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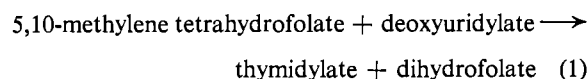
Thyminy! Derivatives of Tetrahydrofolate*

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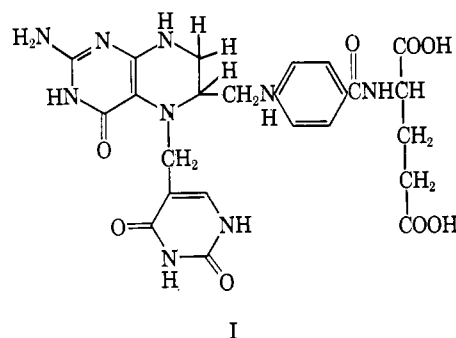
ABSTRACT: Tetrahydrofolate reacted rapidly with 5-chloromethyluracil (unlabeled or 2-¹⁴C) in dioxane-phosphate buffer (pH 7.0) at 5° to yield predominantly 5-thyminyltetrahydrofolate (I). The latter compound was isolated by chromatography on DEAE-cellulose and crystallized as the free acid or the barium salt. Under similar conditions, folate was converted to the 10-thyminy! 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 H₂O₂ in the presence of peroxidase or reduction of II with hydrosulfite gave 10-thyminy!-7,8-dihydrofolate (V). Alternatively, oxidation of I with H₂O₂-peroxidase produced a dihydro compound, presumably 5-thyminy!-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;



Blakley *et al.*, 1963), a mechanism has been proposed (Friedkin, 1959; Huennekens, 1963) which involves 5-thymidy!tetrahydrofolate as an intermediate. Cleavage of this hypothetical intermediate by an internal shift of a hydride ion would yield thymidy!ate 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 under-



taken 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, thymidy!ate, is linked to tetrahydrofolate.

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 N---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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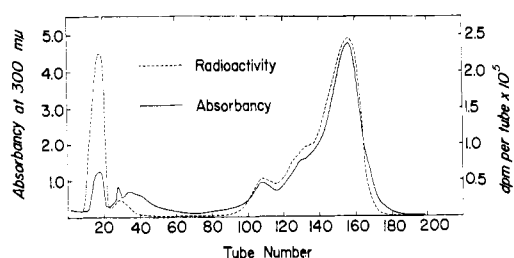
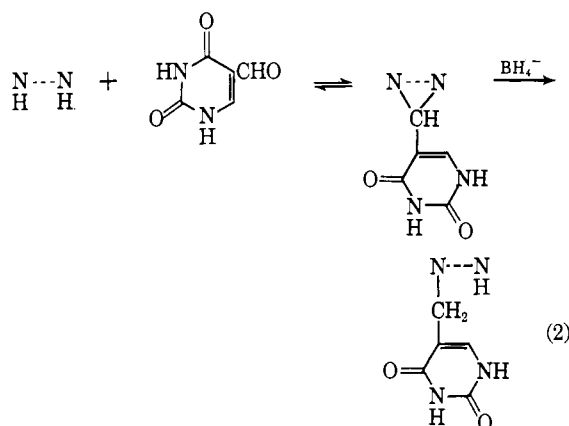


FIGURE 1: Chromatography on DEAE-cellulose of products resulting from interaction of chloromethyluracil-2- ^{14}C with tetrahydrofolate.



and tetrahydrofolate could be demonstrated spectrophotometrically nor could anything resembling the desired product be isolated after treatment of the mixture with borohydride. The reason for the refractory behavior of 5-formyluracil is still not clear, although internal hydrogen bonding between the aldehyde and the adjacent hydroxyl group, similar to that which occurs in salicylaldehyde, is a possible explanation.

Attention was turned to an alternate route for the synthesis of I. The N^5 and N^{10} positions of tetrahydrofolate are nucleophilic centers whose hydrogen atoms are readily displaced by bifunctional alkylating agents (Gupta *et al.*, 1967) or by the methylene group of formaldehyde (Blakley, 1957; Osborn *et al.*, 1960). Thus it seemed likely that a halide derivative of thymine, such as 5-chloromethyluracil, could be used for the introduction of a thyminyl group at the N^5 or N^{10} positions of tetrahydrofolate.

