditions led to speculation about the possibility of hydrogen bonding or actual linkage of the formyl to the 4-hydroxyl group. With this in mind, a new pteridine, 2-amino-4-methyl-6,7-diphenylpteridine (IX), was prepared by the condensation of benzil with 2,4,5-triamino-6-methylpyrimidine.[18]

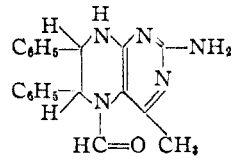
(16) H. S. Forrest, *et al.*, *J. Chem. Soc.*, 3 (1951).

(17) D. B. Cosulich and J. M. Smith, Jr., THIS JOURNAL, **70**, 1922 (1948).

(18) S. Gabriel and J. Colman, *Ber.*, **34**, 1255 (1901).



IX



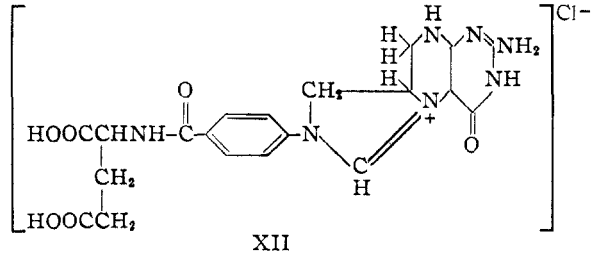
X

IX was hydrogenated and formylated by the same procedure used for V and VI to give 2-amino-4-methyl-5-formyl-6,7-diphenyl-5,6,7,8-tetrahydropteridine (X). Because of its insolubility in alkali, the treatment with hot 0.1 *N* sodium hydroxide was carried out in the presence of alcohol. The formyl compound (X) was recovered unchanged. The only difference between X and members of the 4-hydroxy series lay in the acid lability. X was much more stable to acid hydrolysis, but it could be hydrolyzed by heating in a 2.5 *N* hydrochloric acid solution. These results eliminated the possibility of any involvement of the 4-hydroxyl with the formyl group, and indicated the ketonic nature of the 4-oxygen in pteridines in acid solution, which would make the 5-formyl group more labile under these conditions.

Since the citrovorum factor is inactivated biologically at *p*H 2, a study of the effect of acid upon leucovorin was undertaken, and a number of acid transformation products from leucovorin have been isolated in pure form and characterized.[19] At *p*H 2 leucovorin yielded a yellow precipitate (XI) which was probably a mixture of several substances. However, leucovorin in hydrochloric acid at *p*H 1.3 or below yielded a homogeneous crystalline material containing chlorine, and this was purified by repeated solution in 12 *N* hydrochloric acid and dilution to 2 *N*. The chlorine content varied from one to about two atoms per mole, depending upon the conditions of washing and drying; by drying *in vacuo* over potassium hydroxide, isoleucovorin chloride[19] was obtained for which the empirical formula $C_{20}H_{22}N_7O_6Cl$ was indicated by analysis. The crystallographic constants were identical, and the X-ray diffraction patterns were very similar for preparations containing from one to two chlorine atoms per mole, which indicated that the pure compound contained one chlorine but that under certain conditions incidental hydrogen halide was absorbed. The work of Pfiffner, *et al.*,[20] showed that the crystallographic constants of pteroylglutamic acid were not altered by removal of apparent water of hydration and the situation here appears to be analogous.

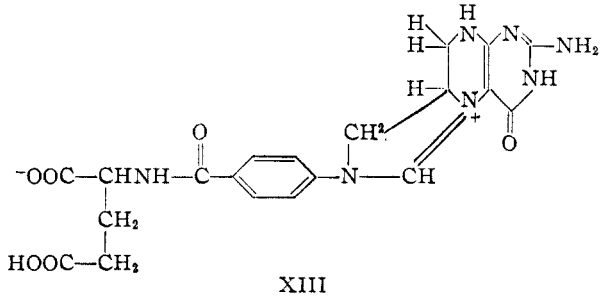
Since isoleucovorin chloride contained a potential formyl group, as shown by the formation of formic acid in the drastic acid hydrolysis of the usual formyl analysis, contained ionic chlorine, and did not react with nitrous acid, the structure suggested was an imidazolinium chloride (XII) in which the N^5- and N^{10}-positions of the parent tetrahydro-PGA structure were joined by a single carbon bridge derived from the formyl group.[19]

When XII was dissolved in boiling water and cooled slowly, anhydroleucovorin-A (XIII)[19] in the form of yellow matted hair-like needles precipitated



XII

from the solution at *p*H 2. XIII was purified by repeated crystallization from 0.01 *N* hydrochloric acid, and had the empirical formula $C_{20}H_{21}N_7O_6 \cdot 4H_2O$. The analytical results showed the loss of a molecule of water, as compared to leucovorin, and the presence of a potential formyl group. XIII did not react with formic acid in the presence of acetic anhydride, and it was concluded, therefore, that it was an imidazolinium "betaine-type" derivative of leucovorin. It should be noted that in these imidazolinium derivatives the conditions for resonance of the quaternary nitrogen between the N^5- and N^{10}-positions are ideal. This undoubtedly accounts for the stability of these compounds.



XIII

By warming in 0.1 *N* hydrochloric acid, XIII was readily reconverted to XII.

When XIII was heated in water or *p*H 4 buffer at the boil, anhydroleucovorin-B (XIV)[19] crystallized from the boiling solution in the form of tablets. The empirical formula, $C_{20}H_{21}N_7O_6 \cdot {}^1/_2H_2O$, indicated that XIV was isomeric with XIII, and that the differences could not be accounted for on the basis of difference of hydration, since the anhydrous forms of XIII and XIV had quite different behavior on heating. XIII melted at 258° (dec.) while XIV did not melt up to 330°. Since XIV could not be formylated further with formic acid in the presence of acetic anhydride, the imidazolinium ring was present. A number of possibilities have been considered, but in the absence of unequivocal evidence to explain satisfactorily the relationship of XIV to its isomer, XIII, we prefer not to postulate a structure for XIV at this time.

It should be noted that XII can also be transformed to XIV in boiling *p*H 4 buffer. This may be reversed by treating XIV with hot 0.1 *N* hydrochloric acid. Attempts to convert XIV to XIII have been unsuccessful, probably because of the extremely low solubility of XIV in 0.01 *N* hydrochloric acid.

Polarographically, these products behaved identically, *i.e.*, they exhibited at *p*H 9 the typical tetrahydropteridine wave, the inference being that, under these mildly alkaline conditions, the imidazolinium ring opened to yield the readily oxidizable

(19) D. B. Cosulich, *et al.*, THIS JOURNAL, **73**, 5006 (1951).
(20) I. J. Pfiffner, *et al.*, *ibid.*, **69**, 1476 (1947).

10-formyl-5,6,7,8-tetrahydro-PGA. The ultraviolet absorption spectra[19] of these three compounds are shown in Fig. 3. Since they were interchangeable by means of changes in acidity, it was necessary to obtain the curves at the acid concentration used for the formation of each. In 0.1 *N* hydrochloric acid XIII and XIV showed curves identical with XII. XIII in *p*H 4 buffer gave the same curve as it did in 0.01 *N* hydrochloric acid but, after standing 24 hours, the spectrum indicated that transformation to XIV had occurred.

