Reaction of Bifunctional Alkylating Agents with Tetrahydrofolate[*]

V. S. Gupta,[†] J. G. Ozols,[‡] and F. M. Huennekens

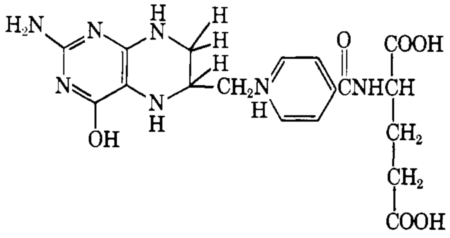
ABSTRACT: The facile *in vitro* alkylation of tetrahydrofolate (I) by Chlorambucil [*p*-*N*,*N*-di(β-chloroethyl)-aminophenylbutyric acid] (II) has been demonstrated under physiological conditions of temperature and pH. Two principal alkylated products have been separated by column chromatography and identified as the 5- and 10-substituted alkyl derivatives of tetrahydrofolates (III and IV). Similarly, folic acid reacted with Chlorambucil to yield the 10-substituted alkyl derivative V which, upon catalytic hydrogenation in acidic solution, consumed 2 moles of hydrogen to yield IV by a separate route. Compounds III and V have been obtained in crystalline form and characterized by elemental analysis; because of its lability toward oxygen, IV could not be crystallized. Compounds III, IV, and V have also been characterized by absorption spectra, paper chromatography, chromatography on DEAE-cellulose, and radioactivity when prepared from ^{14}C-labeled II. Some parallel experiments have been carried out on the alkylation of tetrahydrofolate with Nitromin (methylbis(β-chloroethyl)amine *N*-oxide) and Mustargen (methylbis(β-chloroethyl)amine) and on the alkylation of 10-methyltetrahydrofolate and 5-formyltetrahydrofolate with Chlorambucil.

Biological alkylating agents, such as nitrogen and sulfur mustards, ethylenimines, sulfonic esters, epoxides, etc., are known to produce striking mutagenic and cytotoxic effects at the cellular level (see reviews by Schmidt, 1958; Ross, 1962). Although the above classes of compounds have different chemical structures, they probably share a common mechanism of action (Price, 1958), *i.e.*, an actual or potential carbonium ion (R^+ from RX) attacks an electron-rich group (YH) to form the alkylated product (RY), as shown in eq 1.

$$RX + YH \longrightarrow RY + HX \qquad (1)$$

The nonspecific nature of this reaction accounts for the fact that a wide variety of biologically important compounds containing thiol, amino, hydroxyl, carboxyl, or phosphate groups have been shown to be susceptible to alkylation under *in vitro* conditions. Still uncertain, however, is the identity of the actual compound, or compounds, whose alkylation under *in vivo* conditions is responsible for the observed biological effects (reviewed by Wheeler, 1962). Evidence has been presented that the cytotoxic effects of these agents are due to their alkylation of the guanosyl residues of DNA (Brookes and Lawley, 1963, 1964). Conversely, other investigators have suggested alternate sites of action, *e.g.*, upon precursors of DNA (Haddow, 1953; Syzbalski, 1960; Biesele, 1962).

Tetrahydrofolic acid (I) may be considered, *a priori*, as a potential target for alkylating agents since: (a)



tetrahydrofolic acid (I)

both N^5 and N^{10} are nucleophilic centers[1] in which the hydrogen atoms are readily displaced during adduct formation with formaldehyde (Blakley, 1957; Osborn *et al.*, 1960); (b) the spatial relationship of these two nitrogen atoms make tetrahydrofolate especially susceptible to attack by *bifunctional* agents; and (c) inactivation of tetrahydrofolate, a coenzyme for the biosynthesis of key precursors of nucleic acids and proteins (reviewed by Huennekens *et al.*, 1963), might account for certain of the observed biological effects of alkylating agents.

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[1] N^8 is also a potential nucleophilic center but, for reasons not yet clear, it does not appear to interact with acylating or alkylating reagents.

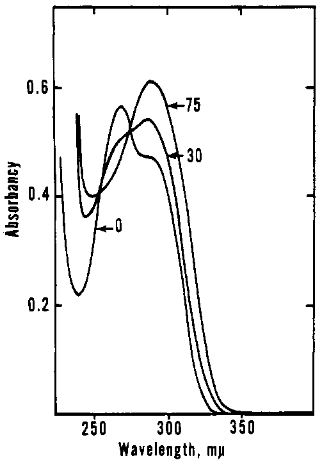


FIGURE 1: Spectrophotometric demonstration of the alkylation of tetrahydrofolate with Chlorambucil (see Experimental Section for details).

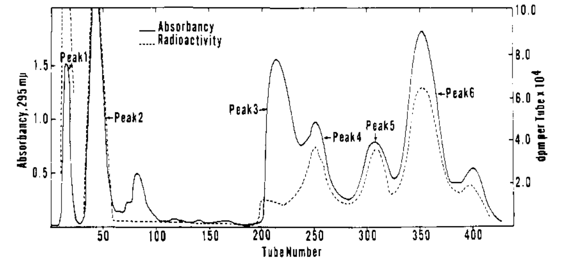
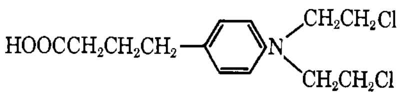


FIGURE 2: Elution profile of the reaction mixture obtained by alkylation of tetrahydrofolate with ^{14}C-labeled Chlorambucil (see Experimental Section for details).

