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Polarographic Determination and Evidence for the Structure of Leucovorin[1a]

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RECEIVED JANUARY 11, 1952

A polarographic method of assay of crude and purified synthetic leucovorin is described. The method yields values that agree, within reasonable limits, with those found by a microbiological assay method. The application of this method to the analysis of natural products or biological fluids was not studied. Polarographic evidence is also reported that the formyl group in the leucovorin molecule is located on the five position of the pyrazine ring. From the polarographic half-wave potentials three successive stages of reduction of compounds containing a pteridine nucleus may be differentiated: unreduced pteridines, dihydropteridines and tetrahydropteridines.

A growth factor for *Leuconostoc citrovorum* 8081 in liver concentrate and other naturally occurring substances was reported by Sauberlich and Baumann.[1b] They also developed a microbiological method of assay which was specific for this factor and defined the "citrovorum unit" in terms of the activity of a standardized liver concentrate (Reticulogen). The synthesis and isolation of a crystalline substance active for *Leuconostoc citrovorum* was later described by Brockman and associates.[2,3] They also determined the weight of the pure synthetic product which has a biological activity equal to one "citrovorum unit." The synthetic product was named leucovorin.

During the course of collaboration in a program of work on the synthesis and chemistry[4] of leucovorin, the authors of this paper developed a polarographic method for its determination which is more rapid and more precise than the microbiological assay. Both methods, when applied to the determination of the calcium or barium salt of leucovorin, agreed within the limitations of their precision. The polarographic method was of value in the routine assay of crude and refined samples. The applicability of the polarographic method to the determination of leucovorin in natural products or biological fluids has not been investigated. In addition, the polarographic study of leucovorin and related compounds yielded information which supported other chemical evidence for the structure 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid for leucovorin. The same structure was proposed recently for "folinic acid-SF" on the basis of certain ultraviolet absorption spectra.[5]

Polarograms of pteroylglutamic acid and related compounds could be obtained at *p*H 9 in the presence of an aqueous phosphate buffer. Leucovorin, on the other hand, was neither reducible nor oxidizable polarographically in such a buffered solution. When, however, an aqueous solution of leucovorin was treated first with dilute hydrochloric acid[4] and then adjusted to *p*H 9, a tetrahydropteridine resulted (presumably by further reaction of an intermediary substance produced by the action of the acid, which has also been noted in other connections). This tetrahydropteridine was easily oxidized by air in the alkaline solution to an unreduced pteridine and a compound reducible in the same voltage region as dihydropteroylglutamic acid. The polarogram (Fig. 1) showed waves for all three compounds. The sum of the anodic and cathodic currents was a function of the concentration and was used as the basis for calculating the leucovorin content of the sample.

Polarographic Assay Method

The *p*H 9.0 buffer consisted of a solution in distilled water of 142 g. of reagent grade anhydrous disodium hydrogen phosphate made up to 2 liters.

The H-type electrolysis cell and reference electrodes[6] are slightly modified forms of the types described by Lingane and Laitinen.[7] A Fisher Elecdropode and a Sargent Model 21 recording polarograph were used. The Elecdropode was suitable for the routine determination of leucovorin.

The reaction vessel consisted of a weighing bottle 50 mm. in height with an inside diameter of 25 mm. A solid glass stopper was used in order to keep the air space above the solution at a minimum.

The polarographic determination of leucovorin consisted of two steps. The first step, procedure A, was a blank determination designed to measure the reducible and oxidizable impurities. Twenty-five mg. of sample was dissolved in a 50-ml. volumetric flask with *p*H 9 buffer and diluted to volume with the buffer. Dissolved oxygen was removed with a stream of nitrogen, and the sum of the anodic and cathodic currents was measured as the difference in current between −0.12 and −1.57 v. *versus* S.C.E. The second step, procedure B, measured leucovorin plus reducible and oxidizable impurities. Twenty-five mg. of sample was dissolved in approximately 13 ml. of distilled water contained in the reaction vessel. Nitrogen was bubbled through this solution for ten minutes and then 1 ml. of approximately 6 *N* HCl, which had previously been swept with nitrogen, was added. Nitrogen was again bubbled through the solution for an additional minute. The bottle was tightly stoppered and placed in a constant-temperature-bath at 25 ± 0.25°. After 2–2.5 hours the solution was transferred rapidly with the aid of a small amount of distilled water to a 50-ml. beaker and adjusted to a *p*H of 9.0 ± 0.5 by dropwise addition of 5 *N* and finally 0.1 *N* sodium hydroxide solution. Excess alkali was avoided, but when added it was neutralized with 0.1 *N* HCl. This solution was transferred to a 50-ml. volumetric flask with 25 ml. of *p*H 9 buffer, diluted to volume with distilled water, swept free of air with nitrogen and then electrolyzed. The current measurement was made immediately after the solution had been treated with nitrogen. A Fisher Elecdropode was used for quantitative work. The total time required for neutralization and exposure of the solution to air did not exceed about five minutes. The

(1a) Presented in part at the Meeting-in-Miniature of the North Jersey Section, American Chemical Society, January 28, 1952.