All three of these compounds when treated anaerobically for one hour with 0.1 *N* sodium hydroxide were converted in good yield to leucovorin. While they, *per se*, were inactive for *Le. citrovorum*, PGA-like activity for *S. faecalis* R. and *L. casei* was retained; some activity in reversing aminopterin toxicity also remained.[3] Leucovorin was added to 0.5 *N* nitric acid and from the solution isoleucovorin nitrate (XV, XII with Cl^- replaced by NO_3^-) slowly precipitated. Analysis showed the empirical formula $C_{20}H_{22}N_8O_9$ for XV. In the biological assay with *Le. citrovorum* XV was essentially inactive, and in the polarograph it showed a major tetrahydropteridine wave. The analogy of XV to isoleucovorin chloride (XII) was further shown by the conversion of XV to anhydroleucovorin-A and -B under the same conditions (Table I), and to leucovorin by treatment with 0.1 *N* sodium hydroxide under anaerobic conditions. The ultraviolet absorption spectrum exhibited the usual strong maximum at 355 mμ in 0.1 *N* acid.

Treatment of a concentrated aqueous solution of leucovorin with 5 *N* nitric acid resulted in the immediate precipitation of a white material, leucovorin nitrate (XVI), which retained full activity for *Le. citrovorum*, when corrected for the difference in molecular weights, as compared to leucovorin. The ultraviolet absorption spectra and polarographic behavior of leucovorin and XVI were also identical. Although leucovorin nitrate was not obtained in an analytically pure state since attempts to purify it led to loss of biological activity, it is reported here because, heretofore, the action of mineral acids on leucovorin has invariably resulted in complete inactivation for *Le. citrovorum*. The explanation for the retention of leucovorin activity by XVI undoubtedly lies in the fact that the active nitric acid salt precipitated before rearrangement to isoleucovorin nitrate could occur.

Treatment of leucovorin nitrate (XVI) with water (to give *p*H 1.5–1.75) resulted in crystalline material (XVII) which had lost all activity for *Le. citrovorum*. Its ultraviolet absorption spectra and polarographic behavior showed it to be an acid transformation product of leucovorin. Its crystallographic constants and the *p*H for its formation were identical with those of anhydroleucovorin-A (XIII) but analysis of the crude material seemed to indicate the presence of nitrate which was not removed by drying *in vacuo* over potassium hydroxide. Two recrystallizations from hot water yielded anhydroleucovorin-B (XIV). The X-ray powder diagram of XVII was different from anhydroleucovorin-A, although there were certain similarities. At present, it is believed that XVII is anhydroleucovorin-A in an impure state, on the basis of the crystallographic data and *p*H of formation, but the possibility of another nitrate derivative of leucovorin cannot be entirely dismissed.

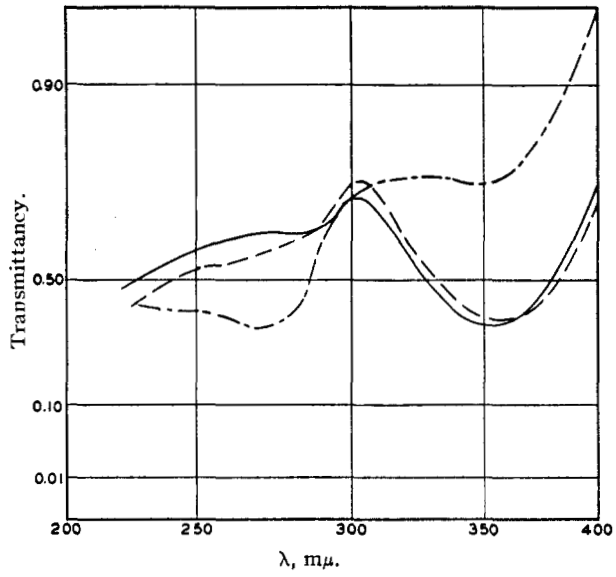


Fig. 3.—Ultraviolet absorption spectra (10 mg./l.): ———, isoleucovorin chloride in 0.1 *N* hydrochloric acid; — — —, anhydroleucovorin-A in 0.01 *N* hydrochloric acid; — · —, anhydroleucovorin-B in *p*H 4 buffer.

The effect of sulfurous acid on leucovorin has been mentioned.[3] Instead of cleavage between the 9- and 10-positions as in the case of pteroylglutamic acid[21] a crystalline product (XVIII) was obtained which had the crystallographic constants and conditions for formation (*p*H 1.7–1.9) of anhydroleucovorin-A (XIII). However, the analysis indicated an impure material containing a variable amount of sulfur, which was not removed by drying over potassium hydroxide. Attempts to purify XVIII by crystallization from water yielded mixtures containing variable amounts of anhydroleucovorin-B plus unidentified products, as was expected. The X-ray powder diagrams of XVIII were almost identical with XVII except that the unit cell of the latter was smaller. As in the case of XVII it is believed that XVIII is impure anhydroleucovorin-A, although the possibility that it could be an "isoleucovorin sulfite" cannot be rejected.

Acknowledgments.—We are indebted to Mr. W. Allen and co-workers for the polarographic studies, to Mr. O. Sundberg and co-workers for the microanalyses, to Mr. J. Morath for the ultraviolet absorption spectra, and Dr. C. E. Maresh for the crystallographic studies. We also thank Dr. H. P. Broquist and co-workers of the Lederle Laboratories, American Cyanamid Company, for the microbiological data, and Dr. L. A. Siegel, Stamford Laboratories, American Cyanamid Company, for the X-ray powder diagrams and their interpretation.

Experimental

Potassium Permanganate Oxidation of Leucovorin.—A solution of 0.5 g. (representing 0.25 g. of anhydrous leuco-

(21) B. L. Hutchings, *et al.*, THIS JOURNAL, **70**, 10 (1948).

vorin) of the barium salt of leucovorin in 50 ml. of 0.1 *N* hydrochloric acid was allowed to stand for five hours. The solution was then made 1 *N* with sodium hydroxide by the addition of 50 ml. of 0.1 *N* and 33.2 ml. of 5 *N* sodium hydroxide and dilution to 166 ml. After warming to 95°, a solution of potassium permanganate was added so that only a slight excess was present at any time. The oxidation was extremely rapid. When a slight excess remained after five minutes, sodium hydrosulfite was added to decolorize, manganese dioxide was removed by filtration, and the filtrate was cooled and adjusted to *p*H 2.5–3.0 with hydrochloric acid. After two days, the precipitated material was centrifuged, washed with a small amount of water and then with acetone, and dried at 100°. Identified as 78% 2-amino-4-hydroxypteridine-6-carboxylic acid by ultraviolet absorption spectra and by chromatographic analysis of the cyclohexylamine salt in butanol–water, the material weighed 0.077 g., representing a 68.1% yield of theoretical.

A 0.6-g. sample of the barium salt of leucovorin placed directly in 166 ml. of 1 *N* sodium hydroxide and then treated with potassium permanganate reacted much more slowly than in the above experiment. When neutralized, as above, no product could be isolated.

2-Amino-4-hydroxy-5-formyl-6-methyl-5,6,7,8-tetrahydropteridine (V).—A solution of 5 g. of 2-amino-4-hydroxy-6-methylpteridine[13] in 150 ml. of 98–100% formic acid was shaken with 0.3 gram of platinum oxide under 41 lb. of hydrogen pressure until there was no more hydrogen absorbed. Then 15 ml. of acetic anhydride was added and the mixture allowed to stand overnight. After removal of the platinum catalyst, a portion of the filtrate was examined polarographically[9] and found to have no waves until treated with acid, whereby a major tetrahydropteridine wave appeared. This filtrate was evaporated *in vacuo* to an oily residue, to which was added 300 ml. of water. The *p*H of the solution was then adjusted to 4–5. After cooling overnight, microscopic clumps of needles separated which were filtered, washed with water, and recrystallized from 65 ml. of boiling water with a charcoal clarification. After a second recrystallization long, shiny needles were obtained which weighed 0.591 g. and melted with decomposition at 282–286°. A small sample was dried for analysis at 100° and 2–3 mm. for 2.5 hours.