It has been shown previously (Blakley, 1957; Osborn *et al.*, 1960) that when tetrahydrofolate (λ_{max} at 298 mμ) is treated with an excess of formaldehyde at neutral pH, the absorption maximum shifts to shorter wavelengths (292–294 mμ) and the absorbancy increases by 20–30%. This spectral change results from the conversion of tetrahydrofolate to 5,10-methylenetetrahydrofolate (eq 2 where $\underset{H}{N}{-}{-}{-}\underset{H}{N}$ represents the 5 and 10 positions of tetrahydrofolate). The spectroscopic changes accompanying eq 2 are more pronounced, however, if acidified aliquots of the mixture are examined. Below pH 4, tetrahydrofolate displays two absorption maxima (272 and 292 mμ), while the 5,10-methylene derivative has only a single maximum at 292 mμ (Osborn *et al.*, 1960; Uyeda and Rabinowitz, 1963).

$$\underset{H}{N}\cdots\underset{H}{N} + CH_2(OH)_2 \rightleftharpoons \underset{CH_2}{N\cdots N} + 2H_2O \qquad (2)$$

Similar spectral changes were observed when tetrahydrofolate was treated at neutral or slightly alkaline pH with alkylating agents such as Chlorambucil (II), Nitromin, or Mustargen. With these reagents, however, it was obligatory to follow the reaction by examination of *acidified* aliquots, since spectra of the reaction products at neutral pH were not appreciably different from that of tetrahydrofolate.[2] In successive aliquots, withdrawn over a 90-min period and acidified to pH 1, the two-banded spectrum of tetrahydrofolate was gradually replaced by a single maximum at 292 mμ characteristic of the reaction products. This experiment is illustrated in Figure 1 in which, for clarity, only the spectra at 0, 30, and 75 min are shown. Under the conditions of this experiment, complete alkylation was obtained in 45–50 min.



Chlorambucil (II)

Essentially the same results were obtained when tetrahydrofolate was treated with Nitromin or Mustargen except that the rate of alkylation was more rapid, being completed in about 30 min. The products obtained with these alkylating agents likewise displayed a single absorption maximum at 290–295 mμ in acid solution. Increasing the concentration of Chlorambucil above the threefold molal excess normally employed did not appear to affect the rate of reaction or the nature of the products, but an excess of Nitromin or Mustargen caused some decomposition of the alkylated products as evidenced by the development of a yellow color; the solution also showed a gradual decrease in absorbancy at 290 mμ and an increase at 277 mμ.

Although subsequent chromatographic analysis revealed that several products were formed in the alkylation reaction, each has an absorption maximum in the 290–295-mμ region. A simple spectrophotometric assay, involving an increase in absorbancy at 290 mμ of acidified aliquots, was used, therefore, to determine optimal conditions for the alkylation of tetrahydrofolate.

Unlike the interaction of tetrahydrofolate and HCHO, which has a pH optimum of 4.5 (Osborn *et al.*, 1960), the maximal rate of alkylation with Chlorambucil was achieved at pH 8–9. In addition, the rate of alkylation with Chlorambucil also depended upon the nature of the buffer. Maximal rates were obtained in bicarbonate or Tris buffers at pH 8–9, while in phosphate buffer at the same pH the rate was depressed by about 10%. Linford *et al.* (1963) have reported an inhibitory effect of phosphate on the alkylation of serum proteins by Chlorambucil and have

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[2] Acidification also repressed the absorbancy owing to the aromatic amine portion of Chlorambucil. For example, at pH 7, Chlorambucil has λ_{max} at 256 mμ (ϵ 15.6 × 10^3 M^{-1} cm^{-1}) while at pH 1, the extinction coefficient drops to about 1 × 10^3 M^{-1} cm^{-1}.

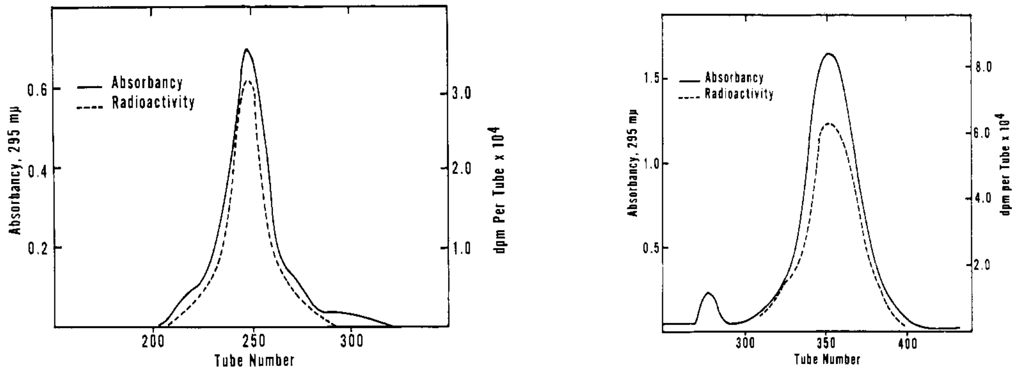

FIGURE 3: Rechromatography of peak 4 (A) (left) and peak 6 (B) (right) on DEAE-cellulose.

suggested that phosphate itself may interact with the alkylating agent.

The effect of Chlorambucil concentration upon the yield of alkylated products was also studied. Reaction mixtures containing two-, three-, four-, and sixfold molal excesses of Chlorambucil were subjected to chromatographic analysis, as described in the Experimental Section. Table I records the amounts of various products (*cf.* peaks 3–6 in Figure 2). In general, the amount of peak 3 (unreacted tetrahydrofolate) decreased progressively and that of the alkylated derivatives (peaks 4–6) increased as the Chlorambucil:tetrahydrofolate ratio was increased.

TABLE I: Effect of Chlorambucil on Yield of Various Alkylated Products.[c]

Molal Excess of Chlorambucil	Amount of Material (mg) in Peak[a]		
	3	4	5 and 6
2	20	5	22
3	10	15	30
3	10	7	5 + 19[b]
3	10	16	32
4	5	11	36
4	5	10	35

[a] Calculated from an average extinction coefficient of 25.0×10^3 M^{-1} cm^{-1} at 295 mμ for these components. [b] In this experiment the ammonium acetate gradient was changed to that used in Figure 2 in order to permit separation of peaks 5 and 6. [c] In each experiment 75 mg of tetrahydrofolate diacetate was mixed with the indicated molal excess of Chlorambucil under standard reaction conditions given in the Experimental Section. Chromatography on DEAE-cellulose was carried out essentially as described in the Experimental Section (see also Figure 2) except that a gradient of 0.1–0.6 M ammonium acetate was employed; under these conditions, peaks 5 and 6 are not completely resolved.

Based upon the optimal conditions described above (Chlorambucil:tetrahydrofolate ratio of 3, pH ~8, 37° for 90 min), tetrahydrofolate was alkylated with ^{14}C-labeled Chlorambucil and the products were separated by chromatography on DEAE-cellulose. Figure 2 is a typical elution profile of the products showing absorbancy at 295 mμ and radioactivity. Excluding minor components centered at tubes 80 and 400, there were six major peaks of ultraviolet-absorbing material. Except for peak 3, each of these contained radioactivity. On the basis of absorption spectra and behavior of authentic standards, peak 1 (tubes 8–24) and peak 2 (tubes 32–60) were identified as unreacted and hydrolyzed Chlorambucil, respectively, contaminated with some impurities originating from tetrahydrofolate. Peak 3 (tubes 200–230) was unreacted tetrahydrofolate by its position in the elution profile and by its absorption spectra at various pH values. In peaks 4–6, which constituted the alkylated products of tetrahydrofolate, radioactivity corresponded closely with absorbancy.