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

(2) John A. Brockman, Jr., Barbara Roth, H. P. Broquist, Martin E. Hultquist, James M. Smith, Jr., Marvin J. Fahrenbach, Donna B. Cosulich, Robert P. Parker, E. L. R. Stokstad and T. H. Jukes, THIS JOURNAL, **72**, 4325 (1950).

(3) B. Roth, M. E. Hultquist, M. J. Fahrenbach, D. B. Cosulich, H. P. Broquist, J. A. Brockman, Jr., J. M. Smith, Jr., R. P. Parker, E. L. R. Stokstad and T. H. Jukes, *ibid.*, **74**, 3247 (1952).

(4) D. B. Cosulich, B. Roth, J. M. Smith, Jr., M. E. Hultquist and R. P. Parker, *ibid.*, **74**, 3252 (1952).

(5) Albert Pohland, Edwin H. Flynn, Reuben G. Jones and William Shive *ibid.*, **73**, 3247 (1951).

(6) William Seaman and William Allen, *Sewage and Industrial Wastes*, **22**, No. 7, 912 (1950).

(7) J. J. Lingane and H. A. Laitinen, *Ind. Eng. Chem., Anal. Ed.*, **11**, 504 (1939).

value found for the blank (A) was deducted from that found after treatment with acid in the absence of air (B). Calculation of leucovorin in samples of the barium or calcium salt was made from a curve of current *vs.* concentration which had been determined for a purified sample of the calcium salt of leucovorin.

Comparison of Polarographic and Microbiological Assay Methods

The microbiological assay has been related to the "citrovorum unit" (based upon liver concentrate) and also to a purified sample of the barium salt of leucovorin.[1,2] A purified sample of barium salt served as the standard for the values obtained by microbiological assay which are given in Table I. A purified sample of the calcium salt of leucovorin, having the following analysis, was used as the standard for the polarographic values reported in this paper. *Anal.* Calcd. for $C_{20}H_{21}N_7O_7Ca\cdot 4H_2O$: C, 40.0; H, 5.20; N, 16.3; Ca, 6.67; HCO, 4.83; H_2O, 12.3. Found: C, 40.6; H, 5.44; N, 16.8; Ca, 6.62; HCO, 4.57; H_2O, 12.5 (water determined by titration with Karl Fischer reagent).

The precision of the polarographic assay method was established from replicate analyses for six samples (with values ranging upward from 35%) on each of which between two and five determinations were made. The weighted average standard deviation was ±2.4% (absolute). The standard deviation of a series of N determinations of a quantity x was calculated from the expression

$$S = \pm\sqrt{\frac{\Sigma X^2 - \frac{(\Sigma X)^2}{N}}{N-1}}$$

The corresponding weighted standard deviation for the microbiological assay was ±7.4% (absolute). This was calculated from analyses of six samples, on each of which five determinations were made.

The relationship between the polarographic and microbiological assays was determined by comparing microbiological values for these six samples of the calcium salt of leucovorin with polarographic values for the samples. Good agreement between the two methods was shown by the coefficient of correlation (r) 0.99. The regression coefficient or slope of the line (b) was 1.21; the Y-intercept (a) was 3.9; and the 95% confidence limit about the regression line was ±6.4% (absolute). In arriving at these relationships the regression line of the bioassay upon the polarographic method (rather than the reverse) was used because of the greater variability of the bioassay method over that of the polarographic method. The confidence limits above and below the regression line were computed according to the expression

$$\text{confidence limit} = \pm tS'_Y\sqrt{1-r^2} = \pm 6.4$$

where t is Student's "t" at the 95% probability level for 4 degrees of freedom, r is the coefficient of correlation and S'_Y is the uncorrected standard deviation of the Y values calculated from the equation

$$S'_Y = \pm\sqrt{\frac{\Sigma Y^2 - \frac{(\Sigma Y)^2}{N}}{N}}$$

This value, ±6.4, represents the agreement which could be expected 95% of the time between a bioassay value calculated from a polarographic value and the corresponding determined bioassay value when the mean of replicates of three or four polarographic determinations is used as a basis for calculating the bioassay value.

In addition to the 6 samples used for the regression analysis, 21 other samples were compared by both methods, most of these being single determinations. Replicate bioassay values were unavailable. The slope of the regression line of the bioassay upon the polarographic method was different for the 6 samples than for the 21 others, indicating that the two groups did not make up a homogeneous population. It would therefore have been difficult to treat all the data statistically as one group. The data are listed in Table I, which also includes the means of the six replicated samples. The entire 27 pairs were plotted and a least squares line drawn. The slope of the regression line (b) and the coefficient of correlation (r) of this, apparently non-homogeneous, group of 27 pairs was 0.98 and 0.90, respectively.