Anal. Calcd. for $C_8H_{11}N_5O_2 \cdot {}^1/_2H_2O$: C, 44.1; H, 5.55; N, 32.1; -CHO, 13.3. Found: C, 44.5; H, 5.4; N, 32.1; -CHO, 13.6.

Polarographically,[9] this material behaved in a manner identical to that of leucovorin, *i.e.*, it showed no waves until treated with mineral acid at *p*H 2, then adjusted to *p*H 9, whereupon a major tetrahydropteridine wave appeared.

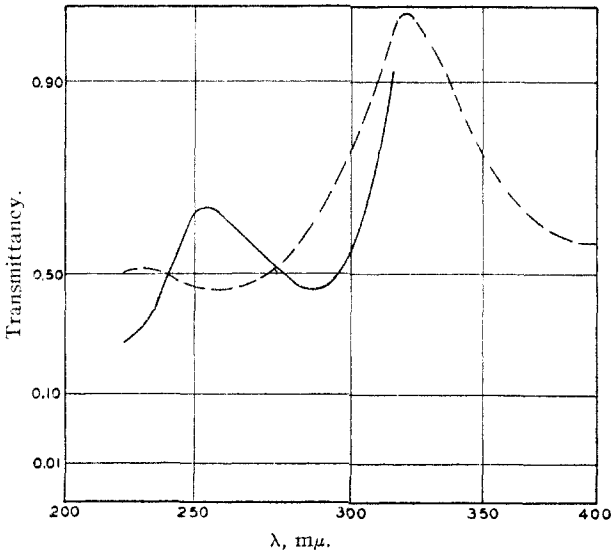


Fig. 4.—Ultraviolet absorption spectra (10 mg./l.) in 0.1 *N* sodium hydroxide: ———, 2-amino-4-hydroxy-5-formyl-6,7-diphenyl-8-ethyltetrahydropteridine; - - - - - -, 2-amino-4-hydroxy-6,7-diphenyl-8-ethyl-7,8-dihydropteridine.

The ultraviolet absorption spectrum in 0.1 *N* sodium hydroxide is shown in Fig. 1.

When a 0.2090-g. sample was placed in 20 ml. of 1 *N* hydrochloric acid at 0–5° and titrated with 0.1 *N* sodium nitrite at once, it was found that there was no absorption of nitrite. This solution was allowed to stand at room temperature overnight. After cooling to 0–5°, 7.7 ml. of 0.1 *N* sodium nitrite was required to reach a starch-iodide paste end-point. Since the theoretical amount for one mole was 9.6 ml., hydrolysis was 81% complete.

A solution of 0.093 g. of V in 10 ml. of 0.1 *N* sodium hydroxide was heated at 95° for 45 minutes, cooled and adjusted to *p*H 4–5. After cooling overnight a filmy white precipitate was present which was not isolated. The mixture at 0–5° was acidified with hydrochloric acid and titrated with 0.1 *N* sodium nitrite. There was no absorption of nitrite, since one drop of solution gave an end-point with starch-iodide paste immediately, and hence no hydrolysis of the 5-formyl group.

2,6-Diamino-4-hydroxy-5-formamidopyrimidine. Acid Lability.—A sample (0.3195 g.) of 2,6-diamino-4-hydroxy-5-formamidopyrimidine[15] was added to 50 ml. of 0.5 *N* hydrochloric acid at 0° and titrated immediately with a total of 0.4 ml. of 0.1 *N* sodium nitrite to a strong starch-iodide paste test. Since the theoretical amount for the absorption of one mole was 18.84 ml. of 0.1 *N* sodium nitrite, it was concluded that the formamidopyrimidine had not reacted *per se*. However, when the same quantity of sample stood in 50 ml. of 0.5 *N* hydrochloric acid at room temperature overnight, 13.2 ml. of the 0.1 *N* sodium nitrite reagent was absorbed, representing 70% hydrolysis.

Alkali Stability.—A solution of 0.3107 g. of 2,6-diamino-4-hydroxy-5-formamidopyrimidine in 50 ml. of 0.1 *N* sodium hydroxide was heated for one hour at 95°. After cooling to 0°, the solution was treated with 5-ml. portions of 1 and 5 *N* hydrochloric acid at 0°, and titrated to a starch-iodide paste end-point with 1.9 ml. of 0.1 *N* sodium nitrite. Therefore, only 10% hydrolysis of the formyl group in alkali had occurred.

2-Amino-4-hydroxy-5-formyl-6,7-diphenyl-8-ethyltetrahydropteridine (VI).—A sample of 2-amino-4-hydroxy-6,7-diphenyl-8-ethyl-7,8-dihydropteridine (VII) was prepared by the method described by Forrest and co-workers,[16] and 0.1017 g. reduced in 15 ml. of 98–100% formic acid over 0.02 g. of platinum oxide in a semimicro hydrogenation apparatus.[22] The hydrogen absorption was 5.8 ml. (STP), corresponding to 87.7% of the theoretical amount for one mole.

To prepare a larger sample of the tetrahydro compound 3.49 g. of VII in 150 ml. of 98–100% formic acid was shaken under pressure with 0.3 g. of platinum oxide for three hours. The reaction vessel was flushed with nitrogen and 15 ml. of acetic anhydride added. After standing 24 hours, the catalyst was removed by filtration, and the filtrate was concentrated, under water-pump vacuum, to a sirup. This was diluted with water and adjusted to *p*H 7. After cooling several hours the crystalline precipitate was isolated and recrystallized six times from 50% aqueous alcohol. The pure white, shiny needles weighed 1.645 g. and melted with decomposition at 248–249°, and were dried at 100° (5 mm.) for 3 hours.

Anal. Calcd. for $C_{21}H_{21}N_5O_2 \cdot {}^1/_2H_2O$: C, 65.7; H, 5.77; N, 18.2; -CHO, 7.55; H_2O, 2.34. Found: C, 65.1; H, 5.69; N, 18.4; -CHO, 7.82; H_2O, 3.0 (by Karl Fischer titration).

The ultraviolet absorption spectra are shown in Fig. 4. Twenty-four hour aging of VI in 0.1 *N* sodium hydroxide showed no pronounced change in the curve; however, aging in 0.1 *N* hydrochloric acid produced a curve identical with VII (also shown in Fig. 4) indicating loss of the formyl group and subsequent oxidation of the tetrahydropteridine.

The stability in alkaline solution was borne out by heating 0.0531 g. of the formyl compound VI for one hour at 95° in a mixture of 5 ml. of alcohol and 5 ml. of 0.1 *N* sodium hydroxide. After cooling and neutralizing, 0.0465 g. (87.7% recovery) of the starting material was obtained. It was identified by mixture melting point and ultraviolet absorption spectra.

When a solution of 0.0601 g. of the formyl compound in 6 ml. of 90% alcohol at 5° was treated with 5 ml. of 1 *N* hydrochloric acid at 0° and then titrated immediately with

(22) C. R. Noller and M. R. Barusch, *Ind. Eng. Chem., Anal. Ed.*, **14**, 907 (1942).

0.01 *N* sodium nitrite, only 1.0 ml. of the reagent was absorbed. After heating an hour on the steam-bath (to prevent crystallization of the starting material), the solution was cooled and titrated with 15.70 ml. of 0.01 *N* sodium nitrite. The end-point was difficult because of the yellow color which had developed. However, assuming that one mole of nitrite is involved, hydrolysis was 91.7% complete. After adjustment to *p*H 3 with sodium hydroxide the yellow crystalline precipitate was isolated and weighed, when dry, 0.0419 g. It was identified as VII by mixture melting point and ultraviolet absorption spectra. The yield was 77.4% of theory. Therefore, this compound behaved in acid much as did leucovorin. It was also shown that the action of nitrous acid on tetrahydropteridines was oxidative in nature.