Fractions 240–258 (peak 4) were combined and rechromatographed on DEAE-cellulose under the same conditions as used previously (Figure 3A). Fractions 336–368 (peak 6) were also rechromatographed (Figure 3B). Each component appeared at its expected position upon rechromatography and, from the profile, each appeared to be reasonably homogeneous. The specific activity of the material in peak 4 (9.5×10^4 dpm/μmole) and in peak 6 (9.4×10^4 dpm/μmole) was the same as that of the original labeled Chlorambucil. No further studies were carried out on peak 5 which appeared to be a mixture of several products including those in peaks 4 and 6.

When examined by paper chromatography, the components in rechromatographed peaks 4 and 6 migrated as discrete entities (R_F values 0.77 and 0.65, respectively). The R_F value of tetrahydrofolate is 0.40 in this solvent system. In the case of peak 4, a small amount of fluorescent material was usually observed below the principal spot.

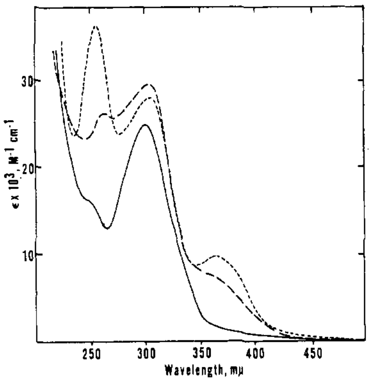

FIGURE 4: Spectra of 10-alkylfolate (IV). For all figures spectra at pH 1.0 (——), 7.0 (– – –), and 13.0 (····).

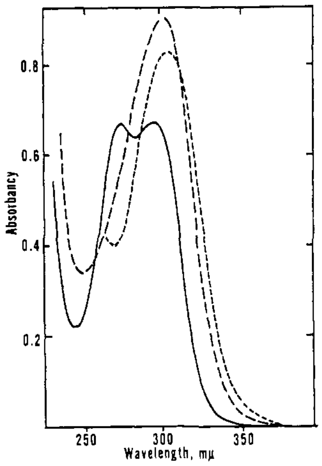

FIGURE 5: Spectra of 10-alkyltetrahydrofolate (V).

Hereafter, III and IV will be used to designate the alkylated products in peaks 6 and 4, respectively. The relatively small amounts of IV available and its lability to air oxidation made it unlikely that it could be crystallized and characterized by conventional analytical techniques. Instead, this product was identified as the N^{10}-alkyl[3] derivative of tetrahydrofolate by indirect methods, *i.e.*, comparison of its absorption spectra to other 10-substituted tetrahydropteridines, susceptibility to oxidation by H_2O_2–peroxidase and synthesis by an alternate and unambiguous route.

The latter synthesis was achieved as follows: folic acid, in which the 10 position is known to be highly reactive toward substitution, was treated with Chlorambucil and the reaction mixture was subjected to chromatography on DEAE-cellulose. Further purification led to crystallization of the 10-alkyl[3] derivative of folic acid (V) which gave good analytical values for $C_{33}H_{38}N_8O_9 \cdot H_2O$. The presence of *one* Chlorambucil residue per molecule of pteridine was established by repeating the synthesis with ^{14}C-labeled Chlorambucil. Attachment of the alkyl group at the N^{10} position of folic acid was further confirmed by the inertness of V toward substitution by formic or nitrous acid and by its absorption spectra. Taking into account the additional absorption maximum at 261 mμ owing to the Chlorambucil moiety, V displays absorption spectra (Figure 4) similar to those of the 10-methyl and 10-thyminyl derivatives of folate (Gupta and Huennekens, 1967a,b).

When the 10-alkylated derivative of folate (V) was hydrogenated over platinum oxide in glacial acetic acid, 2 moles of hydrogen was consumed/mole of starting material and the 10-alkyl derivative of tetrahydrofolate (IV) was isolated as a white, fluffy powder. The absorption spectra of this compound at various pH values, which were similar to those of the material in peak 4 obtained by the direct alkylation of tetrahydrofolate by Chlorambucil, are shown in Figure 5; these spectra are very similar to 10-methyl tetrahydrofolate (Gupta and Huennekens, 1967b). At pH 7, IV is characterized by a principal maximum at 302 mμ but, for reasons not yet clear, the expected shoulder at *ca.* 256 mμ owing to the Chlorambucil residue is not seen (*cf.* Figures 4 and 6 for similar shoulders in spectra of V and III).

By analogy with other tetrahydropteridines, in which the N^5 position is unsubstituted, IV would be expected to be susceptible to oxidation. Treatment of IV at neutral pH with H_2O_2 and peroxidase resulted in a reaction (complete within 5 min) as shown by a decrease in the 302-mμ peak accompanied by an increase in absorbancy at 260 mμ. Although much more stable adducts are formed with HCHO when both the N^5 and N^{10} positions are free, tetrahydropteridines unsubstituted at the N^5 position can also form equilibrium adducts with HCHO (Blakley, 1957; Kisliuk, 1957). When IV was admixed at neutral pH with excess HCHO, however, there was essentially

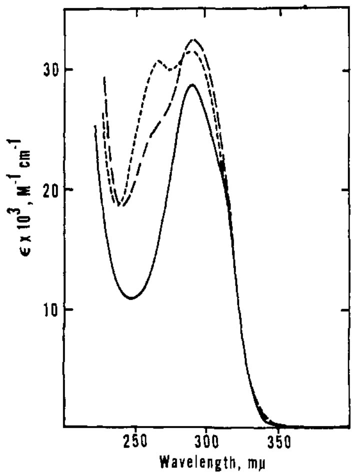

FIGURE 6: Spectra of 5-alkyltetrahydrofolate (III).