5-Chloromethyluracil was synthesized either by chloromethylation of uracil (Skinner *et al.*, 1960; Burckhalter *et al.*, 1960) or by chlorination of hydroxymethyluracil with thionyl chloride (Farakas and Sorm, 1961) and its reaction with tetrahydrofolate was studied. Preliminary experiments established that maximum alkylation of tetrahydrofolate occurred when a twofold molar excess of chloromethyluracil was used and the reaction was carried out in the 0.1 M phosphate buffer (pH 7.0) containing 50% by volume of dioxane at 5° for 10 min under anaerobic conditions. As a marker for the thyminyl group, chloromethyl-

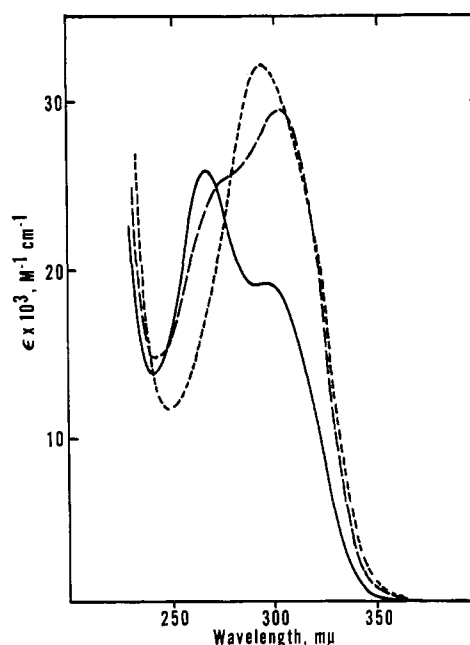


FIGURE 2: Spectra of 5-thyminyltetrahydrofolic acid (I). In the figures, the following solutions were used for control of pH: (—) pH 1.0, 0.1 M HCl; (---) pH 7.0, 0.1 M phosphate buffer; and (...) pH 13.0, 0.1 M NaOH.

uracil-2- ^{14}C was used. The products were chromatographed on DEAE-cellulose in the cold using a gradient (0.1–0.4 M) of ammonium acetate containing mercaptoethanol at a concentration of 0.05 M. A typical elution profile of a chromatographed reaction mixture is shown in Figure 1. Tubes were monitored for radioactivity and for absorbance at 300 $\text{m}\mu$. The labeled component in tubes 8–22 was identified as 5-hydroxymethyluracil by its absorption maximum at 287 $\text{m}\mu$ at pH 13 and by paper chromatography. The principal product (tubes 142–162) was characterized by an absorption maximum at 302 $\text{m}\mu$ plus a distinct shoulder at 275 $\text{m}\mu$ at pH 7 and by a single maximum at 290 $\text{m}\mu$ at pH 13. From the pooled contents of these tubes, a compound was isolated which migrated as a single quenching spot on paper chromatograms and which, upon rechromatography on DEAE-cellulose, yielded a single, symmetrical peak at essentially the same position in the elution profile. After crystallization as the free acid, the material gave analytical values in good agreement with the empirical formula $\text{C}_{24}\text{H}_{27}\text{N}_9\text{O}_8 \cdot 2\text{H}_2\text{O}$. The crystalline barium salt corresponded to the empirical formula $\text{C}_{24}\text{H}_{25}\text{N}_9\text{O}_8\text{Ba} \cdot 5\text{H}_2\text{O}$. The specific activity (1.2×10^5 dpm/ μmole) was the same as that of the labeled precursor, chloromethyluracil, establishing that the product is a *monothyminyl* derivative of tetrahydrofolate. The compound was extremely stable, no decomposition occurring even when samples were stored as the barium salt or the free acid for several months at room temperature. In contrast, 10-methyl- and 10-formyltetrahydrofolates are very labile to oxygen

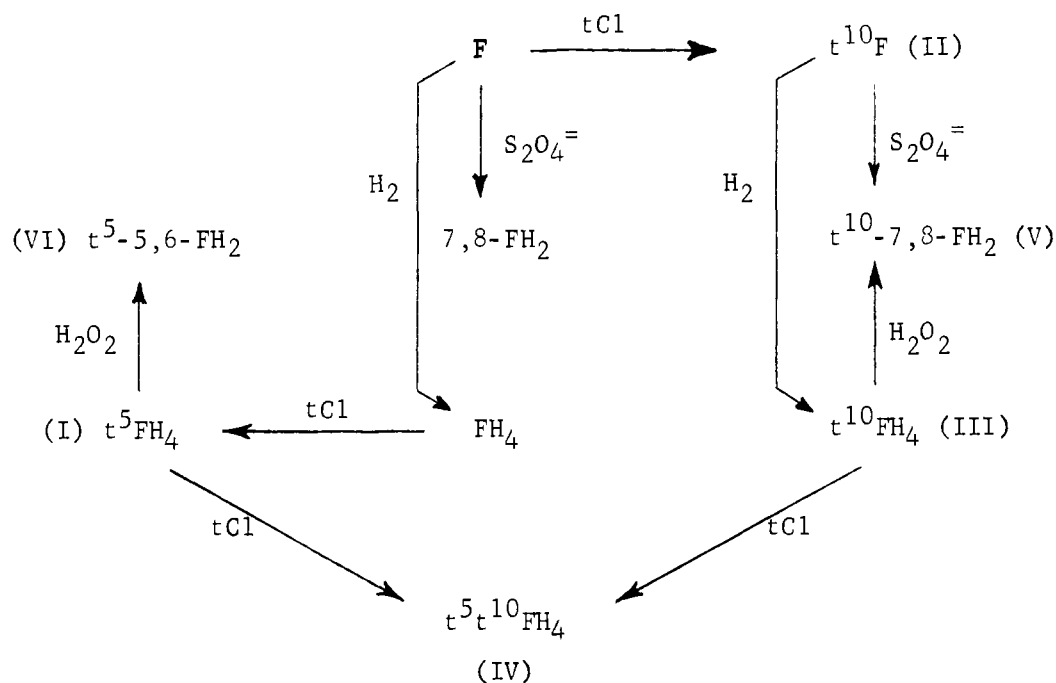
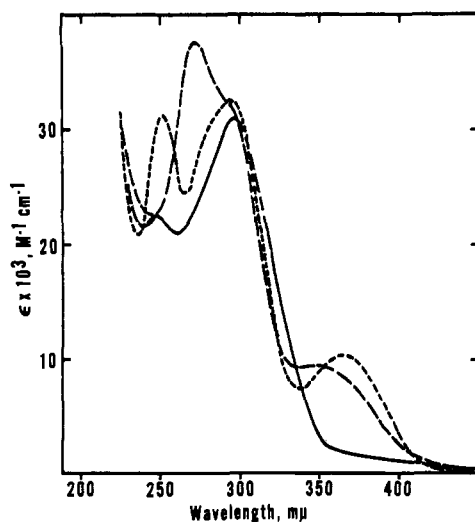