Because of the low water solubility of VI, it was necessary to study the polarographic behavior of this compound in 30% aqueous alcohol. In general, it was analogous to that of leucovorin, except that because of the ease of oxidation of the tetrahydro form only a dihydropteridine wave was observed.

Nitrous Acid Reaction (A) Leucovorin.—To 5 ml. of 5 *N* hydrochloric acid at 0° was added 0.0495 g. of calcium leucovorin. Immediately the solution was titrated with 7.92 ml. of 0.01 *N* sodium nitrite to a positive test on starch–iodide paste. The amount required was 94% of the theoretical for one mole.

(B) Acid-Treated Leucovorin.—A solution of 0.0455 g. of calcium leucovorin (0.0000775 mole) in 5 ml. of 5 *N* hydrochloric acid was allowed to stand at room temperature for three hours. After cooling to 0° only 0.4 ml. of 0.01 *N* sodium nitrite was required to give an excess of the reagent, indicating no nitrite absorption.

(C) 10-Formylpteroylglutamic Acid.—To 5 ml. of 5 *N* hydrochloric acid at 0° was added 0.0487 g. of 10-formylpteroylglutamic acid[6] (0.000104 mole). Upon titrating immediately, only 0.3 ml. of 0.01 *N* sodium nitrite was required to reach the usual end-point.

(D) 10-Methylpteroylglutamic Acid.[14]—A solution of 0.5510 g. of 10-methyl PGA[17] in concentrated hydrochloric acid was cooled to 0°. One drop of 0.1 *N* sodium nitrite gave a positive starch–iodide test.

(E) Dihydropteroylglutamic Acid.—A cold solution of 0.5174 g. of dihydropteroylglutamic acid[8] in 5 *N* hydrochloric acid was titrated with 17.5 ml. of 0.1 *N* sodium nitrite. The end-point in this case was indefinite and difficult to obtain. Assuming that 2 moles of nitrite should be absorbed, 74.5% of theory was used.

(F) Tetrahydropteroylglutamic Acid.—A solution of 0.55 g. of PGA (0.00112 mole) in 40 ml. of acetic acid was hydrogenated over 0.15 g. of platinum oxide in the usual manner.[8] After the absorption of the customary two moles of hydrogen, the catalyst was removed by anaerobic filtration, and the filtrate added to fairly concentrated hydrochloric acid. While cooling to 0°, a slow stream of nitrogen was bubbled through the solution. This was maintained throughout the titration with 0.1 *N* sodium nitrite. The end-point in this case was even more indeterminant than with dihydro-PGA. However, approximately 25–30 ml. of the reagent was used, which corresponds to about 2.5 moles.

10-Nitrosoleucovorin.—About 185 ml. of 1.0 *N* hydrochloric acid was cooled to 0° and 2.5 g. of barium leucovorin (0.00291 mole) added. To the partially dissolved mixture was started immediately the addition of 0.202 g. (0.00293 mole) of sodium nitrite dissolved in a little water. After adding a few drops of 0.1 *N* sodium nitrite to assure a purple spot with starch–iodide paste, the solution was allowed to stand at 0° for 15–20 minutes. Sodium hydroxide was then added with cooling to adjust to *p*H 3, precipitating a white solid. After cooling overnight, this was filtered and washed with water to give 0.8615 g. of material, or a 59% yield. This was purified for analysis by two recrystallizations from water. The pure material melted with decomposition at 240–245°, gave a positive Liebermann nitroso test, and consisted of elongated plate crystals of negative character with an obtuse optic axial angle and oblique extinction. The refractive indices were: α, 1.492 ± 0.004; β, 1.720 ± 0.005. The ultraviolet absorption spectrum in 0.1 *N* sodium hydroxide exhibited one maximum at 278 mμ and one minimum at 246 mμ; in 0.1 *N* hydrochloric acid, maximum 282 mμ, minimum 242 mμ.

Anal. Calcd. for $C_{20}H_{22}N_8O_8 \cdot 2H_2O$: C, 44.6; H, 4.87; N, 20.8; H_2O, 6.54. Found: C, 44.3; H, 4.64, N, 20.7; H_2O, 6.3 (by Karl Fischer titration). The presence of the nitroso group interfered in the formyl analysis.

10-Nitrosoleucovorin had leucovorin-like activity for *Le. citrovorum* to the extent of 0.213 mg./mg., using leucovorin as the standard. In the reversal of aminopterin toxicity for *S. faecalis* R., 10-nitrosoleucovorin was found to be twice as active as pteroylglutamic acid.

5-Formyl-10-methyltetrahydropteroylglutamic Acid (VIII).—A solution of 24.8 g. of 10-methylpteroylglutamic acid[17] in 350 ml. of 98–100% formic acid was reduced over 0.75 g. of platinum oxide catalyst at 0°. The absorption of two moles of hydrogen was completed in an hour. The reaction mixture was treated with 35 ml. of acetic anhydride, and allowed to stand 24 hours under a nitrogen atmosphere. After removal of the catalyst by filtration, the formic acid solution was added with stirring to 3.5 liters of ether. The solid was allowed to settle and the supernatant decanted. The material was washed with ether several times by decantation, filtered and washed well with ether. The air-dried solid weighed 14.6 g. and, by polarographic methods,[9] was shown to contain about 50% of a 5-formyltetrahydropteridine derivative.

The crude calcium salt was prepared by dissolving 12.8 grams of the above material in 70 ml. of 1 *N* sodium hydroxide, and after adjusting the slightly turbid solution to about *p*H 7.5, adding a solution of 3.9 g. of calcium chloride in 12.8 ml. of water. To remove considerable colored impurity, the solution was stirred about five minutes with 5.1 g. of Magnesol.[23] The filtered solution was added slowly with stirring to 420 ml. of alcohol. The precipitated calcium salt was filtered, washed and dried to yield 10.8 g.

For purification a method similar to that employed in the chromatographic purification of leucovorin[3] was advantageous. A 10% aqueous solution of 4.3 g. of the crude calcium salt was placed on a Magnesol column (100 g.), about 2 inches deep. Elution was accomplished by water, and the presence of the desired material was determined by addition of a few drops of barium chloride and excess alcohol to a portion of the eluate; a flocculent precipitate was considered a positive test. About 800 ml. of eluate was collected before any flocculence was detected in the test. A total of 1550 ml. was obtained which gave a positive test. This was concentrated *in vacuo* to a small volume, and the concentrate, after being made alkaline, was filtered to remove silicates. After adjusting the *p*H of the filtrate to 7–8, 1.3 ml. of 33% aqueous calcium chloride solution was added. The resulting solution (11 ml.) was added with stirring to 65 ml. of alcohol. After a short cooling period, the calcium salt was filtered, washed and dried to yield 0.285 g.

A small portion of this calcium salt was converted to the free acid by adjustment of its aqueous solution to *p*H 3.48, and cooling. The acid precipitated in the form of small beads collected in aggregates.

A total of 8.0 g. of the crude calcium salt was chromatographed as indicated above, and the products were combined to give 2.0 g. of purified calcium salt.

To effect further purification, 1.4 g. of the calcium salt obtained thus was purified on a column, 1.75 inches deep, as before, using 20 g. of Magnesol. The eluates containing the desired material were concentrated and the calcium salt isolated as described. This calcium salt was recrystallized twice by warming in water and diluting slowly with alcohol to give small tablets aggregated in rosettes. The dried material weighed 0.434 g. The polarographic behavior of this purified material was similar to that of leucovorin.[9] The ultraviolet absorption spectrum is shown in Fig. 2.