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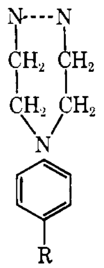
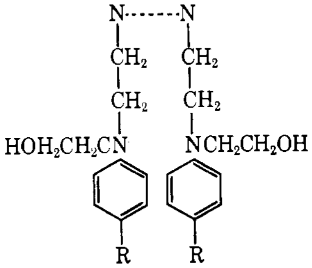
[3] Alkyl refers to a Chlorambucil residue.

no change (<5%) in the 302-mμ absorption band. This does not necessarily show a lack of reactivity since 10-methyltetrahydrofolate likewise showed no change in absorption spectrum when treated with HCHO (Gupta and Huennekens, 1967b).

Compound III, the major product resulting from the alkylation of tetrahydrofolate by Chlorambucil, was examined by the same techniques that were applied to IV. The absorption spectra of III at various pH values (Figure 6) closely resemble those of 5-methyltetrahydrofolate (Gupta and Huennekens, 1967b). At pH 7.0, III is characterized by an absorption maximum at 290 mμ and a shoulder at 265 mμ; the latter band, owing to the Chlorambucil residue, largely disappears upon acidification (*cf.* footnote 2).

One of the most distinctive properties of III was its extreme stability. No decomposition was observed when III was stored at 4° in neutral or slightly alkaline solutions or frozen in the presence of 0.05 M mercaptoethanol. Alkylation of tetrahydrofolate at the N^5 position would be expected to produce a compound having greater stability than the N^{10}-substituted analog. However, an attack by the bifunctional groups of Chlorambucil at *both* the N^5 and N^{10} positions of tetrahydrofolate could give rise to either the monoalkylated bridge compound (VI) or the dialkylated derivative (VII). Both of these compounds would be expected to show the stability observed for III and it was necessary to consider them as possibilities.


VI


VII

The dialkylated structure (VII) was ruled out by the analytical data since III, isolated as the crystalline barium salt, conformed to the empirical formula, $C_{33}H_{40}N_8O_9Ba \cdot 5H_2O$. The presence of only *one* alkyl residue per reduced pteridine was further shown by use of ^{14}C-labeled Chlorambucil. The specific activity (9.4×10^4 dpm/μmole) of III under these circumstances was the same as that of the initial Chlorambucil. The bridge structure (VI) was more difficult to eliminate. There are no relevant reference compounds for such a compound, except for 5,10-methylenetetrahydrofolate. The methylene group is somewhat atypical, however, since it permits a degree of interaction between the N^5 and N^{10} nitrogens. 5,10-Methylenetetrahydrofolate is resistant to peroxidation whereas III, when treated with H_2O_2 and peroxidase, underwent a change in spectrum similar to that seen for the oxidation of 5-methyltetrahydrofolate to 5-methyldihydrofolate (Larrabee *et al.*, 1963). Thus, it appears most likely that the single alkyl residue in III is located at the N^5 position of the tetrahydrofolate. This is also in accord with the observation (Gupta and Huennekens, 1967a) that alkylation of tetrahydrofolate with chloromethyluracil occurs most readily at the 5 position.

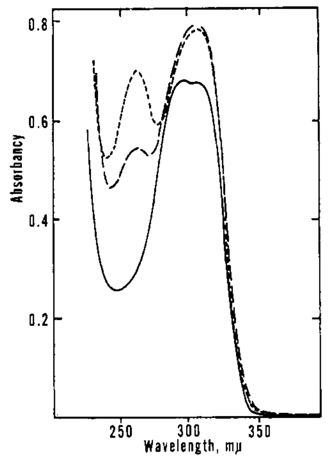


FIGURE 7: Spectra of 5-alkyl-10-methyltetrahydrofolate (VIII).

In order to obtain further information about dialkylated compounds similar to VII, 10-methyltetrahydrofolate was alkylated with ^{14}C-labeled Chlorambucil. When the reaction mixture was chromatographed on DEAE-cellulose, three principal peaks were observed in the elution profile. The first two were hydrolyzed and unhydrolyzed Chlorambucil, respectively. The third peak, corresponding in position to that of peak 6 in Figure 2, had a specific activity (9.5×10^5 dpm/μmole) identical with that of the labeled Chlorambucil. The absorption spectra of this product, 5-alkyl-10-methyltetrahydrofolate (VIII), are shown in Figure 7.

Upon rechromatography on DEAE-cellulose, the dialkylated product appeared to be reasonably homogeneous except for a slight shoulder on the leading edge of the peak. When examined by paper chromatography, VIII showed only a single quenching spot (R_F 0.72). Compound VIII did not react with HCHO but was oxidized with H_2O_2 and peroxidase. These results demonstrate that when the 10 position of tetrahydrofolate is blocked, alkylation by Chlorambucil occurs only at N-5, rather than at N-8 or the 2-amino group.

5-Formyltetrahydrofolate (folinic acid) also serves as a useful model compound since, in this case, introduction of an alkyl substituent at N^{10} would prevent the formyl group from being able to cyclize under acidic conditions to yield the 5,10-imidazolinium derivative (λ_{max} at 355 mμ). Treatment of folinic acid with labeled Chlorambucil and subsequent work-up of the reaction mixture by DEAE chromatography yielded one major radioactive peak which was eluted with 0.4 M ammonium acetate. The material was homogeneous upon rechromatography and showed a quenching spot at R_F 0.66 upon paper chromatographic

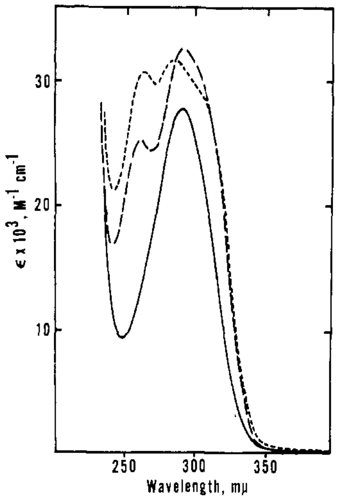


FIGURE 8: Spectra of 5-formyl-10-alkyltetrahydrofolate (IX).

examination. The product had an absorption maximum at 292 mμ (Figure 8) and a specific activity of 1.5 × 10^5 dpm/μmole, the same as that of the Chlorambucil. The product was also inert toward both HCHO and peroxidation, and did not show any increase in absorbancy at 355 mμ upon treatment with acid. The compound was judged to be, therefore, 5-formyl-10-alkyltetrahydrofolate (IX).