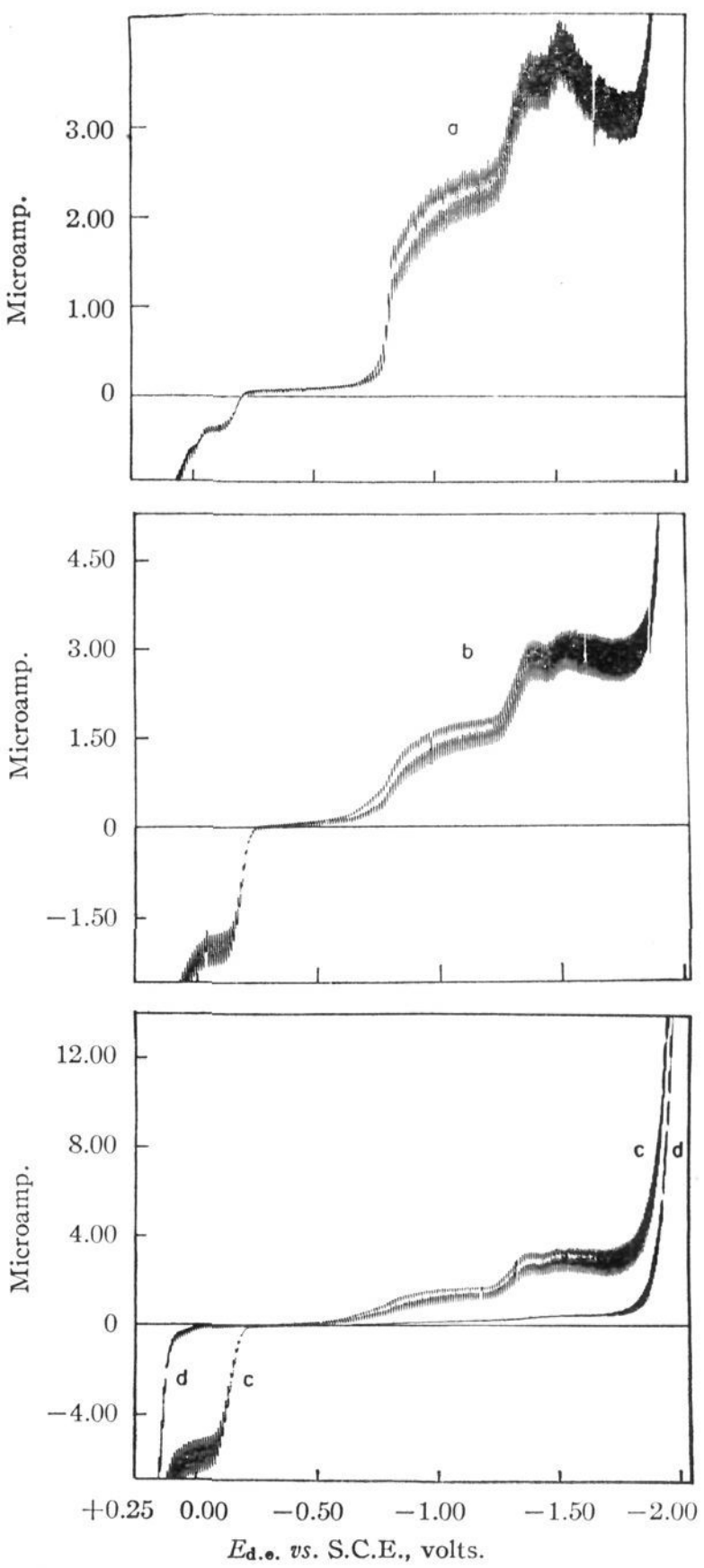

Fig. 1.—Polarograms showing the effect of leucovorin concentration on the relative magnitude of the current observed for each of the three stages of reduction of pteroylglutamic acid. Polarograms a, b and c were recorded by procedure B for concentrations of 1.02, 1.71 and 3.42 millimoles leucovorin per liter, respectively. Polarogram d was obtained by procedure A (blank) at a concentration of 3.42 millimoles leucovorin per liter.

Polarographic Behavior of Leucovorin and Related Compounds

Polarography of Pteridines.—Rickes, Trenner, Conn and Keresztesy[8] reported that folic acid and rhizopterin are reducible polarographically in a solution containing 0.1 M $Li_2B_4O_7$ at a pH of 9.12. Carpenter and Kodicek[9] studied

(8) E. L. Rickes, N. R. Trenner, J. B. Conn and J. C. Keresztesy, THIS JOURNAL, **69**, 275 (1947).

(9) K. J. Carpenter and E. Kodicek, *Biochem. J.*, **43**, ii (1948).

TABLE I

POLAROGRAPHIC *vs.* MICROBIOLOGICAL ASSAY VALUES

Sample	Leucovorin, % Polarographic method (*X*)	Microbiological method (*Y*)
1	70	64
2	64	47
3	63	57
4	56	55
5	52	52
6	34	31
7	3	6.4
8	75	45
9	40	60
10	61	60
11	56	46
12	60	56
13	31	35
14	6	5.8
15	38	32
16	47	42
17	76	69, 71 av. 70
18	70	71, 66 av. 69
19	64	72, 55 av. 64
20	71	94
21	3	1.5
22	77, 72, 72, 77, av. 75	76, 78, 100, 96, 101 av. 90
23	80, 80, 78, 84, 81 av. 81	80, 88, 93, 94, 98 av. 91
24	36, 37 av. 37	35, 35, 43, 41, 42 av. 39
25	40, 38 av. 39	41, 44, 48, 44, 42 av. 44
26	52, 47 av. 50	47, 58, 58, 59, 54 av. 55
27	79, 83, 79 av. 80	84, 91, 99, 93, 92 av. 92

polarographic method for the determination of leucovorin. Data are given in Table II. Reduction of pteroylglutamic acid took place on the pyrazine ring of the pteridine portion of the molecule. The pyrimidine ring and *p*-aminobenzoylglutamic acid were not reducible, whereas pyrazinoic acid was reducible in the same approximate voltage region as were the pteridines. Pyrazine and aminopyrazine (not included in the table because of lack of quantitative data) were also found to be reducible.

