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

Synthesis of Leucovorin

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The synthesis of leucovorin, a formyltetrahydropteroylglutamic acid active for *Leuconostoc citrovorum*, is described. Leucovorin is obtained from pteroylglutamic acid or formylpteroylglutamic acid by hydrogenation in formic acid, alkaline treatment, and isolation of the active material.

A previous communication from these laboratories[1] reported the synthesis and isolation of a crystalline acid and its barium salt which have biological properties similar to those of the naturally occurring citrovorum factor (CF) of Sauberlich and Baumann.[2] It is the purpose of the present paper to describe in detail the preparation and purification of this synthetic substance, which we have designated leucovorin.[3]

It was reported in 1948 by Sauberlich and Baumann[2a] that the organism *Leuconostoc citrovorum* 8081 requires a growth factor which is present in concentrates from natural materials such as liver, rice bran and yeast extract. A delayed and submaximal growth response was obtained with massive doses of pteroylglutamic acid (PGA). It was demonstrated that CF was different from vitamin B_{12}.[4,5] A functional relationship between thymidine, PGA, and CF was suggested by Broquist, *et al.*,[5] which was emphasized by findings that naturally occurring CF would reverse the inhibitory effect of 4-aminopteroylglutamic acid (Aminopterin)[6] for *Le. citrovorum*.[7] Administration of large doses of PGA to rats or human subjects increased the urinary excretion of CF as much as 200-fold.[8]

It was observed by Broquist, *et al.*, that CF on mild treatment with hydrochloric acid lost activity for *Le. citrovorum*, but retained PGA-like activity for *Streptococcus faecalis* R.[9a] Similar results have been obtained by others.[9b,c]

All these observations suggested that the citrovorum factor was closely related biologically to pteroylglutamic acid. The distribution coefficients of CF and PGA for the system *n*-butanol–water were indistinguishable[9a] thus indicating also that CF was probably closely related to PGA chemically.

It has been suggested that PGA is concerned in the metabolism of formate and other one-carbon fragments in the living cell,[10] and it was found[10a] that "formylfolic acid" was more effective than PGA in preventing the toxicity of "x-methyl PGA"[11] for *S. faecalis* R. In the present investigation 10-formyl-PGA and PGA were compared for anti-Aminopterin potency on the basis of equal PGA activity as measured by separate assay with *S. faecalis* in the absence of Aminopterin. It was found that the inhibitory effect of Aminopterin for *S. faecalis* was reversed more readily by 10-formyl PGA than by PGA. It has been suggested that PGA may function through an oxidation–reduction system in the cell,[12] and therefore the reduction of 10-formylpteroylglutamic acid was undertaken. Reduction by sodium amalgam in aqueous bicarbonate solution did indeed result in a product which gave an increased growth response with *Le. citrovorum*, and a variety of chemical reducing agents including aluminum amalgam, sodium borohydride, magnesium amalgam and magnesium or zinc and ammonium chloride, were found to have similar effect. Attention was then turned to a study of the catalytic reduction of 10-formylpteroylglutamic acid.

In preliminary experiments, the catalytic reduction of 10-formylpteroylglutamic acid in aqueous medium gave increased yields of activity, which, however, were still only 1% or less. These experiments included high pressure reductions with Raney nickel catalyst at 120°, as well as reductions with platinum at room temperature and atmospheric pressure. PGA in aqueous medium absorbs one mole of hydrogen to form a dihydro PGA when reduced catalytically[12] and it appeared likely that the 10-formyl derivative behaved similarly under these conditions. In a different approach, the formylation of dihydro- and tetrahydropteroylglutamic acids[12] was studied. The reaction of dihydropteroylglutamic acid with ethyl formate in the presence of sodium methylate at 150° gave products with an activity of about 1%. However, when the experiment was repeated using freshly prepared tetrahydropteroylglutamic acid,[12] the activity of the product was increased fivefold. This indicated that the substance with activity for *Le. citrovorum* was probably a tetrahydro-, rather than a dihydro-, formylated pteroylglutamic acid. It therefore appeared highly desirable to reduce 10-formylpteroylglutamic acid under conditions such that a tetrahydro derivative would be formed. O'Dell, *et al.*, reported that pteroylglutamic acid formed a tetrahydro derivative when reduced over platinum in glacial acetic acid.[12] It seemed logical to expect that 10-formylpteroylglutamic acid would

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

(2) (a) H. E. Sauberlich and C. A. Baumann, *J. Biol. Chem.*, **176**, 165 (1948); (b) H. E. Sauberlich, *ibid.*, **181**, 467 (1949).

(3) H. P. Broquist, E. L. R. Stokstad and T. H. Jukes, *Federation Proc.*, **10**, 167 (1951).