Experimental Section

Materials. Chemicals were obtained from the following commercial sources: folic acid and Nitromin, California Corp. for biochemical research; DEAE-cellulose, Schleicher & Schuell, Inc.; 2-mercaptoethanol Eastman Organic Chemicals; platinum oxide, Engelhard, N. J.; [1,2-^{14}C]ethylene oxide (sp act. 16 mc/μmole), International Chemical and Nuclear Corp.; calcium folinate, General Biochemicals, Inc.; and peroxidase, Worthington Biochemical Corp. Mustargen was a gift from the Merck Sharp and Dohme Laboratories.

dl-L-Tetrahydrofolic acid was prepared by the catalytic hydrogenation of folic acid according to the method of Hatefi *et al.* (1960). 5-Methyltetrahydrofolic acid and 10-methyltetrahydrofolic acid were prepared by the procedures of Gupta and Huennekens (1967b).

Methods. A Beckman spectrophotometer, Model DU, was used for measurements involving changes in absorbancy at a single wavelength and the Cary recording spectrophotometer, Model 14, was employed to measure the complete spectra of compounds. Weighed amounts of crystalline compounds of known analytical composition were used for determination of extinction coefficients. Melting points (uncorrected) were determined with a Fisher-Johns apparatus and microanalyses were performed by Spang Microanalytical Laboratories, Ann Arbor, Mich. Radioactivity was measured with a Nuclear-Chicago decade scaler, Model 181 A, equipped with an automatic sample changer, Model C 110 B. Samples were plated on copper planchets to infinite thickness.

DEAE-cellulose was purified according to Mathews and Huennekens (1960) and stored as an aqueous suspension. In the cold room (*ca.* 4°) columns of DEAE-cellulose were washed with approximately 2 l. of water followed by 2 l. of 0.05 M ammonium acetate containing 0.05 M mercaptoethanol. Reaction mixtures from the alkylation of folate compounds were added to the column and elution was carried out with ammonium acetate buffers of increasing molarity as stated elsewhere.

Ascending paper chromatography at 4° was carried out on Whatman No. 1 paper using 0.1 M phosphate buffer (pH 7.0), which also contained mercaptoethanol at a concentration of 0.5%. Compounds were detected as quenching spots when viewed under ultraviolet light.

Oxidation of Reduced Pteridines with H_2O_2–Peroxidase.[4] The experimental cuvet contained 0.1 μmole of the reduced pteridine compound and 10 μmoles of mercaptoethanol (omitted in the case of 5-substituted compounds) in 3 ml of 0.1 M phosphate buffer (pH 7.0). The blank cuvet was identical except for the omission of the pteridine. The reaction was initiated by the addition to each cuvet of 0.1 mg of peroxidase and 0.05 ml of 0.03% H_2O_2 and the spectrum of the oxidized product was recorded after 5 min.

Reaction of Reduced Pteridines with HCHO. The experimental cuvet contained 0.1 μmole of the pteridine compound in 3 ml of phosphate buffer (pH 7.0). The blank cuvet contained no pteridine. The reaction was initiated by the addition of 0.5 mmole of HCHO and the absorption spectrum was taken at various times.

Purification of 5-Formyltetrahydrofolic Acid. The calcium salt of commercial folinic acid (200 mg) was dissolved in 300 ml of water, the pH was adjusted to 7.0, and the solution was chromatographed on a 3 × 36 cm column of DEAE-cellulose. Elution was carried out with a gradient of ammonium acetate (0.1–0.4 M, pH 7, 1 l. each). The first 550 ml of the effluent was discarded and the column was then transferred to an automatic fraction collector. Fractions (10 ml) were collected and monitored for absorbancy at 290 mμ. The major component of the elution profile, characterized by an absorption maximum at 285 mμ and a minimum at 245 mμ, was present in tubes 90–105. The contents of these tubes were pooled and lyophilized to yield a light cream-colored powder (120 mg), R_F 0.56, pH 7.0, λ_{max} 286 mμ.

^{14}C-Labeled Chlorambucil was synthesized by the following modification of the procedure of Everett *et al.* (1953).

Methyl Ester of p-Aminophenylbutyrate. The methyl

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[4] We are indebted to Dr. P. P. K. Ho for the development of this procedure.

ester of *p*-nitrophenylbutyrate (10 g), dissolved in 50 ml of methanol containing palladium–strontium carbonate (1 g, 2%), was hydrogenated at 10 lb of pressure for 45 min. The catalyst was removed by filtration and washed with methanol. The combined filtrate and washings were concentrated under suction to give an oil which crystallized from petroleum ether (bp 30–50°) as fine needles, mp 41–42°.

The methyl ester of *p*-aminophenylbutyrate (4 g) was dissolved in 10 ml of 1 N acetic acid. The solution was cooled to 4° and 10 ml of ethylene oxide containing 2 mc of [1,2-^{14}C]ethylene oxide (sp act. 16 mc/μmole) was added slowly over a period of 30 min. The suspension was stirred at room temperature for 4 hr (complete miscibility occurred after 2 hr), excess ethylene oxide was removed under reduced pressure, and the oily hydroxyethyl compound was extracted with cold ether and washed with 2 N Na_2CO_3. The residue obtained after evaporation of the solvent was dissolved in 60 ml of benzene and the last traces of water were removed by distilling 30 ml of the solvent. $POCl_3$ (6 ml) was added to this solution and the mixture was refluxed for 1 hr. The solvent and excess $POCl_3$ were then removed under suction and the residue was heated cautiously for 1 hr with 16 ml of concentrated HCl. The solution was cooled, diluted with 80 ml of water, extracted with ether (6 × 25 ml), and dried. Ether was removed by distillation and the residue was crystallized from petroleum ether as colorless needles: mp 64–66°, sp act. 1.6×10^5 dpm/μmole, $R_F = 0.50$, (in ethanol), λ_{max} at 258 mμ (ϵ 19.3×10^3 M^{-1} cm^{-1}) and (at pH 7.0) λ_{max} at 256 mμ (ϵ 15.6×10^3 M^{-1} cm^{-1}).