Pteridines of different degrees of reduction could be distinguished alone or in admixture by the separation of their polarographic waves. Compounds such as 6-methylpteridine and pteroylglutamic acid, containing an unreduced pyrazine ring, were reduced more readily than the partially reduced pteridine, dihydropteroylglutamic acid, while the pteridines which are more thoroughly reduced, 10-formyltetrahydropteroylglutamic acid and 6-methyltetrahydropteridine, gave well defined anodic waves at a potential indicating an easily oxidizable substance. The tetrahydropteridine formed on treatment of leucovorin with acid followed by alkali was actually completely oxidized to a mixture of a dihydropteridine and an unreduced pteridine by passage of air through a weakly alkaline solution of the compound for a short time.

Polarography of Leucovorin.—Leucovorin was completely inert polarographically when examined in a solution buffered at *p*H 9. In dilute hydrochloric acid in the absence of air (according to the procedure given) an acid-transformation product of leucovorin was formed.[4] When a solution of this alkali-labile substance was made alkaline, a tetrahydropteridine was formed. A polarogram (Fig. 1) of a solution of the latter buffered at *p*H 9 (as in the procedure given) showed the presence of oxidation products as well as of a tetrahydropteridine.

Leucovorin, allowed to stand for two hours in solution in air-free dilute hydrochloric acid, failed to show a cathodic or anodic wave in the acid solution. When, however, this acid solution of leucovorin was allowed to stand for two

TABLE II

POLAROGRAPHIC DATA BY PROCEDURE A FOR COMPOUNDS RELATED TO LEUCOVORIN

Compound[a]	$(E_{1/2})_1$ *vs.* S.C.E.	$(id)_1$ μa.	$(E_{1/2})_2$ *vs.* S.C.E.	$(id)_2$, μa.[d]
p-Aminobenzoylglutamic acid[e]		No waves found		
2,4-Diamino-6-hydroxypyrimidine[f]		No waves found		
2,4,5-Triamino-6-hydroxypyrimidine[g]		No waves found		
Pyrazinoic acid[h]	−0.96	28		
2-Amino-4-hydroxy-6-methylpteridine[i]	− .88	17.3	−1.54	8.1
Pteroylglutamic acid (folic acid)[j]	− .88	5.4	−1.56	1.1
10-Formylpteroylglutamic acid[k]	− .88	4.5	−1.54	0.6
Dihydropteroylglutamic acid[l]	−1.41	4.7		
10-Formyltetrahydropteroylglutamic acid[m]	−0.20 (anodic)[b]			
2-Amino-4-hydroxy-6-methyltetrahydropteridine[n]	− .22 (anodic)[c]			
1,2,3,4-Tetrahydroquinoxaline[o]	+ .05 (anodic)	−28		

[a] The concentration of samples was 500 mg./liter except where indicated. The purity of some of the compounds is not established although it is believed to be high. The data were obtained from polarograms recorded with a Sargent Model XXI polarograph. For the capillary used the value of $m^{2/3}t^{1/6}$ was 1.941 mg.$^{2/3}$ sec.$^{-1/2}$; t was 3.02 seconds/drop; m was 2.05 mg./second; and the measurement was made in *p*H 9 buffer at −1.57 v. *vs.* S.C.E. Half-wave potentials have been corrected for *IR* drop. The precision of measuring $E_{1/2}$ is about ±0.01 v. [b] This sample was a catalytically reduced solution of pteroylglutamic acid in formic acid which had conusmed four equivalents of hydrogen. [c] This sample was a catalytically reduced solution of 6-methylpteridine in formic acid which had consumed four equivalents of hydrogen. [d] The currents indicated are presented only for their relative magnitudes and show the position of the major wave in each case. The Sargent Model XXI recording polarograph which was used in this work was known to give slightly erroneous current values. [e] J. Van der Scheer and K. Landsteiner, *J. Immunol.*, **29**, 371 (1935). [f] W. Traube, German Patent 134,984 (1902). [g] W. Traube, *Ber.*, **33**, 1371 (1900). [h] C. Stoehr, *J. prakt. Chem.*, [2] **51**, 449 (1895). [i] J. H. Mowat, *et al.*, THIS JOURNAL, **70**, 14 (1948). [j] Robert B. Angier, *et al.*, *Science*, **103**, 667 (1946). [k] Malcolm Gordon, *et al.*, THIS JOURNAL, **70**, 878 (1948). [l] B. L. O'Dell, J. M. Vandenbelt, E. S. Bloom and J. J. Pfiffner, *ibid.*, **69**, 250 (1947). [m] Reference 3. [n] Reference 4. [o] G. Kranzlein, W. Eckert and E. Besler, German Patent 495,101 (1927).

the effect of ultraviolet light on folic acid in $N/10$ K_2HPO_4 solutions by means of a polarographic procedure. Mader and Frediani[10] reported a method for the determination of folic acid in reasonably pure preparations and pointed out that the wave for folic acid was a general wave for pteridines and that other pteridines would be included as folic acid.