(4) C. N. Lyman and J. M. Prescott, *J. Biol. Chem.*, **178**, 523 (1949).

(5) H. P. Broquist, *et al.*, *Proc. Soc. Exp. Biol. and Med.*, **71**, 549 (1949).

(6) D. R. Seeger, J. M. Smith, Jr., and M. E. Hultquist, THIS JOURNAL, **69**, 2567 (1947).

(7) H. E. Sauberlich, *Federation Proc.*, **8**, 247 (1949).

(8) H. E. Sauberlich, *J. Biol. Chem.*, **181**, 467 (1949).

(9) (a) H. P. Broquist, E. L. R. Stokstad and T. H. Jukes, *ibid.*, **185**, 399 (1950); (b) T. J. Bond, *et al.*, THIS JOURNAL, **71**, 3852 (1949); (c) J. C. Keresztesy and M. Silverman, *J. Biol. Chem.*, **183**, 473 (1950).

(10) (a) M. Gordon, *et al.*, THIS JOURNAL, **70**, 878 (1948); (b) H. P. Broquist, *et al.*, *ibid.*, **73**, 3535 (1951).

(11) M. E. Hultquist and J. M. Smith, Jr., unpublished data; cited by A. L. Franklin, *et al.*, *J. Biol. Chem.*, **169**, 427 (1947).

(12) B. L. O'Dell, *et al.*, THIS JOURNAL, **69**, 252 (1947).

yield a tetrahydro derivative in acetic acid, and that formic acid, which is a better solvent for PGA and formyl PGA, would produce similar results. The latter procedure would have the added advantage that PGA could be formylated and the product hydrogenated in the same medium, thus eliminating the isolation and purification of the intermediate 10-formyl derivative. The one apparent difficulty in obtaining citrovorum factor activity by such a procedure was that the activity of the natural factor was reported to be completely destroyed by mild acid treatment at *p*H 2.[9] The use of an anhydrous medium was expected to reduce this hazard, and immediate neutralization of the formic acid with alkali would further minimize it. In the initial experiments PGA was formylated in 98–100% formic acid followed by reduction with hydrogen over a platinum catalyst, and two moles of hydrogen were absorbed indicating the formation of a tetrahydro derivative. The reaction mixture was buffered in excess aqueous sodium bicarbonate, and a 10 to 15% yield of citrovorum factor activity resulted. The activity of the solution increased on standing with excess bicarbonate at room temperature, and heating the solution caused a further increase in activity. The optimum yield (40–50%) of citrovorum factor activity was obtained by adjusting the bicarbonate solution to *p*H 12 and heating one hour on the steam-bath, and material prepared in this way was used in the purification and isolation of leucovorin. The addition of acetic anhydride to the starting formic acid–PGA mixture to ensure anhydrous conditions during the reduction had an adverse effect on the yield, and acetic onhydride had no effect on the yield when added after the reduction. It was found that commercial 87–90% formic acid readily formylated pteroylglutamic acid, and when used in the synthesis of leucovorin gave yields equivalent to those obtained with 98–100% formic acid.

The initial product obtained by the reduction of 10-formylpteroylglutamic acid in formic acid differed from leucovorin, but was converted to the active compound by aqueous alkali, as indicated above. The initial product of reduction was isolated by diluting the formic acid solution into absolute ether, and the yellow substance thus obtained had very little activity for *Le. citrovorum*. When the substance was treated with 0.1 *N* sodium hydroxide at 100° for one hour, under anaerobic conditions, the yield of leucovorin activity was about 40%. The presence of ascorbic acid during the synthesis had no effect upon the yield of leucovorin.

Pteroylglutamic acid was reduced in formic acid at 0° without prior heating to effect formylation; two moles of hydrogen were absorbed as before, and upon immediate treatment of the cold reduced solution with alkali, as above, similar yields of leucovorin activity were obtained. This indicated that formylation occurred with ease during or subsequent to the reduction process. Tetrahydropteroylglutamic acid[12] was prepared by reduction of PGA in acetic acid and isolated. It was readily formylated by either 87–90% or 98–100% formic acid, and by the usual alkaline treatment of the product a high yield of leucovorin activity was obtained comparable to that by the other process. Dihydropteroylglutamic acid[12] when treated with formic acid and subsequently with alkali gave a product which was essentially devoid of activity for *Le. citrovorum*.