Alkylation of Tetrahydrofolate by ^{14}C-Labeled Chlorambucil. Tetrahydrofolate diacetate (75 mg, 133.3 μmoles) was dissolved in 24 ml of a freshly prepared solution of 0.1 M sodium bicarbonate–carbonate buffer (pH 9.2) that had been cooled previously to 5° and deoxygenated with a continuous stream of nitrogen. ^{14}C-labeled Chlorambucil (125 mg, 400 μmoles), dissolved in 6 ml of absolute ethanol (also flushed with nitrogen), was added to the tetrahydrofolate solution and the mixture was allowed to stand at 37° for 90 min under a continuous stream of nitrogen; at the end of the incubation period, the pH had dropped to about 8. The solution was diluted with 50 ml of ice water, 2-mercaptoethanol was added to a final concentration of 0.1 M, the pH was adjusted to 7.0 by the dropwise addition of 0.1 M HCl, and the resulting solution was chromatographed on a 3 ×17 cm column of DEAE-cellulose. The elution profile is shown in Figure 2. The column was first washed with 300 ml of water. Gradient elution was then applied using ammonium acetate (0.1–0.3 M, pH 7.5, 1 l. each) followed by a gradient of ammonium acetate (0.3–0.6 M, pH 7.5, 1 l. each). All of these solutions contained mercaptoethanol at a concentration of 0.05 M. Fractions (10 ml) were monitored at 295 mμ using the Vanguard automatic analyzer, Model 1056. Absorbancies at 295 mμ of the contents of alternate tubes in the peak regions were determined with a Beckman spectrophotometer, Model DU. One-half milliliter from alternate tubes was used for measurement of radioactivity.

The labeled components in tubes 10–60 (peaks I and 2) were identified as unreacted and hydrolyzed Chlorambucil, respectively. Unreacted tetrahydrofolate (peak 3) was found in tubes 205–235. This material had a single absorption maximum at 298 mμ (pH 7.0) and contained essentially no radioactivity. Fractions 240–258, constituting peak 4, were characterized by λ_{max} at 295 mμ. The contents of these tubes, characterized by absorbancy ratios (295:262) between 1.20 and 1.35, were pooled and lyophilized. The solid material was dissolved in 10 ml of 0.01 M ammonium acetate containing 0.05 M mercaptoethanol and rechromatographed. One principal peak was found at essentially the same place in the above profile; small peaks occasionally found earlier in the profile were breakdown products.

Peak 5 (tubes 290–320) was a mixture of several compounds, including materials found in peaks 4 and 6, and was not examined further. Peak 6, the principal product in the elution profile (tubes 335–365), was characterized by λ_{max} of 290 mμ and a shoulder at 265 mμ. The contents of these tubes were pooled and lyophilized. The solid material was dissolved in 10 ml of water containing 0.05 M mercaptoethanol and the pH was adjusted to 7.0. Upon rechromatography, this solution yielded a single symmetrical peak at essentially the same position and moved as a single quenching spot (R_F 0.65). The specific activity (9.5×10^4 dpm/μmole) of the isolated material[5] was the same as that of the labeled Chlorambucil used. Based on absorbancy, the yield of this component was about 50–60% of the total alkylated products. The material in the small peak following peak 6 was not examined further.

Large-Scale Preparation of Crystalline III. Tetrahydrofolate diacetate (1.4 g) was suspended in 100 ml of a cooled and deaerated 0.5 M bicarbonate–carbonate buffer (pH 9.2). Chlorambucil (2.5 g), dissolved in 25 ml of absolute ethanol, was added to the former solution and the reaction mixture was incubated at 37° for 90 min under a continuous stream of nitrogen. The solution was then diluted with ice water to 1 l., 3.5 ml of mercaptoethanol was added to give a final concentration of 0.05 M, and the pH was adjusted to 7.5. The above reaction mixture was passed through a 3.8 × 42 cm column of DEAE-cellulose that had been washed previously and equilibrated with 0.05 M ammonium acetate (pH 7.5) containing 0.05 M mercaptoethanol. The column was eluted with varying concentrations of ammonium acetate (pH 7.5) as described below, and absorbancy of the effluent was monitored at 295 mμ. Elution was initiated with 2 l. of 0.1 M ammonium acetate to remove Chlorambucil. This was followed by gradient elution with ammonium acetate (0.1–0.4 M, 1 l. each) and then with 3 l. of 0.4 M ammonium acetate. When about 2.8 l. of the 0.4 M solvent had passed through, the column was transferred to a

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[5] Assuming a molar extinction coefficient at 295 mμ of 25×10^3 M^{-1} cm^{-1}.

fraction collector and 10–12-ml fractions were collected. The elution was completed by a gradient of ammonium acetate (0.4–0.6 M, 1 l. each). All of these solutions also contained 0.05 M mercaptoethanol. The desired material (peak 6) began to be displaced when about 3 l. of the final gradient (0.4–0.6 M) had passed through the column and was completely removed by about 1.6 l. The contents of these latter tubes (*ca.* 1300 ml) were combined and lyophilized to yield the 5-alkyltetrahydrofolate (III) as a white fluffy powder (300 mg).

From two of the above preparations, 500 mg of partially purified III was dissolved in 100 ml of deaerated water containing 0.05 M mercaptoethanol and the pH was adjusted to 7.5. The solution was rechromatographed on a 3.8 × 36 cm column of DEAE-cellulose using the above elution procedures beginning with the 0.1–0.4 M gradient. Peak tubes (1.2 l.) were pooled and lyophilized to yield a white powder (375 mg). This material (300 mg) was dissolved in 60 ml of deaerated water containing 0.02 M mercaptoethanol and 200 mg of NaCl, the pH was adjusted to 7.0, and the solution was filtered. $BaCl_2$ (400 mg) was added to the filtrate and the barium salt was precipitated by the addition of 300 ml of absolute ethanol. After refrigeration overnight, filtering, washing with ethanol, and drying at 25° in a dessicator, 250 mg of a nearly white product was obtained. The above purification was repeated to yield a barium salt that was crystallized from water–methanol as microcrystalline platelets and quickly dried under vacuum (125 mg); R_F 0.64, at pH 7.0, λ_{max} at 290 mμ (ϵ 32 × 10^3 M^{-1} cm^{-1}) with a shoulder at 265 mμ (ϵ ~25 × 10^3 M^{-1} cm^{-1}). *Anal.* Calcd for $C_{33}H_{40}N_8O_9 \cdot Ba \cdot 5H_2O$: C, 43.07; H, 5.47; Ba, 14.92; N, 12.18. Found: C, 42.8; H, 5.5: Ba, 14.8 N, 12.1. The dried material was completely stable for several months when stored under anhydrous conditions at −20° but samples stored at room temperature under identical conditions slowly decomposed as evidence by darkening and changes in the absorption spectrum.