A study of the polarographic behavior of pteridines was made in connection with this work on the development of a

(10) W. J. Mader and H. A. Frediani, *Anal. Chem.*, **20**, 1191 (1948).

hours and was then neutralized and buffered at *p*H 9, anodic and cathodic waves were obtained which indicated that the acid transformation was complete, because the same total diffusion current was obtained upon an additional three hours standing. A reaction time of 1.5 hours or less was not sufficient.

Considerable differences were found for the relative height of the three waves corresponding to the three stages of oxidation of the pyrazine ring when the concentration of leucovorin was varied (see Fig. 1). Weights of leucovorin which

varied from 40 to 80 mg. (3.42 to 6.84 millimolar) gave polarograms in which a tetrahydropteridine (anodic wave at −0.22 v.) represented the major portion of the total current. Samples of leucovorin weighing 20 mg. (1.71 millimolar) yielded polarograms in which a much smaller fraction of the total current was due to the tetrahydro form. When less than 12 mg. (1.02 millimolar) of leucovorin was used, a very small anodic wave, or none, was found. The explanation for this is believed to be that a small amount of air was probably present in the reaction vessel and oxidized a small fixed amount of the tetrahydropteridine. A second source of oxidation arises from the neutralization of the acid-treated leucovorin during preparation of the test solution, whereby the solution is exposed to air for about five minutes.

The polarograms of leucovorin following acid treatment, which are given in Fig. 1, show an anodic wave with a half-wave potential of −0.22 v., and cathodic waves at −0.87 v., and at −1.36 v. followed by a small rounded wave at about −1.5 volts. By comparison with data given in Table II it was concluded that the first wave is due to the oxidation of a tetrahydro compound and the second to reduction of an unreduced pteridine. The third wave indicates the presence of a compound which is slightly more easily reduced than dihydropteroylglutamic acid; this is also believed to be a dihydro compound.

Study of Variables in the Polarographic Assay Method

Interferences.—As will be brought out, the polarographic method measures tetrahydropyrazine compounds containing the formyl group in the five position. Certain compounds other than leucovorin would interfere in the determination. 5-Formyl-6-methyltetrahydropteridine and 5,-10-diformyltetrahydropteroylglutamic acid would interfere if present. For the samples studied in the present investigation it is unlikely that either compound could be formed in the synthesis described.[2]

Current *vs.* Leucovorin Concentration.—Under the conditions of the determination, a fixed concentration of leucovorin gave a reproducible value for the sum of the anodic and cathodic currents. The polarogram of leucovorin showed the presence of three different compounds and, as shown in Fig. 1, the relative amounts of these compounds varied with the concentration of sample. A linear current *vs.* concentration relationship was not found, indicating that the compounds present did not have identical diffusion current constants. A plot of diffusion current constant *vs.* concentration showed this effect more clearly than the usual current *vs.* concentration plot of the data. Data for a purified sample of the calcium salt of leucovorin from which $i_d/Cm^{2/3}t^{1/6}$ was calculated are given in Table III. In the concentration range of 3.4 to 6.8 millimoles of leucovorin per liter the polarogram showed a large anodic wave and a smaller cathodic wave. The relative currents for the two waves remained constant, so that a plot of $i_d/Cm^{2/3}t^{1/6}$ *vs.* concentration in this range yielded a straight line parallel to the concentration axis. Polarographic assay of leucovorin in this range would avoid the calibration curve necessary for smaller amounts, but has not been possible due to the limited quantity of samples available. Concentrations between 0.34 and 3.4 millimolar gave polarograms in which the ratio of the current due to the oxidation of tetrahydropteridine to the total current due to reduction products varied and smaller relative amounts of the tetrahydro compound were found as the concentration decreased, thus necessitating a calibration curve in this region. In the region 0.10 to 0.34 millimolar concentrations of leucovorin, where only waves due to reduction products were found, a calibration curve would also be needed because of the rapid change in the value of the diffusion current constant with changes in concentration.

TABLE III

CURRENT AND CONCENTRATION DATA

d(μa.)	Leucovorin, mg.	Millimoles leucovorin/liter	$i_d/Cm^{2/3}t^{1/6}$[a]
14.2	80.8	6.84	1.20
7.15	40.4	3.42	1.21
3.88	20.2	1.71	1.32
2.52	12.1	1.02	1.43
1.05	4.03	0.34	1.79
0.75	2.01	.17	2.56
.50	1.21	.10	2.89

[a] The value $m^{2/3}t^{1/6}$ was 1.719 mg.$^{2/3}$ sec.$^{-1/2}$ by measurement in *p*H 9 phosphate buffer at −1.57 volts *vs.* S.C.E. The height of the mercury column was 33.7 cm., m was 1.59 mg./second and t was 3.94 seconds/drop.