In a study of the isolation of leucovorin from the dilute aqueous solutions obtained in the synthesis, it was found that at *p*H 7 to 8 Magnesol[13] adsorbed much of the colored impurities from the solution. It also removed substances which absorbed in the 365 mμ region of the spectrum, and were probably pteridines, but very little leucovorin was lost in the process. In order to separate the active compound from the large quantities of sodium formate and inorganic material present, it was adsorbed on Darco G-60[14] at *p*H 4, eluted with an alcohol–ammonia mixture, and then after concentration of the eluates leucovorin was isolated as a neutral calcium or barium salt. Final purification was accomplished by chromatography on Magnesol columns. At first the cyclohexylamine salt of leucovorin was employed, with cyclohexylamine–butanol–water used to develop the column. Subsequently it was found that good separation could be obtained using the calcium or barium salts with water as the developing medium. Approximately fourteen bands were observed in the chromatographic separation; some were visible in ordinary daylight and others were fluorescent under the ultraviolet lamp. Colored impurities were retained at the top of the column, and the leucovorin appeared as a non-fluorescent, rapidly moving band between two blue fluorescent bands. The leucovorin fraction could be recognized by the precipitation of the characteristic white calcium (or barium) salt when small portions of the eluate were diluted with alcohol.

After one passage through a Magnesol column leucovorin was obtained sufficiently pure to be isolated as the crystalline free acid at *p*H 3.5. For analytically pure material leucovorin was chromatogaphed a second time on Magnesol, then isolated as the barium or calcium salt, and precipitated at *p*H 3.5. In precipitating the free acid thus, various hydrates were formed, which contained 8 to 14% water of hydration and differed in their solubility in dry pyridine. Water was lost *in vacuo* at 100° without loss in activity or change in color. The acid is practically colorless, but when heated at 155° for four hours under high vacuum it turned a bright yellow and lost about one-half of its activity. At 200° the loss of activity was complete. The elementary composition of the free acid, $C_{20}H_{23}N_7O_7$, corresponded to a tetrahydroformylpteroylglutamic acid, and in the *Le. citrovorum* assay,[1] half-maximal growth response was obtained with 0.15 mγ of the acid per ml. of test medium. The ultraviolet absorption spectrum of leucovorin is shown in Fig. 1.

Leucovorin formed di- and triequivalent alkaline earth salts. It was stable to heating in 0.1 *N* sodium hydroxide solution, but on standing overnight in 5 *N* sodium hydroxide all activity for *Le. citrovorum* was lost and a mixture of readily oxidizable substances was obtained. In acid solution at *p*H 2 or lower leucovorin lost almost completely the ac-

(13) Syntbetic magnesium silicate, the Westvaco Chemical Division, Food Machinery and Chemical Corp., New York, N. Y.

(14) Activated carbon, Darco Corporation, New York, N. Y.

tivity for *Le. citrovorum*, but retained PGA-like activity for *S. faecalis* R. and *L. casei*. Leucovorin was stable to oxidation by molecular oxygen in neutral to mildly alkaline solution, and it was also stable to oxidation or reduction at the dropping mercury electrode in the polarograph at *p*H 9.[15] The chemical oxidation of a tetrahydroformylpteroylglutamic acid with iodine in sodium bicarbonate solution has been reported by others.[16] Titration of leucovorin under these conditions gave an indeterminate end-point, and reaction of leucovorin with iodine or potassium dichromate in weakly acid solution was slow. The chemical assay of Hutchings, *et al.*,[17] for PGA did not appear to be applicable to leucovorin. Although pteroylglutamic acid is cleaved between the 9- and 10-positions by sulfurous acid,[18] treatment of leucovorin with this reagent yielded a yellow crystalline substance which is apparently a rearrangement product of leucovorin. The chemistry of this substance is discussed in another paper.[19]

During the course of our work, and subsequent to the isolation of analytically pure leucovorin,[1] Shive, *et al.*, reported the preparation of a reaction mixture from pteroylglutamic acid, by treatment with formic acid followed by reduction, which was active for *Le. citrovorum*.[20] Flynn and co-workers[21a] have more recently described the synthesis of a compound, "Folinic acid–SF," with biological properties similar to the citrovorum factor. The structure 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid has been proposed for "folinic acid–SF."[21b,c]

Our investigations have shown that leucovorin probably has the structure 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid, and the details of this work are reported in another paper.[19]

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Experimental

Microbiological Methods.—Citrovorum factor activity was determined by microbiological assay with *Leuconstoc citrovorum* 8081 following the procedure of Sauberlich.[2b] The synthetic crystalline free acid, leucovorin, was used as a standard, and all results are reported in terms of the anhydrous free acid, 0.15 mγ of which is equivalent to one "unit"[3] as defined by Sauberlich.[2a]

Reversal of Aminopterin Inhibition.—PGA and 10-formyl PGA were compared with a concentrate of CF in reversing the inhibitory effect of Aminopterin for *S. faecalis*.