Anal. Calcd. for $C_{21}H_{23}N_7O_7Ca \cdot 4^1/_2H_2O$: C, 41.6; H, 5.32; N, 16.2; Ca, 6.61; -CHO, 4.78; H_2O, 13.4. Found: C, 42.0; H, 5.28; N, 15.6; Ca, 6.77; -CHO, 4.92; H_2O, 13.4 (by Karl Fischer titration).

To determine the absorption of nitrite, 41.8 mg. of the pure calcium salt was added to 5 ml. of 5 *N* hydrochloric acid at 0°, and addition of 0.01 *N* sodium nitrite begun immediately. The solution absorbed only 0.23 ml. of the reagent before giving a test with starch–iodide paste. Since 8.2 ml. is the theoretical amount for 1 mole it was concluded that this compound did not react with nitrite. However, after the solution had stood at room temperature for three

(23) Synthetic magnesium silicate, Westvaco Chemical Division, Food Machinery and Chem. Corp., New York, N. Y.

and a half hours, 12.2 ml. of nitrite was absorbed. The end-point was not sharp, as was found in general in titrating tetrahydropteridines. The acid instability of this compound, as with leucovorin, was apparent.

The alkali stability of this compound was demonstrated by treating 43 mg. in 0.1 *N* sodium hydroxide at 95° for 50 minutes. After cooling to 5°, 5 ml. of 5 *N* hydrochloric acid at 5° was added, and titration with 0.01 *N* sodium nitrite begun at once. Only 2.8 ml. was absorbed, indicating that hydrolysis had occurred to the extent of 23%. It was concluded that the formyl group in this compound had a stability similar to leucovorin.

2-Amino-4-methyl-6,7-diphenylpteridine (IX).—A solution of 0.81 g. (0.00583 mole) of 2,4,5-triamino-6-methylpyrimidine[18] and 1.35 g. (0.00641 mole) of benzil in 70 ml. of alcohol was refluxed for a total of 14 hours. The solution was cooled overnight to yield 1.3 g. of crystalline material which was recrystallized from alcohol to give 1.03 g. of large yellow prisms, melting with decomposition at 283–285°.

Anal. Calcd. for $C_{19}H_{16}N_6$: C, 72.8; H, 4.83; N, 22.4. Found: C, 72.7; H, 4.48; N, 22.5.

2-Amino-4-methyl-5-formyl-6,7-diphenyltetrahydropteridine (X).—A solution of 0.7065 g. of 2-amino-4-methyl-6,7-diphenylpteridine in 31 ml. of 98–100% formic acid was reduced with hydrogen over 0.05 g. of platinum oxide. After adding 3.1 ml. of acetic anhydride, the reaction mixture was allowed to stand in a nitrogen atmosphere for 24 hours at room temperature. The catalyst was filtered and the filtrate concentrated *in vacuo*. After diluting with 35 ml. of water and adjusting to *p*H 5, the mixture was cooled for some time to yield a pale yellow solid which, when filtered, washed and dried, weighed 0.576 g. After two recrystallizations from dilute acetone, the shiny, pale yellow plates weighed 0.281 g. and melted, in a bath preheated to 200°, with decomposition at 253.5–262.5°.

Anal. Calcd. for $C_{20}H_{19}N_5O\cdot{}^1/_2H_2O$: C, 67.8; H, 5.69; N, 19.8; -CHO, 8.19. Found: C, 67.8; H, 5.54; N, 19.9; -CHO, 8.35.

The ultraviolet absorption spectrum of X is shown in Fig. 5 with that of the parent pteridine (IX).

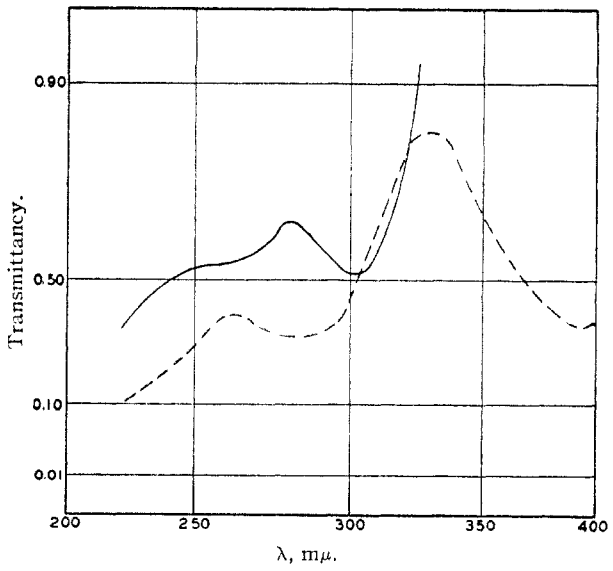


Fig. 5.—Ultraviolet absorption spectra (10 mg./l.) in 0.1 *N* sodium hydroxide: ———, 2-amino-4-methyl-5-formyl-6,7-diphenyltetrahydropteridine; - - - - - -, 2-amino-4-methyl-6,7-diphenylpteridine.

A solution of 0.0306 g. X in 5 ml. of alcohol and 2 ml. of water at 0° was treated with 5 ml. of 5 *N* hydrochloric acid at 0°. Titration with 0.01 *N* sodium nitrite was begun at once. Only 0.7 ml. was absorbed before a positive test with starch–iodide paste resulted. The solution was then heated at 95° for an hour and a quarter. After cooling to 0°, the solution absorbed 6.52 ml. of 0.01 *N* sodium nitrite. The end-point was difficult to obtain because of the yellow color which developed and the usual difficulty with tetrahydropteridines. However, assuming that one mole of nitrite is involved in the reaction, the hydrolysis was 76.5% complete. These results corresponded to the behavior of leucovorin, except that X appeared to be considerably more stable to acid.

The polarographic[9] data for **X** bore this out. In the usual procedure using 0.5 *N* hydrochloric acid with 30% alcohol (for solution), there was no evidence of hydrolysis even after 16 hours. To obtain any hydrolysis it was necessary to heat a 5 *N* hydrochloric acid solution for an hour at 95°, and even then the reaction was probably not complete.

The alkali stability was demonstrated by heating a solution of 0.0242 g. of the formyl compound **X** in 5 ml. of alcohol and 5 ml. of 0.1 *N* sodium hydroxide at 95° for one hour. After adjusting to *p*H 6 with acetic acid, the mixture was cooled for some time. The precipitate which appeared was isolated and weighed 0.0206 g. It was shown to be unchanged **X** by mixture melting point and ultraviolet absorption spectra. The recovery was 85%.

Crude Isoleucovorin Chloride (A) Using 0.1-0.5 *N* Hydrochloric Acid.—A solution of 1.05 g. of barium leucovorin in 50 ml. of 0.5 *N* hydrochloric acid was allowed to stand 2–3 days. A buff-colored crystalline precipitate appeared in the form of rosettes of short needles which were filtered, washed and dried to yield 0.5566 g.

Anal. Calcd. for $C_{20}H_{22}N_7O_6Cl\cdot 2H_2O$: C, 45.5; H, 4.93; N, 18.6; Cl, 6.73; -CHO, 5.5. Found (cor. for 2.81% ash): C, 45.7; H, 4.87; N, 18.3; Cl, 6.83; -CHO, 5.93. (The hydrochloric acid was precipitated with silver sulfate in the digestion mixture.)

The same results were obtained if the acid solution was adjusted to *p*H 1.1–1.2 (*i.e.*, equivalent to 0.1 *N* hydrochloric acid).

(B) ***p*H 1.5–2.0.**—A solution of 2.5 g. of calcium leucovorin in very dilute hydrochloric acid (*p*H 1.62) was allowed to stand for 24 hours. The material which precipitated was crystalline but it was approximately an equal mixture of rosettes of short needles and long, curved hair-like needles. These were isolated to yield 1.94 g. of material (**XI**).