Half-wave Potential *vs.* Concentration.—The total diffusion current for the anodic plus cathodic waves found for leucovorin, according to the procedure given, was measured as the difference in current readings taken at −0.12 and −1.57 v. *vs.* S.C.E. The cathodic waves did not undergo measurable shifts in half-wave potential with changes in concentration. But the anodic wave for the tetrahydro compound which was formed on treatment of leucovorin with hydrochloric acid did undergo a large shift with changes in concentration. The half-wave potential was −0.10 v. for a 6.8 millimolar solution, −0.16 v. for a 3.4 millimolar solution, and −0.19 v. for a 1.7 millimolar solution of leucovorin. Measurement of the limiting current for this wave for samples containing no more than 20 mg. of leucovorin (25 mg. of the calcium salt) was made at −0.12 v. *vs.* S.C.E. This shift in half-wave potential with changes in concentration was also observed qualitatively for the anodic wave found for tetrahydroquinoxaline; this compound was analogous to tetrahydropteroylglutamic acid in having a tetrahydropyrazine ring.

Correction for Reducible Impurities in Leucovorin.—Since reducible impurities present in the sample of leucovorin were also subjected to treatment with dilute hydrochloric acid, it was necessary to show that these compounds did not undergo any change which would invalidate a quantitative correction based on the measurement of the polarogram obtained in *p*H 9 buffered solution (blank determination). Polarographically oxidizable impurities were not found to be present in leucovorin samples. Samples of 10-formylpteroylglutamic acid, dihydropteroylglutamic acid and 6-methylpteridine, likely impurities in leucovorin, were analyzed by both polarographic procedures. The diffusion currents were found to be the same for each compound by both procedures, thus showing that these compounds were stable under the conditions of the experiment.

Temperature during Acid Treatment.—Variations of temperature during acid treatment from 25 to 35° did not affect the values found for the calcium salt of leucovorin. The restriction, given in the procedure, to 25 ± 0.25° is not essential.

Stability of Test Solution.—After neutralization and addition of phosphate buffer, the test solution (procedure B) was immediately transferred to the polarographic cell and dissolved oxygen was removed by passing nitrogen through the solution. Delay in removing dissolved oxygen appeared

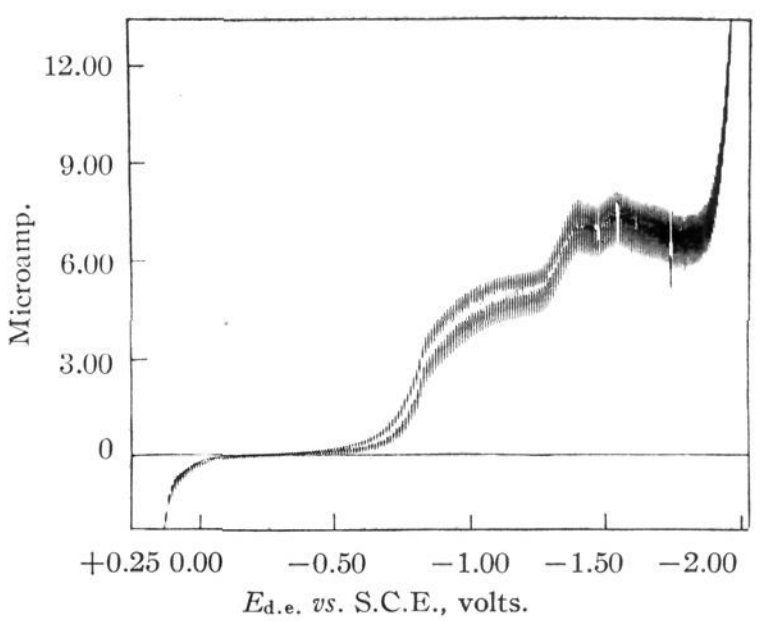

Fig. 2.—Effect of air on compounds present in test solution after treatment of leucovorin by procedure B. A rapid stream of air was passed through the test solution which gave curve b in Fig. 1. After removing dissolved oxygen this polarogram was recorded.

TABLE IV

POLAROGRAPHIC BEHAVIOR OF LEUCOVORIN AND ANALOGOUS COMPOUNDS

Compound	Procedure	$E_{1/2}$ vs. S.C.E. $(E_{1/2})_1$	$(E_{1/2})_2$	$(E_{1/2})_3$
Diformyltetrahydroquinoxaline[d]	A	No waves found		
	B	+0.06 (anodic)[a]		
5-Formyl-2-amino-4-hydroxy-6-methyltetrahydropteridine[e]	A	No waves found		
	B	−0.21 (anodic)	[−0.87][b]	−1.52
5-Formyltetrahydropteroylglutamic acid (leucovorin)[f]	A	No waves found		
	B	−0.22 (anodic)	−0.87	−1.36[c]
5,10-Diformyltetrahydropteroylglutamic acid[g]	A	No waves found		
	B	−0.22 (anodic)	−0.9 (small wave)	−1.37[c]