10-Alkylfolic Acid (V). Folic acid (40 mg, 100 μmoles) was dissolved in 20 ml of 0.1 M bicarbonate buffer (pH 8.2). ^{14}C-labeled Chlorambucil (sp act. 9.4 × 10^4 dpm/μmole, 90 mg, 300 μmoles) dissolved in 5 ml of absolute ethanol was added to folate solution and the reaction mixture, covered with aluminum foil to prevent photodecomposition, was incubated at 37° for 90 min in a nitrogen atmosphere. The color of the solution gradually changed from orange-yellow to reddish-brown. The reaction mixture was diluted with 25 ml of cold water, adjusted to pH 7.0, and chromatographed on a 3 × 18 cm column of DEAE-cellulose. The column was washed with 350 ml of water and then with 800 ml of 0.1 M ammonium acetate to remove both unreacted and hydrolyzed Chlorambucil. The column was then eluted with the following ammonium acetate solutions, 9-ml fractions being collected in all cases: 600 ml of 0.1–0.3 M (tubes 110–234), 1200 ml of 0.3 M (tubes 235–367), and 1 l. of 0.4 M (tubes 368–460). Fractions were monitored for absorbancy at 262 mμ and for radioactivity; most of the radioactive material was present in tubes 260–350. From the radioactivity and absorbancy profiles, two alkylated products were seen in this region. Fractions 305–315, which showed a two-banded absorption spectrum (λ_{max} at 262 and 286 mμ), were mixed and lyophilized. The unknown material present had an R_F value of 0.61.

The contents of tubes 324–344, containing the bulk of the radioactivity and having a two-banded absorption spectrum (λ_{max} at 261 and 302 mμ), were also pooled and lyophilized. The specific activity (9.44 × 10^4 dpm/μmole) of this product (V) was the same as that of labeled Chlorambucil. Compound V moved as a single quenching spot (R_F 0.68) on paper chromatograms and did not react with formic or nitrous acids showing that the 10 position was blocked.

Large-Scale Preparation of 10-Alkylfolic Acid (V). Folic acid (441 mg) was dissolved with stirring in 80 ml of 0.1 M sodium bicarbonate–carbonate buffer (pH 9.2). Chlorambucil (909 mg) in 20 ml of absolute ethanol was added to the solution and the reaction mixture was kept at 37° for 2 hr under a continuous stream of nitrogen. The initial turbidity disappeared after about 15 min and thereafter the solution was clear and homogeneous. The red solution (pH 8.8) was cooled to 5°, and the pH was adjusted to 7.3 with 2 N HCl and diluted with water to 200 ml. Further purification of the crude product was accomplished by chromatography on a 3.8 × 42 cm column of DEAE-cellulose using a slight modification of the procedure described above. After the solution had been placed on the column, elution was begun with 6 l. of 0.25 M ammonium acetate; most of the colored material had been removed after about 3.6 l. had passed through the column. At this stage, the column was transferred to an automatic fraction collector and elution was continued with the same buffer. Fractions of 10–12 ml were collected and the complete spectrum was taken of every tenth tube. Most of the unknown product characterized by absorption maxima at 262 and 286 mμ had been displaced by the time 5 l. of ammonium acetate had passed through the column; complete elution of this component was achieved in the next 400–500 ml (40–50 tubes). The contents of tubes showing absorption maxima at 262 and 286 mμ were pooled and lyophilized (25 mg). Further work-up of this component will be described below.

The molarity of ammonium acetate was increased to 0.3 M and the elution was continued. Compound V appeared in the next 500–700 ml and those fractions having the correct spectral characteristics (λ_{max} 300 mμ and with a shoulder at 261 mμ) were mixed and lyophilized (35 mg). Several such columns yielded an amorphous brownish powder (150 mg) which was dissolved in 150 ml of water containing NaCl (1.0 g), adjusted to pH 3.5 with 1 N HCl, and cooled overnight; compound V separated out as a microcrystalline solid (110 mg). The product was filtered with suction, washed with several 5-ml portions of water, and dried quickly under vacuum at room temperature. A small sample of this material was crystallized twice to yield V as glistening pale reddish microcrystalline flakes; mp

175–185° (sample introduced in preheated bath at 155°); at pH 7, λ_{max} at 303 mμ (ϵ 32.5 $\times$ 10^3 M^{-1} cm^{-1}) and 260 mμ (ϵ 28.3 $\times$ 10^3 M^{-1} cm^{-1}). *Anal.* Calcd for $C_{33}H_{38}N_8O_9 \cdot H_2O$: C, 55.92; H, 5.68; N, 15.80. Found: C, 55.9; H, 5.72; N, 15.3. Compound V could be stored in brown bottles under anhydrous conditions for several months without decomposition.

The unknown component (100 mg pooled from 4 runs), eluted prior to V on the DEAE-cellulose column, was dissolved in 15 ml of water, adjusted to pH 7.0, and chromatographed on a 3 $\times$ 18.5 cm column of DEAE-cellulose. The column was eluted with a gradient of ammonium acetate (0.1–0.4 M, 1 l. each). Fractions of 11 ml were collected and every fourth tube was scanned for absorbancy at 260 mμ. The bulk of the material having the correct spectral characteristics was eluted in tubes 164–186. These were combined, lyophilized, and crystallized by adjusting the pH to 2.8 after addition of NaCl (100 mg). The product separated out as a microcrystalline solid (70 mg). A sample was recrystallized twice and dried rapidly under vacuum over P_2O_5. At pH 1, λ_{max} at 250 (shoulder) and 296 mμ (ϵ 12.2 and 18.8 $\times$ 10^3 M^{-1} cm^{-1}); at pH 7, λ_{max} at 262 and 285 mμ (ϵ 23.2 and 23.5 $\times$ 10^3 M^{-1} cm^{-1}). *Anal.* Calcd for $C_{33}H_{38}N_8O_9 \cdot H_2O$: C, 55.92; H, 5.68; N, 15.80. Found: C, 55.7; H, 5.80; N, 15.2.