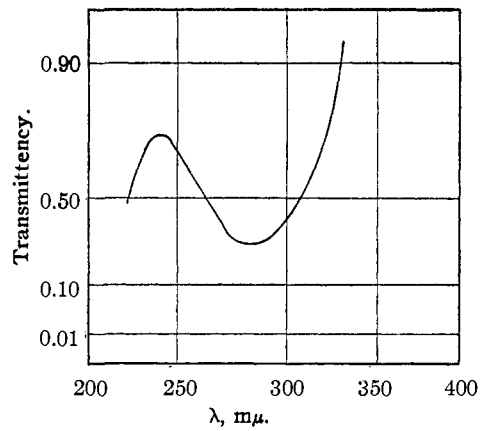

Fig. 1.—Ultraviolet absorption spectrum of leucovorin (10 mg./l.) in 0.1 *N* sodium hydroxide.

The concentrate of CF was prepared as described elsewhere[9a] and was standardized with *Le. citrovorum* and *S. faecalis*. It was used in the assay on the basis of its potency when tested in comparison with PGA using *S. faecalis*.[9a] The results are shown in Table I and indicated that 10-formyl PGA occupied a position intermediate between PGA and CF in reversing the toxic action of Aminopterin.

Chemical Reduction of 10-Formylpteroylglutamic Acid.—Ten mg. of 10-formyl PGA prepared by a method similar to that described for 10-formylpteroic acid[22] was suspended in 1 ml. of an aqueous 30% solution of ammonium chloride and an excess of zinc dust was added. The mixture was shaken for one hour and then heated on a steam-bath for 10 minutes. After cooling, the solution was diluted to 100 ml. in *p*H 7 phosphate buffer and assayed with *Le. citrovorum*. The results, shown in Table II, indicate that 10-formyl PGA was no more potent than PGA, but zinc reduction increased the potency about 1000-fold, thus indicating the production of a new growth factor.

TABLE I

COMPARATIVE EFFECTS OF CERTAIN AGENTS IN REVERSING THE INHIBITION OF *S. faecalis* BY AMINOPTERIN

Preparation tested (0.1 γ added per tube)	Aminopterin added per tube: 0	0.1 γ	1.0 γ	10 γ
	Optical density			
PGA	0.93	0.14	0.01	0
10-CHO PGA	.91	.67	.13	0
CF concentrate[a]	.98	.93	.54	.09

[a] The CF concentrate was added at a level corresponding to 0.1 γ by biological assay against PGA with *S. faecalis*.

TABLE II

RESPONSE OF *Leuconostoc citrovorum* TO PTEROYLGLUTAMIC ACID (PGA), 10-FORMYL PGA AND ZINC-REDUCTION PRODUCT OF 10-FORMYL PGA

Amount of compound added per tube, γ	Compound tested: (a) PGA	(b) 10-formyl PGA	Zn-reduction[a] product of (b)
	Optical density		
0	0	0	0
.01	0	0	.05
.1	0	0	.46
1	0	0	1.40
10	.04	.05	1.70
100	.45	.38	1.70

[a] The amounts of the zinc-reduction product were calculated in terms of the starting material (10-formyl PGA).

Synthetic Methods for Obtaining Leucovorin Activity from Pteroylglutamic Acid

A. Hydrogenation of Formylated Pteroylglutamic Acid in Formic Acid. (1) Typical Procedure.—A mixture of 100

(15) W. Allen, *et al.*, THIS JOURNAL, **74**, 3264 (1952).

(16) F. Weygand, *et al.*, *Zeitschrift für Naturforschung*, **5B**, 413 (1950).

(17) B. L. Hutchings, *et al.*, *J. Biol. Chem.*, **168**, 705 (1947).

(18) B. L. Hutchings, *et al.*, THIS JOURNAL, **70**, 10 (1948); C. W. Waller, *et al.*, *ibid.*, **72**, 4630 (1950).

(19) D. B. Cosulich, *et al.*, *ibid.*, **74**, 3252 (1952).

(20) W. Shive, *et al.*, *ibid.*, **72**, 2818 (1950).

(21) (a) E. H. Flynn, *et al.*, *ibid.*, **73**, 1979 (1951); (b) A. Pohland, *et al.*, *ibid.*, **73**, 3247 (1951); (c) M. May, *et al.*, *ibid.*, **73**, 3067 (1951).