Isoleucovorin Chloride[19] (XII).—Material from either (A) or (B) above could be used in the purification. The crude material (3.0 g.) was dissolved in 65 ml. of concentrated hydrochloric acid and decolorized with charcoal. The filtered solution was diluted with 425 ml. of water and then cooled. The boat-shaped crystals, with tendencies for cluster formation, which precipitated were recrystallized in this manner, using decreasing volumes, four times more. The precipitate was filtered and washed with 1 *N* hydrochloric acid and then with acetone. After drying *in vacuo* over calcium chloride, 0.935 g. of the boat-shaped lamellar crystals was obtained, decomposing at 250–251°.

Anal. Calcd. for $C_{20}H_{22}N_7O_6Cl\cdot HCl$: C, 45.4; H, 4.35; N, 18.6; Cl, 13.4; -CHO, 5.49. Found (corrected for 4.19% H_2O, by Karl Fischer titration): C, 45.4; H, 4.34; N, 18.4; Cl, 13.3; -CHO, 5.58.

If the above material was washed successively with 0.1

TABLE I

CRYSTALLOGRAPHIC CONSTANTS OF ACID TRANSFORMATION PRODUCTS

Compound	Extinction	Refractive indices n_S	n_L
Isoleucovorin chloride (XII)	Parallel	1.84	1.51
Cl, 9.8%	Parallel	1.84	1.51
Cl, 13.26%	Parallel	1.84	1.51
Anhydroleucovorin-A (XIII)	Parallel (0°)	1.63	1.87
	Oblique (29°)	1.48	>1.90
Hydrolysis product of leucovorin nitrate (XVII)	Parallel (0°)	1.63	1.87
	Oblique (29°)	1.48	>1.90
Product of leucovorin and sulfurous acid (XVIII)	Parallel (0°)	1.63	1.87
	Oblique (29°)	1.48	>1.90
Anhydroleucovorin-B (XIV)	Parallel	1.90	1.57
Isoleucovorin nitrate (XV)	Oblique (12°)	1.77	1.47±

TABLE II

ISOLEUCOVORIN CHLORIDE

6.88% Cl		9.8% Cl		13.26% Cl	
Å.	I/I_m[a]	Å.	I/I_m	Å.	I/I_m
12.3	1.0	12.4	0.8	12.5	0.8
9.5	0.7	9.4	1.0	9.5	1.0
9.0	.4	9.0	0.2		
7.5	.2	7.2	.3	7.3	0.2
6.6	.2	6.6	.2	6.7	v.w.
6.4	.1	6.2	.3	6.3	0.3
6.1	.1	6.2	.1		
5.83	.6	5.79	.6	5.87	.2
				5.72d	.2
5.64	.4				
5.29	.6	5.32	.2	5.28	.1
5.13	v.w.[b]	5.11d[c]	.2	5.10	.2
4.91	0.3	4.91	.1	4.90	.2
4.73	.2	4.72	.4	4.75	.4
4.61	.2	4.60	v.w.		
		4.48d	0.2	4.48d	.2
4.45	.2	4.44	0.1	4.35	.1
4.31	.1	4.29	v.w.		
4.11	.4	4.15	0.3	4.11	.3
		4.16	.1		
3.99d[c]	v.w.				
3.87	0.1	3.86	.1	3.88	v.w.
3.79	v.w.	3.75	.1	3.75	0.1
3.65	0.1	3.62	.1		
3.56	0.2	3.58	.1		
3.48	v.w.	3.47	v.w.	3.47	v.w.
3.41	1.0	3.41	1.0	3.41	1.0
3.33	0.7	3.32d	0.3	3.33	0.2
3.23	.3	3.22	.2	3.27	.2
3.19	.1	3.16	.3	3.16	.3
3.10	.5	3.11	.3	3.09	.2
3.03	.1	3.06	.3		
2.96	.1			2.95d	.1
2.88d	.2	2.86	.2	2.87	.1
2.77	.1	2.77	.1		
2.65	v.w.	2.63d	.2	2.64	.2
2.61	v.w.				
2.52	0.1	2.50d	.1		
2.42	.1	2.41	v.w.		
2.35	.1	2.34	v.w.		
2.27	.1	2.26	v.w.		
2.20	.1	2.21	0.1		
2.13	.2	2.13	.1		
2.06	.2	2.05	.1		
2.02	.1				

[a] The data are the interplanar spacings, in ångströms and the estimated relative intensities, a maximum intensity of 1.0 being arbitrarily assigned to the strongest line of each pattern. The radiation used was Ni-filtered CuKα. [b] v.w., very weak line. [c] d, diffuse line.

and 0.01 *N* hydrochloric acid and then acetone, the chlorine analysis fell to 9.8%. Drying of this product at 100° (2–3 mm.) over potassium hydroxide for two hours gave material which contained 8.76% chlorine. Drying under these conditions for 8 hours gave the pure monochloride.

Anal. Calcd. for $C_{20}H_{22}N_7O_6Cl$: C, 48.8; H, 4.51; N, 19.9; Cl, 7.21; -CHO, 5.9. Found (corrected for 6% water, by Karl Fischer titration): C, 49.4; H, 4.67; N, 20.2; Cl, 7.32; -CHO, 5.74.

These various products were examined crystallographically and by X-ray powder diagrams (Tables I and II) and were found to be almost identical. The differences between columns two and three may be due to the poorer pattern obtained for the latter.

Measured as a growth factor for *Le. citrovorum* XII had an activity of only 0.2%. It was three times as active as PGA in reversing the aminopterin toxicity for *S. faecalis* R.

The ultraviolet absorption spectra are shown in Fig. 3.

Polarographic analysis[9] at *p*H 9 under strictly anaerobic conditions revealed a major tetrahydropteridine wave.

A solution of 0.2890 g. of XII in concentrated hydrochloric acid was diluted slightly with water and cooled to 0°. A considerable amount of crystallization occurred. Nevertheless, titration with 0.1 *N* sodium nitrite was undertaken; however, none was absorbed.

Leucovorin from Isoleucovorin Chloride.—A mixture of 4.3 ml. of 1 *N* and 45.7 ml. of 0.1 *N* sodium hydroxide was boiled and cooled under a strong nitrogen stream. Then 0.5 g. of XII was added quickly. The solution was heated under a steady stream of nitrogen for 1 hour at 95–100°. After cooling, filtering and adjusting to *p*H 8, a small sample was assayed with *Le. citrovorum* and was found to contain 10.1 mg./ml.; this indicated that conversion was 100%. The remainder of the filtrate was adjusted slowly to *p*H 3.7 with dilute formic acid. After cooling, the precipitate, which appeared in the form of spherulites typical of leucovorin, was filtered, washed and dried to yield 0.192 g. of material which contained 0.846 mg./mg. of leucovorin activity by bioassay. Cooling the filtrate from this first fraction gave an additional 0.039 g. By ultraviolet absorption spectra analysis, it was 90% pure I.

Anhydroleucovorin-A[19] (XIII).—Isoleucovorin chloride (XII) (3.1 g.), when added to 378 ml. of boiling water, dissolved immediately to yield a solution (*p*H 2) which was treated with charcoal, filtered, and allowed to cool slowly. The mats of hair-like bright yellow needles which crystallized were filtered when the temperature reached 35°, washed with 0.01 *N* hydrochloric acid, and dried to yield 1.3 g. Further cooling of the filtrate gave 0.72 g. of gelatinous material containing undifferentiated crystals.