[a] The half-wave potential of this anodic wave becomes more positive with increasing concentration. The current found for this sample was about the same as was found for the unformylated tetrahydroquinoxaline listed in Table II. The half-wave potentials of the two compounds are in reasonably good agreement. [b] This wave was not found originally but appeared when air was passed through the cell for a short time. The height of the anodic wave decreased as the height of this new wave increased. [c] This wave is followed by a second small rounded wave. The position of the major wave indicates a dihydro compound but this compound apparently is not dihydropteroylglutamic acid. [d] This preparation was carried out by Dr. D. B. Cosulich as follows: A solution of 5 g. of tetrahydroquinoxaline in 100 ml. of 98–100% formic acid containing 100 ml. of acetic anhydride was allowed to stand overnight. After concentrating *in vacuo* to a sirupy liquid, the residue was frozen to permit crystallization and then triturated with water and sodium bicarbonate to give *p*H 7.5. The crude crystalline diformyl compound was isolated by filtration and recrystallization three times from 95% alcohol with charcoal decolorizations to yield 3.8 g. of white needles melting at 126.7–128.3°, with preliminary shrinking at 125°. *Anal.* Calcd. for $C_{10}H_{10}N_2O_2$: C, 63.1; H, 5.27; N, 14.7; CHO, 3.05. Found: C, 63.1; H, 5.36; N, 14.7; CHO, 3.09. [e] Reference 4. [f] Reference 2. [g] Reference 3.

to give slightly higher values than were found when the dissolved oxygen was removed immediately. The high values were found to be the result of oxidation of the tetrahydro form by air. The current due to the reducible compounds formed on oxidation was considerably larger than the initial total anodic plus cathodic current. The polarogram reproduced in Fig. 2 shows the results of accelerated oxidation by air. A large increase in the sum of the anodic and cathodic currents was observed after 15 minutes of oxidation with a rapid stream of air. From the half-wave potentials it was thought that the products of oxidation contain an unreduced pteridine and a dihydropteridine.

After removal of the dissolved oxygen from the test solution, it was found that the current readings decreased slowly so that it was essential that the current measurement be made immediately after the removal of dissolved oxygen, an operation requiring ten minutes. The cause of this instability in air-free solution has not been determined.

Polarographic Evidence for the Structure of Leucovorin

Leucovorin was completely inert polarographically when examined in a solution buffered at *p*H 9. An explanation of this unexpected stability of the pyrazine ring was found through a study of model compounds. As shown in Table IV, the presence of a formyl group in the five position on the tetrahydropyrazine portion of the pteridine nucleus resulted in stabilization of the pyrazine ring to reduction or oxidation at the dropping mercury electrode. Treatment of the compounds listed in Table IV with dilute hydrochloric acid in the absence of air resulted in the same type of behavior as was found for leucovorin. Certain qualitative differences were observed, particularly with reference to the ease of oxidation of the tetrahydro form of the molecules concerned. For example, formylated tetrahydroquinoxaline was found to yield a stable tetrahydroquinoxaline when treated with dilute hydrochloric acid. The latter compound could not be oxidized by air to a more unsaturated compound. The same behavior was observed to a lesser extent with 5-formyl-6-methyltetrahydropteridine, although in this case it was possible by treatment with a rapid stream of air of the solution buffered at *p*H 9 to convert the tetrahydropteridine into a compound containing a pyrazine ring. A wave was also found (prior to air-oxidation) with a half-wave potential of −1.52 v. which was believed to be due to reduction of a dihydro compound. The mechanism whereby the pyrazine ring is stabilized by the introduction of a formyl group is not known. The stabilization of the pyrazine ring in a tetrahydropteridine by introduction of a formyl group in the five position, is sufficiently characteristic behavior to show that leucovorin contains the formyl group in the five position. The formyltetrahydropteridines examined polarographically were all formylated in the five position. The work of Cosulich, *et al.*,[4] has shown that 8-formyltetrahydropteridines are not formed in the synthesis of leucovorin or simpler pteridines. Tetrahydropteroylglutamic acid, which was prepared in an acetic acid solution and therefore could not be substituted in the five position, and 10-formyltetrahydropteroylglutamic acid, which was available as a formic acid solution, failed to show a stabilized pteridine nucleus. Both compounds yielded an anodic wave in the region expected when the polarogram was obtained in *p*H 9 buffered solution (procedure A). Treatment of these compounds with mineral acid (procedure B) had no effect as the polarograms obtained after acid-treatment were identical with the original polarograms.

The usefulness of the polarographic method in structural studies of leucovorin and related compounds was due to its ability to indicate specifically a tetrahydropteridine containing a 5-formyl group, which imparted polarographic stability to the compound. The polarogram obtained on the original sample prior to treatment with acid was used to determine the presence of reducible or oxidizable impurities in the sample and from their half-wave potentials to determine whether the impurities were tetrahydropteridines, dihydropteridines or unreduced pteridines. It was not possible to distinguish polarographically between an unreduced pteridine and folic acid or a similar analog since the

half-wave potentials were practically identical. The same was true of the tetrahydro compounds. Considerable differences were observed for the dihydro compounds in that half-wave potentials of -1.36 to -1.52 v. *vs.* S.C.E. were found for the dihydro form of different molecules containing pteridines, indicating that the ease of reduction of the dihydropteridine varied with the over-all structure of the molecule.