(22) D. E. Wolf, *et al.*, *ibid.*, **69**, 2753 (1947).

g. of pteroylglutamic acid (90%) (0.204 mole) and 650 ml. of commercial 87–90% formic acid was heated at 50° for one hour. It was then cooled to 15°, and 3 g. of platinum oxide catalyst and 5 g. of ascorbic acid were added. The mixture was hydrogenated at atmospheric pressure in a 1-liter flask using a high speed tantalum stirrer (approx. 1500 r.p.m.) for agitation. The hydrogen was contained in calibrated cylindrical tanks over water in the usual manner, so that the hydrogen take-up could be measured accurately. At the end of 65 minutes, 8400 ml. (S.T.P.) of hydrogen had been absorbed, or 92% of the theoretical for two moles. The catalyst was filtered and the solution was then allowed to stand for three days under nitrogen at room temperature, after which it was poured into 9 liters of water containing 1400 g. of sodium bicarbonate (solution A). Sodium hydroxide was added to about *p*H 13, and the mixture was heated at 90–95° for one hour in an open container. It was then cooled and neutralized to *p*H 7.5 (solution B). The dark brown solution was then stirred for 15 minutes with 450 g. of Magnesol and filtered, giving a light yellow filtrate (solution C). This was acidified to *p*H 4, and 400 g. of Darco G-60 was added. After stirring 15 minutes, the mixture was filtered, and the cake washed well with water (filtrate, solution D). The Darco cake was extracted with a hot mixture of 1 liter of alcohol, 250 ml. of ammonium hydroxide, and 750 ml. of water (solution E). A second extraction was carried out using one-half the above volumes (solution F). The combined extracts were concentrated to a volume of 385 ml. and the solution adjusted to *p*H 7. Then 50 g. of barium chloride in 100 ml. of hot water was added, which gave no precipitation. Sodium hydroxide was added to *p*H 13, yielding a yellow-tan precipitate, which was filtered off and discarded. The filtrate was neutralized to *p*H 7, and 2500 ml. of alcohol added, thus precipitating crude barium leucovorin as a light yellow product; dry weight 73.5 g. (G). *Leuconostoc citrovorum* assays on the above samples were as shown in Table III.

TABLE III

Sample	Volume or wt.	Bioassay, mg./ml.	Total leucovorin content, g.
A	10 l.	1.44	14.4
B	10 l.	3.68	36.8
C	9.7 l.	3.36	32.6
D	11 l.	0.34	3.7
E	1.9 l.	11.30	21.5
F	0.8 l.	6.28	5.02
G	73.5 g.	0.29	21.3

The calcium salt of leucovorin was prepared similarly. Further purification of leucovorin was accomplished by chromatography.

(2) **Effect of *p*H and Temperature.**—One gram of pteroylglutamic acid (90%) was heated one hour at 50° in 20 ml. of 87–90% formic acid, cooled to 25°, and hydrogenated at 30 lb. pressure in the shaker in the presence of 0.10 g. of platinum oxide catalyst until approximately two moles of hydrogen were absorbed. The catalyst was filtered, and portions of the filtrate (21.2 ml.) were treated as follows: (a) 1.0 ml. was poured into 10 ml. of water and diluted to 15 ml. of solution, *p*H 1.8. Bioassay of this solution indicated a yield of 3.9% leucovorin activity. (b) 10.0 ml. was poured into 100 ml. of water containing 20 g. of sodium bicarbonate, and diluted to 150 ml. The bioassay showed a 14.9% conversion to leucovorin. (c) A portion of (b) was heated on the steam-bath 30 minutes. The yield of leucovorin by bioassay was 35.1%. (d) A portion of (b) was made alkaline to Clayton Yellow test paper (*ca.* *p*H 13) and allowed to stand at room temperature 1 hour, followed by neutralization to *p*H 7. The yield of leucovorin activity was 5.8%. (e) A sample of (d) was allowed to remain at *p*H 13 for three days before assaying. The yield of leucovorin activity was 25.5%. (f) Another sample of (d) was heated 1 hour on the steam-bath at *p*H 13, followed by neutralization to *p*H 7. The yield of leucovorin activity was 46.8%.

(3) **Isolation of Intermediate Reduced Formylpteroylglutamic Acid.**—The solution from another hydrogenation of formylated pteroylglutamic acid, carried out as in (2) was divided in half, and one portion was immediately poured into ten volumes of absolute ether, yielding a yellow precipitate (a). The second portion was allowed to stand at room temperature for three days, and then treated in the same fashion. This precipitate (b) had a reddish hue not present in (a). These products were analyzed for formyl content as follows: (a), 5.86% CHO; (b) 6.87% CHO. In the bioassay for *Leuconostoc citrovorum*, (a) had an activity of 0.093 mg./mg., and (b) had an activity of 0.031 mg./mg. Samples of 0.2 g. of (a) and (b) were then dissolved in 30 ml. each of oxygen-free 0.1 *N* sodium hydroxide and heated at 100° under nitrogen for 1 hour. The solutions were neutralized and assayed; (a) had a bioassay indicating 34.6% conversion to leucovorin, and (b) 53%.