XIII was purified by dissolving 1.3 g. in 0.01 *N* hydrochloric acid (180 ml.) at 96° and after charcoal decolorization, it precipitated as mats of hair-like needles. After a second recrystallization 0.5282 g. of material was obtained. Since the material dried *in vacuo* over calcium chloride was hygroscopic, no attempt to dry it to an anhydrous form for analysis was made. The water content was determined by drying a sample at 100° over phosphorus pentoxide and weighing rapidly. The Karl Fischer titration for water content was unsuccessful because the matted character of the material prevented dispersion in the Karl Fischer reagent, in which XIII was largely insoluble.

Anal. Calcd. for $C_{20}H_{21}N_7O_6 \cdot 4H_2O$: C, 45.5; H, 5.42; N, 18.6; CHO, 5.5; H_2O, 13.7. Found: C, 45.4; H, 5.44; N, 18.6; CHO, 5.68; H_2O, 13.4.

The melting point of the dried sample was, with preliminary shrinking at 245°, 250–257° (dec.).

The crystallographic data of the acicular crystals are shown in Table I. The material after drying had the same crystallographic indices and essentially the same X-ray powder diagram (Table III) as the hydrated form.[19]

In the polarograph[9] at *p*H 9 under anaerobic conditions XIII was found to exhibit a major tetrahydropteridine wave. The ultraviolet absorption spectrum in 0.1 *N* hydrochloric acid was the same as that of isoleucovorin chloride, as would be expected, since these are the conditions for the transformation to the latter. In 0.01 *N* hydrochloric acid the spectrum was quite similar to that of XII. In aging in *p*H 4 buffer, the spectrum was the same as XIV.

Biologically, XIII had 0.0024 mg./mg. of leucovorin activity, but was three times as active as PGA in reversing the aminopterin toxicity for *S. faecalis* R.

To a mixture of 4.1 ml. of 98–100% formic acid and 0.41 ml. of acetic anhydride was added 0.1375 g. of XIII. After allowing the solution to stand overnight at room temperature, it was poured into 50 ml. of ether. The bright yellow precipitate was filtered, washed with ether and dried *in vacuo* over calcium chloride to yield 0.104 g. of solid which was similar to the starting material, by both ultraviolet absorption spectrum analysis and polarographic behavior. The formyl analysis (4.79%) indicated that a second formyl group had not been introduced (theory for one formyl, 6.37%; theory for two, 12%).

For the conversion to leucovorin, XIII (6.1 mg.) was heated under nitrogen with 6 ml. of air-free 0.1 *N* sodium hydroxide at 95° for one hour. After adjustment to *p*H

TABLE III

ANHYDROLEUCOVORINS

A		B	
Å.	I/I_m[a]	d	I/I_m
12.2	1.0	11.2	0.2
8.0	.5	9.7	.1
7.5	v.w.[b]	7.6	.3
7.0	v.w.	6.2d	.4
6.6	v.w.	5.64	.3
6.2	0.1	5.33	.2
5.87	1.0	5.08	v.w.
5.57	0.1	4.86	0.3
5.28	.4	4.62	0.5
4.60	.3	4.40	v.w.
4.48	.1	4.21	0.1
4.27d[c]	.2	3.97	.2
4.00	.8	3.76	.5
3.86	.8	3.66	1.0
3.72	.1	3.55	0.5
3.62	.1	3.33	.2
3.49	.4	3.18	.2
3.34	.7	2.93	.5
3.12	.2	2.76	.1
3.05	.2	2.66	.2
2.93	.2	2.59	.1
2.83	.1	2.54	.1
2.78	v.w.	2.47	.1
		2.44	.1
		2.16d	.2
		2.10	.1
		2.07	.1
		2.02	.2

7–8, the solution was found, by bioassay, to contain 0.376 mg./ml. of leucovorin representing a 44% conversion.

XIII (0.0495 g.) was converted to XII by dissolving in 2 ml. of warm 0.5 *N* hydrochloric acid. On cooling, 0.045 g. of XII was isolated, the identity being established by crystallographic comparison.

Anhydroleucovorin-B[19] **(XIV).**—(A) Anhydroleucovorin-A (XIII) (0.17 g.) was heated to the boil in 22 ml. of water. A heavy granular precipitate appeared which was filtered from the boiling solution and dried at 100°. It weighed 0.115 g. and did not melt below 330°. It was extremely insoluble in water or 0.01 *N* hydrochloric acid.

Anal. Calcd. for $C_{20}H_{21}N_7O_6 \cdot {}^1/_2H_2O$: C, 51.7; H, 4.78; N, 21.1; -CHO, 6.25; H_2O, 1.94. Found: C, 51.7; H, 4.68; N, 21.1; -CHO, 6.22; H_2O, 1.6 (by Karl Fischer titration, and also weight loss at 150° *in vacuo*). The anhydrous material also did not melt below 330°.

The crystallographic constants of the equant-tabular crystals are shown in Table I.

Polarographically,[9] XIV exhibited a major tetrahydropteridine wave anaerobically in *p*H 9 buffer. The ultraviolet absorption spectrum in 0.1 *N* hydrochloric acid was, as expected, similar to isoleucovorin chloride. However, it was considerably different when run in *p*H 4 buffer, as can be seen in Fig. 3. The X-ray powder diagram was quite different from that of XIII (Table III).

By bioassay, this compound had 0.0163 mg./mg. of leucovorin activity for *Le. citrovorum*.

A solution of XIV (0.0206 g.) in 20 ml. of air-free 0.1 *N* sodium hydroxide was heated under nitrogen at 95° for one hour. After adjustment to *p*H 7–8 the solution contained 1 mg./ml. of leucovorin activity, representing a 99.5% conversion.

To convert XIV to XII, 0.25 g. was heated to solution in 15 ml. of 0.1 *N* hydrochloric acid at 98°. After cooling, 0.215 g. was isolated which was found to be identical crystallographically with XII.

An attempt was made to transform 0.12 g. of XIV to XIII by heating with 18 ml. of 0.01 *N* hydrochloric acid under reflux at 95° for two hours. By filtering the mixture while hot, 0.0678 g. of XIV was recovered unchanged. The cooled filtrate yielded a trace of amorphous material.

Attempted formylation of 0.15 g. of XIV in a mixture of 4.5 ml. of 98–100% formic acid and 0.45 ml. of acetic anhydride in the usual manner yielded 0.1185 g. of bright yellow solid whose formyl analysis (5.62%) and polarographic behavior[9] (no leucovorin-like activity) indicated that no further formylation had occurred.

(B) Crude isoleucovorin chloride (0.5 g.) was heated to boiling in 63 ml. of *p*H 4 buffer. The heavy insoluble precipitate was isolated to give 0.27 g. which was shown to be anhydroleucovorin-B by crystallographic analysis.

Isoleucovorin Nitrate XV.—A solution of 5 g. of calcium leucovorin in 25 ml. of water was added slowly to 250 ml. of 0.5 *N* nitric acid. The resulting amber solution, after standing at room temperature for 24 hours, was cooled for several days to yield a yellowish tan crystalline precipitate which weighed 1.68 g. For purification this material was recrystallized three times from about 50 ml. of 0.5 *N* nitric acid by warming to 60° and decolorizing with charcoal to give 0.88 g. of light yellow, sharply pointed needles, melting, with pre-heating to 90°, at 194.5–197° (dec.). This material was dried for 8 hours (2–3 mm.) over potassium hydroxide at 100°.

The crystals were lamellar in habit and had the constants shown in Table I. The ultraviolet absorption spectrum in 0.1 *N* nitric acid was essentially that of XII in 0.1 *N* hydrochloric acid. The X-ray powder diagram is shown in Table IV.

Anal. Calcd. for $C_{20}H_{22}N_8O_9 \cdot H_2O$: C, 44.8; H, 4.51; N, 20.9; H_2O, 3.36. Found: C, 45.0; H, 4.24; N, 21.1; H_2O, 2.8 (by Karl Fischer titration). The formyl analysis was omitted since nitric acid would interfere.