10-Alkyltetrahydrofolate (*IV*). 10-Alkylfolic acid monohydrate (14.2 mg, 20 μmoles) suspended in 7 ml of glacial acetic acid was hydrogenated over platinum oxide (25 mg) in a microhydrogenation apparatus until no more hydrogen was consumed. 10-Alkyltetrahydrofolic acid (IV) was isolated after lyophilization as a white fluffy powder (10 mg). The product was homogeneous and showed a single quenching spot (R_F 0.78) on paper chromatograms along with a trace of fluorescent impurities. The absorption spectra of 10-alkyltetrahydrofolic acid (Figure 5) prepared by this route was very similar to that of the material in peak 4 of Figure 2. Compound V was extremely labile to oxidation and deteriorated rapidly in solution or when stored as a solid.

5-Alkyl-10-methyltetrahydrofolate (*VIII*). 10-Methyltetrahydrofolate (30 mg) was suspended in 12 ml of a previously cooled and deaerated 0.1 M bicarbonate–carbonate buffer (pH 9.2). ^{14}C-labeled Chlorambucil (60 mg) dissolved in 3 ml of absolute ethanol was added and the mixture was incubated at 37° for 90 min. The reaction mixture was cooled, 2-mercaptoethanol was added to a concentration of 0.05 M, the pH was adjusted to 7.0, and the solution was chromatographed on a 3 $\times$ 16 cm column of DEAE-cellulose at 4°. The column was eluted with a gradient of ammonium acetate (0.1–0.4 M, pH 7.5, 1 l. each). The solutions also contained mercaptoethanol at 0.05 M. Fractions of 10–12 ml were collected and every alternate tube was scanned for absorbancy at 295 mμ and for radioactivity. The contents of tubes comprising the major peak (corresponding to peak 6 of Figure 2) were pooled and lyophilized. The residue was dissolved in water, adjusted to pH 7, and rechromatographed under similar conditions. Compound VIII was isolated as a symmetrical, homogeneous peak at the same position. The specific activity of VIII was 9.4 $\times$ 10^4 dpm/μmole, the same as that of Chlorambucil. Compound VIII was also moved as a single quenching spot on paper chromatography (R_F 0.72). The absorption spectra of VIII are shown in Figure 7.

5-Formyl-10-alkyltetrahydrofolate (*IX*). Purified folinic acid (30 mg) was alkylated with labeled Chlorambucil (60 mg) under conditions described above for the synthesis of VIII. The reaction mixture was purified by chromatography on a DEAE-cellulose column at 25°, mercaptoethanol was omitted from the buffers. Compound IX was isolated as a homogeneous compound after rechromatography of the lyophilized, pooled fractions on a second DEAE-cellulose column. The absorption spectra of IX are shown in Figure 8. The product had the same specific activity (9.4 $\times$ 10^4 dpm/μmole) as that of the Chlorambucil. Compound IX, like its precursor folinic acid, was unreactive toward formaldehyde and resistant to peroxidation. There was no change in absorbancy at 355 mμ when IX was acidified with 2 N HCl.

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Thyminyl Derivatives of Tetrahydrofolate*

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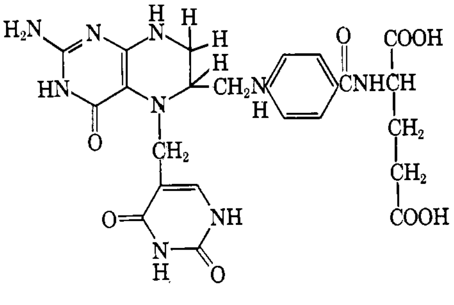
ABSTRACT: Tetrahydrofolate reacted rapidly with 5-chloromethyluracil (unlabeled or 2-^{14}C) in dioxane–phosphate buffer (pH 7.0) at 5° to yield predominantly 5-thyminyltetrahydrofolate (I). The latter compound was isolated by chromatography on DEAE-cellulose and crystallized as the free acid or the barium salt. Under similar conditions, folate was converted to the 10-thyminyl derivative (II) which was also isolated as the crystalline acid. Catalytic hydrogenation of II in glacial acetic acid over platinum oxide yielded 10-thyminyltetrahydrofolate (III). In contrast to I, III was labile to air oxidation and could not be crystallized. 5,10-Dithyminyltetrahydrofolate (IV) was synthesized by further treatment of either I or III with chloromethyluracil and isolated as the crystalline barium salt. Oxidation of III with H_2O_2 in the presence of peroxidase or reduction of II with hydrosulfite gave 10-thyminyl-7,8-dihydrofolate (V). Alternatively, oxidation of I with H_2O_2–peroxidase produced a dihydro compound, presumably 5-thyminyl-5,6-dihydrofolate (VI). Compounds I–VI were further characterized by their absorption spectra at various pH values.

Based upon the observation that tritium is transferred from the reduced pyrazine ring of tetrahydrofolate to the methyl group of thymidylate during synthesis of the latter *via* eq 1 (Pastore and Friedkin, 1962;

$$\text{5,10-methylene tetrahydrofolate} + \text{deoxyuridylate} \longrightarrow \text{thymidylate} + \text{dihydrofolate} \quad (1)$$

Blakley *et al.,* 1963), a mechanism has been proposed (Friedkin, 1959; Huennekens, 1963) which involves 5-thymidyltetrahydrofolate as an intermediate. Cleavage of this hypothetical intermediate by an internal shift of a hydride ion would yield thymidylate and 7,8-dihydrofolate as the products. As part of our general study on 5- and 10-alkylated derivatives of tetrahydrofolate (Gupta *et al.,* 1967; Gupta and Huennekens, 1967), and specifically as a prelude to investigating the feasibility of the above mechanism, we have undertaken the synthesis of 5-thyminyltetrahydrofolate (I), a model compound which contains the key structural feature of the proposed intermediate, namely, a methylene bridge connecting C-5 of the pyrimidine and N-5 of the reduced pyrazine ring. The model compound differs from the hypothetical intermediate only insofar as the base, thymine, rather than the deoxyribonucleotide, thymidylate, is linked to tetrahydrofolate.



I

Preliminary experiments, performed in collaboration with Dr. Peter Ho, attempted to prepare I by condensation of 5-formyluracil with tetrahydrofolate, followed by reduction of the adduct with borohydride. This route, shown in eq 2 where $\underset{H}{N}$---$\underset{H}{N}$ represents the 5- and 10-nitrogen atoms of tetrahydrofolate, would be analogous to that used for the synthesis of 5-methyltetrahydrofolate from tetrahydrofolate and formaldehyde (Sakami and Ukstins, 1961; Keresztesy and Donaldson, 1961). Under a variety of experimental conditions, however, no reaction between formyluracil

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