(4) **Hydrogenation of Highly Purified PGA.**—A solution of 5 g. of pteroylglutamic acid, which had been purified by repeated crystallization as the magnesium salt, in 150 ml. of 98–100% formic acid was hydrogenated at room temperature in the presence of 0.3 g. of platinum oxide. After standing overnight, the catalyst was removed by filtration, and a sample of the filtrate was analyzed by assay with *Le. citrovorum*. Since the volume of solution was 126 ml. and the assay showed 0.677 mg./ml., the yield of active material at this point was 2.04%.

One-fourth of the formic acid solution was poured slowly with stirring into 300 ml. of ether. After cooling a short while, the light yellow precipitate was filtered, washed well with ether, and dried *in vacuo*. This material weighed 0.945 g. Polarographically[15] and biologically, there was no appreciable leucovorin activity. The microbiological assay with *Le. citrovorum* showed 0.014 mg. of leucovorin activity per mg. of solids. Analysis for formyl was 8.24% (theory for one formyl, 6.13%). A solution of 20 mg. of this yellow intermediate in 20 ml. of air-free 0.1 *N* sodium hydroxide was heated on a steam-bath for 45 minutes in a nitrogen atmosphere. The solution, after adjustment to *p*H 7 with acetic acid, gave a 34% yield of leucovorin activity by microbiological assay.

Another one-fourth aliquot of the formic acid reduction mixture was added to 200 ml. of distilled water containing an excess of sodium bicarbonate. This solution, when assayed, indicated a 13.9% yield. After heating at *p*H 12, an assay of the solution indicated a 36.7% yield.

(5) **Effect of Acetic Anhydride.**—An experiment similar to (4), using 5 g. of the same highly purified pteroylglutamic acid, was made. However, upon completion of the reduction, 15 ml. of acetic anhydride was added and the solution allowed to stand overnight. This formic acid solution, when assayed microbiologically, indicated a 1.18% yield.

The leucovorin intermediate was isolated from an aliquot as described in (4) above. It weighed 0.85 g. Polarographically,[15] it showed slight leucovorin activity. Microbiologically, it contained 20 γ/mg. of leucovorin activity. The formyl analysis was 10.1% (theory for two formyl groups, 11.5%). This material when treated in 0.1 *N* alkali just as described above yielded a solution containing 0.566 γ/ml. of leucovorin activity, which represented an over-all yield of 38.5%.

A second one-fourth aliquot was poured into a sodium bicarbonate solution and heated at *p*H 12 just as described in (4) above. The assay by *Le. citrovorum* on this solution indicated a 41.2% yield.

B. **Hydrogenation of Pteroylglutamic Acid in Formic Acid.**—Seventeen grams of pteroylglutamic acid dihydrate was mixed with 170 ml. of 98–100% formic acid. Platinum oxide catalyst (0.5 g.) and 1 g. of ascorbic acid were added, and the mixture was cooled to 2°. It was then hydrogenated at this temperature at atmospheric pressure until the hydrogen take-up had ceased, which took 45 minutes. The volume of hydrogen absorbed was 1542 ml. (S.T.P.), which corresponds to 97.5% of two moles. The catalyst was filtered, and the solution, which had a total volume of 188 ml., divided into aliquot portions for further reactions. An 11-ml. portion was poured into 100 ml. of water containing 40 g. of sodium bicarbonate, made alkaline to Clayton Yellow test paper with sodium hydroxide, and heated on the steam-bath 30 minutes. An assay of the neutralized solution, after dilution to 300 ml., indicated an 11.5% yield of leucovorin.

A second portion of the reduced solution was allowed to stand for three days at room temperature and then treated as above. The assay showed a 27% yield of leucovorin.

A third 11-ml. portion was treated with 3 ml. of acetic anhydride immediately after the reduction. After 10 minutes it was treated with alkali as above. The assay showed an 8.3% yield.

C. Hydrogenation of 10-Formylpteroylglutamic Acid in Formic Acid.—Using a semi-microanalytical hydrogenation apparatus,[23] 0.21 g. of 10-formylpteroylglutamic acid in 10 ml. of 98–100% formic acid was reduced with hydrogen at atmospheric pressure with the aid of 0.02 g. of platinum oxide catalyst. The compound absorbed 19.5 ml. of hydrogen (S.T.P.), which was 97.3% of the theoretical amount for two moles; reduction was complete in 18 minutes. After removal of the catalyst by filtration, the solution was added slowly to 250 ml. of distilled water containing an excess of sodium bicarbonate. The faintly yellow bicarbonate solution was adjusted to *p*H 12 with sodium hydroxide and heated on the steam-bath for an hour, which gave an almost water-white solution containing 0.429 mg./ml. of leucovorin activity when assayed with *Le. citrovorum*. This represented a 15.3% yield.