Acknowledgments.—Grateful acknowledgment is made to Drs. R. P. Parker and J. M. Smith, Jr., for their helpful advice and interest, to Drs. Donna B. Cosulich, M. J. Fahrenbach and Barbara Roth of the Calco Pharmaceutical Research Department for furnishing the compounds described in this paper and for their coöperation in preparing special compounds for this study, to Dr. Harry P. Broquist of the Lederle Laboratories for performing the microbiological assays; to Mr. Joseph B. Lombardo of the Calco Analytical Research Laboratories for the statistical study of the relationship between the polarographic and microbiological assay methods; to Mr. F. B. Dorf of the Calco Development Department for preparing the sample of leucovorin which was used as the polarographic standard; to the Microanalytical Laboratory, under the direction of Mr. O. E. Sundberg, for performing the combustion analysis of the standard sample, and to Mr. A. Pollara of the Calco Analytical Research Laboratories for assisting in the preparation of the figures.

BOUND BROOK, NEW JERSEY

[CONTRIBUTION FROM THE SHELL DEVELOPMENT COMPANY]

Hydrogen Exchange and Isomerization of Saturated Hydrocarbons with Sulfuric Acid

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RECEIVED NOVEMBER 12, 1951

Isomerization and hydrogen exchange reactions of saturated hydrocarbons with sulfuric acid have been studied using deuterium as a tracer and the mass spectrometer as the analytical tool. These reactions are interpreted as occurring by means of an ionic chain. The reactions observed are considered to be the reactions of the ions. The probability that an ion will isomerize or will undergo hydrogen exchange is determined by the rates of such reactions relative to the rate of reconversion of the ion to the hydrocarbon. Only isoparaffins (defined as saturated hydrocarbons with tertiary carbon atoms) undergo any observable reaction, and only isoparaffins are formed as a result of isomerization. The isoparaffin products invariably contain methyl groups attached to the tertiary carbon. The hydrogen atoms exchanging with sulfuric acid always include at least those attached to carbon atoms adjacent to the tertiary carbon. The exchange of others more distant from the tertiary carbon appears to result from isomerization of the ion, and the pattern of such exchange depends upon the molecular structure and, to some extent, upon reaction conditions. With certain isoparaffins all but one of the hydrogen atoms in the molecule invariably exchange with acid, and a study of the mass spectrum of the C_nHD_{2n+1} species furnishes a direct method for the determination of the relative rate of production from the activated state of all isomers, including the species with the same carbon skeleton as the reactant. Results of this work provide an explanation for the facts that only isoparaffins react and only isoparaffins are produced in the sulfuric acid-catalyzed alkylation reaction. The results also demonstrate that the carbonium ions are true intermediates with finite lifetimes, and that their mode of reaction depends upon the degree of their activation.

This is the second of two papers presenting the results of studies of the reactions of alkanes and cycloalkanes in the presence of concentrated sulfuric acid. In the previous paper[2a] it was shown that isobutane exchanges its primary hydrogens[2b] with sulfuric acid by means of an ionic chain reaction. The exchange of primary hydrogens is believed to occur during the residence of the hydrocarbon as an ion[3] in the sulfuric acid. The chain propagating reaction, which is rate-determining in the steady state, is the transfer of a tertiary hydride ion[4] from the hydrocarbon to the ion, regenerating a hydrocarbon molecule and producing a new ion. In order to account for the kinetics, a slow chain initiating reaction (oxidation by sulfuric acid) was postulated, as well as a chain terminating reaction that produces non-volatile, non-reactive products.

This paper describes studies of the reactions of various C_6–C_7 alkanes and cycloalkanes by methods similar to those employed in the studies of the isobutane reactions. It is shown that these new observations fit the same general model that describes the behavior of isobutane with the additional feature that the more complex carbonium ions are able to undergo certain skeletal rearrangements as well as hydrogen exchange reactions. The interrelationship of the reactions of the ions in sulfuric acid have been studied in detail.

Discussion

The experiments described in Tables I–IV are of four types: (1) The reactions of paraffins (and cycloparaffins) with sulfuric acid-d_2, (2) the reactions of paraffins with sulfuric acid, (3) the reactions of various monodeutero-2-methylpentanes with sulfuric acid, and (4) the reactions of isopentane with sulfuric acid-d_2 under various reaction conditions. For the most part, conditions were chosen such that only a small fraction of the molecules underwent reaction. This assures that

(1) Deceased, July 5, 1950.

(2) (a) J. W. Otvos, D. P. Stevenson, C. D. Wagner and O. Beeck, THIS JOURNAL, **73**, 5741 (1951). (b) Hydrogens designated primary, tertiary, etc., designate the type of carbon atom to which they are attached.

(3) A term used to designate either the solvated ion, $R^+ \cdot (H_2SO_4)_n$ or the complexed, polarized ester, $(R^+ - OSO_3H^-) \cdot (HS_2O_4)_n$.

(4) Hydride ion as used here denotes a hydrogen nucleus with an electron pair. It is not an ion in the sense that it is free from the influence of the rest of the hydrocarbon molecule.