TABLE IV

NITRIC ACID PRODUCTS OF LEUCOVORIN

XV		XVII	
d	I/I_m[a]	d	I/I_m
9.6	1.0	18.6	v.w.[b]
7.6	0.3	16.9	0.4
6.5	.2	11.0	.1
5.95	.4	9.9	.9
5.72	.4	9.0	v.w.
5.42	.1	8.1	0.2
5.23	.2	7.5	0.3
5.08	.1	6.5	v.w.
4.82	.3	6.1	0.1
4.60	.4	6.0	.3
4.23	.4	5.49	.1
4.00	.2	5.22	.4
3.66	.1	4.97	.2
3.53	.3	4.68d	.2
3.40	.8	4.40	.1
3.36	.3	4.18d	.1
3.28	.4	3.74	.3
3.16	.3	3.56	.5
3.08	.3	3.43	1.0
3.05	.1	3.20	0.1
2.96	.2	3.00	.3
2.82	.1	2.87	.1
2.75	.2	2.73	v.w.
2.62	.2	2.62	v.w.
2.52	.2	2.56	v.w.
2.37	.1		
2.23	.2		
2.17	.2		

When run anaerobically in the polarograph at *p*H 9 this material showed a major tetrahydropteridine wave, and was inactive biologically (0.016 mg./mg.). It was converted to leucovorin by heating 20 mg. in 20 ml. of 0.1 *N* sodium hydroxide, which had been freed of air, for one hour at 95° under an atmosphere of nitrogen. After adjustment to *p*H 7.5, the solution was found to contain 11.2 mg. of leucovorin representing 63.5% conversion.

XV (0.1 g.) was converted to anhydroleucovorin-A (0.0472 g.) by dissolving in 15 ml. of boiling 0.01 *N* nitric

acid. The identification was made by means of the crystallographic properties.

Anhydroleucovorin-B (0.021 g.) was obtained by boiling 0.1 g. of XV with 12.6 ml. of *p*H 4 buffer for five minutes. Identification was made by melting point (> 330°) and crystallographic data.

Leucovorin Nitrate (XVI).—To 0.50 g. of leucovorin in 3 ml. of water was added sufficient ammonium hydroxide to dissolve the compound, followed by 13 ml. of 5 *N* nitric acid. A white precipitate formed, which was filtered off, washed with 5 *N* nitric acid, alcohol, and acetone, and dried *in vacuo* over calcium chloride to yield 0.35 g.

Anal. Calcd. for $C_{20}H_{23}N_7O_7 \cdot 2HNO_3 \cdot H_2O$: C, 38.9; H, 4.41; N, 20.4; H_2O, 2.92. Found: C, 38.8, 39.9; H, 4.49, 4.88; N, 20.0, 18.5; H_2O, 4.0, 4.2 (by Karl Fischer titration).

The analyses given are for two different preparations.

The ultraviolet absorption spectrum in 0.1 *N* sodium hydroxide was identical with that of leucovorin. The bioassay was the same as that obtained for leucovorin, when corrected for molecular weight differences. The polarograph[9] showed no oxidation or reduction waves at *p*H 9, indicating that the substance retained the leucovorin structure. This compound could not be purified by recrystallization from water without substantial loss of the biological activity.

Hydrolysis Product of Leucovorin Nitrate (XVII).—Five grams of calcium leucovorin was converted to the nitrate (XVI) by dissolving in water and adding 5 *N* nitric acid as above. The product was isolated and placed in 100 ml. of water, which caused it to dissolve in part, and form some gummy material. This mixture was allowed to stand at room temperature for 24 hours, at the end of which time the gummy material had changed to a heavy yellow crystalline precipitate, which appeared as needles under the microscope. The *p*H of the solution was 1.75. The product was isolated and dried *in vacuo;* weight 2.2 g.

Anal. Calcd. for $C_{20}H_{22}N_8O_9$: C, 46.3; H, 4.14; N, 21.6. Found (corrected for 3.3, 12.5 and 7.0% H_2O, respectively, by Karl Fischer titration): C, 46.2, 47.6, 46.4; H, 4.17 3.87, 4.00; N, 21.4, 21.5, 21.7.

The analyses shown represent two preparations; the last series of figures represent the material obtained by drying the second preparation over potassium hydroxide for eight hours at 100° (2–3 mm.).

No leucovorin activity was present in XVII either by polarographic or biological assay (0.0098 mg./mg.). Crystallographically, XVII was identical with anhydroleucovorin-A (Table I). The ultraviolet absorption spectrum was similar to the other acid-transformation products. The X-ray powder diagram (Table IV) was quite different from isoleucovorin nitrate and somewhat different from anhydroleucovorin-A (Table III).

When 0.8 g. of XVII was recrystallized twice from 80–100 ml. of boiling water 0.293 g. of material which did not melt below 330° was obtained. The crystallographic properties were identical with those of anhydroleucovorin-B (XIV).

Product from Leucovorin and Sulfurous Acid (XVIII).—A 1.00-g. sample of leucovorin was mixed with 125 ml. of water at room temperature, and sulfur dioxide was bubbled in until a saturated solution (*ca.* 2 *N*) was obtained. The leucovorin dissolved, and a yellow solution was obtained, which stood at room temperature overnight. The excess sulfur dioxide was then removed by applying a slight vacuum to the solution, over a five-hour period. A light yellow crystalline product separated slowly, which consisted of long hair-like needles. After the five hours, the mixture was cooled, the product isolated, and dried *in vacuo* over calcium chloride; weight, 0.393 g. An additional 0.195 g. was obtained from the mother liquor on standing. XVIII decomposed about 248°, and was inactive in the bioassay (0.019 mg./mg.) with *Le. citrovorum* and by polarographic assay.[9] The ultraviolet absorption spectrum was similar to other acid transformation products of leucovorin and the infrared absorption did not indicate the presence of sulfite. Crystallographically, it was identical with anhydroleucovorin-A (Table I).

Anal. Calcd. for $C_{20}H_{22}N_7O_6 \cdot {}^1/_2SO_3$: C, 48.4; H, 4.47; N, 19.8; S, 3.23. Found (corrected for 7.0, 16.3, 11.5% H_2O, respectively); C, 48.8, 50.4, 53.8; H, 3.97, 3.5, 3.53; N, 20.0, 20.9, 21.9; S, 3.24, 2.12, 1.72.

The first set of analyses represents one preparation, the water content being determined by Karl Fischer. The second series represent another preparation, the water being determined by weight loss (the sulfur analysis did not change on drying). The third set of analyses resulted after drying the second preparation over potassium hydroxide for 8 hours at 100° *in vacuo* (the water content was by Karl Fischer titration). The X-ray powder diagram of XVIII is shown in Table V. The pattern resembled that given by XVII (hydrolysis product of leucovorin nitrate), the principle difference being that the lines of XVII appeared at consistently smaller spacing than equivalent lines of XVIII. This would seem to indicate that the structures of XVII and XVIII are essentially the same, but that the unit of XVII is smaller.

TABLE V

XVIII

Å.	I/I_m[a]	Å.	I/I_m[a]
15.5	0.4	4.82	0.2
12.2	.2	4.46	.2
10.2	.9	4.17	.2
8.3	v.w.[b]	4.00	.1
7.9	0.1	3.76	.5
7.5	.1	3.56	.8
6.8	v.w.	3.41	1.0
6.5	v.w.	2.97	0.1
5.95	0.5	2.85	.1
5.37	.6	2.69	.1
5.07	.1	2.62	v.w.

BOUND BROOK, NEW JERSEY