TABLE IV

HYDROGENATION OF FORMYLATED PTEROYLGLUTAMIC ACID (A), PTEROYLGLUTAMIC ACID (B) AND 10-FORMYLPTEROYLGLUTAMIC ACID (C) IN FORMIC ACID

A		B		C	
Time, minutes	Hydrogen molar equiv.	Time, minutes	Hydrogen molar equiv.	Time, minutes	Hydrogen molar equiv.
5	....	5	0.115	1	0.199
8	0.0402	10	.139	2	.369
19	.222	15	.358	3	.638
26	.442	20	.692	4	.977
30	.662	25	1.04	5.5	1.28
34	.870	30	1.42	6	1.40
36	1.092	35	1.79	8	1.72
40	1.312	40	1.94	9.5	1.84
44	1.530	45	1.95	12	1.92
51	1.752			18	1.95
65	1.836			23	1.95
				34	1.95

D. Hydrogenation of 10-Formylpteroylglutamic Acid in Water (Raney Nickel).—Two grams of 10-formylpteroylglutamic acid was dissolved in 100 ml. of water with the aid of sodium hydroxide. The *p*H was adjusted to 7.0, and the product was hydrogenated in the presence of Raney nickel in a nickel autoclave at 120° and 1470 lb. pressure for two hours. The solution was clarified and diluted to 178 ml. This gave a microbiological assay of 0.393 mg./ml.

E. Dihydropteroylglutamic Acid plus Ethyl Formate.—A mixture of 0.5 g. of dihydropteroylglutamic acid,[12] 5 ml. of ethyl formate, and 0.15 g. of sodium methylate was heated in a sealed tube at 150° for four hours, in an atmosphere of nitrogen. The light gray product was filtered from the reaction mixture in quantitative yield. This was found to contain 0.019 mg./mg. of leucovorin activity in the assay for *Le. citrovorum*. When the reaction was carried out at 100°, an assay of 0.006 mg./mg. was obtained. Without sodium methylate, the products were inactive or contained only a trace of activity. A reaction using glycol formate at 150° gave a product containing 0.004 mg./mg.

F. Dihydropteroylglutamic Acid Plus Formic Acid.—A mixture of 0.50 g. of dihydropteroylglutamic acid,[12] 10 ml. of 98–100% formic acid, and 1 ml. of acetic anhydride was heated at 50° for 30 minutes and allowed to stand at room temperature 4 hours. It was then poured into 150 ml. of water containing 25 g. of sodium bicarbonate. This solution was adjusted to *p*H 13 with sodium hydroxide and heated one hour at 100°. After neutralization to *p*H 7, the solution was assayed and found to be essentially inactive for *Le. citrovorum*.

Similar experiments without acetic anhydride, in which the reaction temperature and alkali treatment were varied, also gave essentially inactive products.

G. Tetrahydropteroylglutamic Acid plus Ethyl Formate.—Tetrahydropteroylglutamic acid[12] was prepared by hydrogenating pteroylglutamic acid in a mixture of glacial acetic acid and glycol over a platinum catalyst until two moles of hydrogen were absorbed, and then pouring the mixture into methyl acetate. This precipitated tetrahydropteroylglutamic acid as a white solid, which was used immediately in the following reactions.

A mixture of 1.0 g. of tetrahydropteroylglutamic acid, 12 ml. of ethyl formate and 0.2 g. of sodium methylate was heated in a sealed tube at 150° for four hours under nitrogen. The cream colored product was filtered (1 g.) and found to contain 0.073 mg./mg. of leucovorin activity. The reaction was repeated using glycol formate, and the product contained 0.096 mg./mg.

H. Tetrahydropteroylglutamic Acid plus Formic Acid.—Three samples, 0.80 g. each of tetrahydropteroylglutamic acid, were dissolved in 20 ml. each of 98–100% formic acid. The first sample (1) was allowed to stand at 0° for one hour. The second (2) stood at 25° one hour, and the third (3) was heated at 60° one hour. Each sample was then poured into 300 ml. of distilled water containing 50 g. of sodium bicarbonate. The volume of the resultant solutions were 315, 320 and 325 ml., respectively. These solutions gave the following assays *vs. Le. citrovorum:* (1) 0.171 mg./ml.; (2) 0.345 mg./ml.; (2) 0.467 mg./ml.

Purification of Leucovorin by Chromatography on Magnesol.—Six grams of crude barium leucovorin prepared as described in (A-1) above, was dissolved in 30 ml. of warm water, and the cloudy solution, after cooling to 20°, was clarified. Magnesol (120 g.) was slurried in 300 ml. of water and poured into a glass column 3.75 inches in diameter. The slurry was sucked down just short of dryness using vacuum, and the 30-ml. solution of barium salt was put on the column and developed with water, using water-pump vacuum. The eluate was collected in 200-ml. portions. The first portion was colorless, but the next two began to show a yellowish color. By this time, a fluorescent band visible under ultraviolet light was just starting to come off the column. A fairly broad, non-fluorescent area in which the desired product was found was just above the fluorescent band. Above the non-fluorescent area were several zones, showing blue or yellow fluorescence under ultraviolet light, as well as some yellow and brown zones as seen in daylight. In the fifth 200-ml. portion, some of the leucovorin was starting to come through. Presence of leucovorin was determined by adding to a 5-ml. portion of eluate two drops of 20% barium chloride solution and 10 ml. of ethanol. A flocculent precipitate was obtained with the leucovorin fraction; this varied in quantity, so that the peak of concentration could be immediately ascertained. With the exception of *p*-aminobenzoylglutamic acid, which was sometimes present, other fractions did not give this characteristic precipitate. Fractions five through nine showed positive tests and further elution started to bring through one of the blue fluorescent zones. The eluates 5-9 were combined and evaporated under reduced pressure to 80 ml.; sodium hydroxide was added to *p*H 10–11, and the precipitate was filtered and discarded. After adjusting to *p*H 7.0–7.5, 10 ml. of 25% barium chloride was added, and the barium salt of leucovorin was precipitated by the addition of 270 ml. of ethanol. On cooling, filtering, washing with ethanol and drying at 50°, there was obtained 2.0 g. of nearly white material showing an assay by polarograph of 94.5% as the barium salt pentahydrate.

For preparation of an analytical sample, the purification procedure above was repeated, and the barium salt obtained was dissolved in water at about 10% concentration and precipitated with three volumes of ethanol. After drying overnight at 25°, there was obtained 0.8 g. of barium leucovorin. The ultraviolet absorption curve is shown in Fig. 1.

Anal. Calcd. for $C_{20}H_{21}N_7O_7Ba \cdot 5H_2O$: C, 34.37; H, 4.47; N, 14.03; Ba, 19.65; CHO, 4.15. Found: C, 34.7; H, 4.31; N, 14.1; Ba, 20.2; CHO, 3.80.

Calcium Leucovorin.—The calcium salt of leucovorin was prepared similarly, and purified by chromatography on Magnesol.

Anal. Calcd. for $C_{20}H_{21}O_7N_7Ca \cdot 4H_2O$: C, 41.2; H, 4.98; N, 16.9; Ca, 6.86; CHO, 4.97. Found: C, 41.1; H, 5.20; N, 16.6; Ca, 7.40; CHO, 4.61.

Tri-equivalent Barium Salt of Leucovorin.—A mixture of 1.00 g. of barium leucovorin (di-equivalent salt) and 0.23 g. of barium hydroxide octahydrate was dissolved in 10 ml. of warm water and filtered from traces of insoluble material. Then 25 ml. of alcohol was added, yielding a heavy precipitate, which was isolated and dried *in vacuo*.

Anal. Calcd. for $C_{20}H_{20}Ba_{3/2}N_7O_7 \cdot 5H_2O$; Ba, 26.9; N, 12.8. Found: Ba, 27.1; N, 12.5.

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

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

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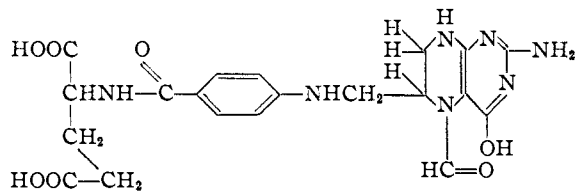
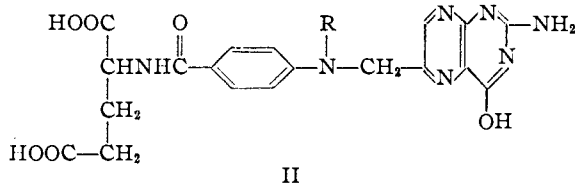
From chemical studies on leucovorin and related formyltetrahydropteridines it is concluded that leucovorin has the structure 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid. Leucovorin nitrate, which is active for *Leuconostoc citrovorum* 8081, is described. Four acid transformation products of leucovorin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