FIGURE 3: Synthetic routes for preparation of thymine derivatives of folate compounds. Abbreviations used: FH_2 , dihydrofolate; FH_4 , tetrahydrofolate; t , thymine (superscript indicates site of attachment to tetrahydrofolate; $t\text{Cl}$, chloromethyluracil).

either in solid form or in solution. Furthermore, the thymine group was not lost by heating the material at pH 7.0 at 100° for 30 min. These properties strongly suggested that the thymine group was located at the 5 position and that the material was in fact, 5-thymine-tetrahydrofolate (I). Spectra of I at various pH values are shown in Figure 2.

In order to confirm this assignment, and to obtain further information about alkyl derivatives of folate

compounds, the synthesis of several related thymine derivatives of folic acid was undertaken. Figure 3 illustrates the synthetic routes used and the interrelationship of these compounds. Folate, in which the N^{10} position is most susceptible to alkylation, was treated with chloromethyluracil and the product, 10-thyminefolate (II), was isolated by chromatography



2170 FIGURE 4: Spectra of 10-thyminefolic acid (II).

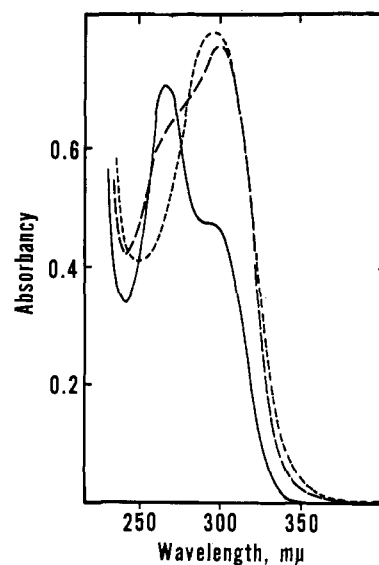


FIGURE 5: Spectra of 10-thymine-tetrahydrofolic acid (III).

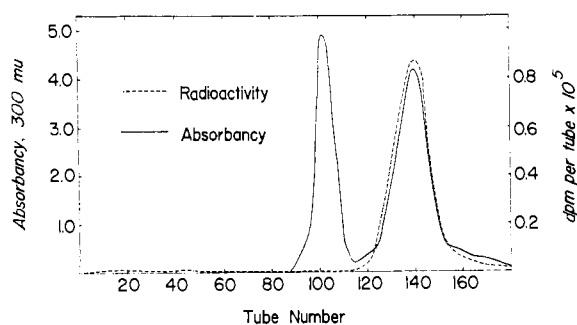


FIGURE 6: Chromatographic separation of ^{14}C -labeled 5-thyminyltetrahydrofolic and unlabeled 10-thyminyltetrahydrofolic acid.

on DEAE-cellulose, crystallized as the free acid and characterized spectrally (Figure 4). Like 10-methylfolate, II displays a bathochromic shift in its spectrum relative to folate, *i.e.*, at pH 13, the absorption maximum occurs at $300\text{ m}\mu$ while the corresponding value for folate is $283\text{ m}\mu$. The compound gave good analytical values for $\text{C}_{24}\text{H}_{23}\text{N}_9\text{O}_8 \cdot \text{H}_2\text{O}$ and the presence of one thyminy residue was confirmed when the synthesis was carried out with chloromethyluracil- $2\text{-}^{14}\text{C}$. That the 10 position was occupied by the thyminy group was further shown by the inertness of the compound toward formic or nitrous acids, both of which react readily at N^{10} of folate. The thyminy group was lost on heating the material at 100° and pH 7; equimolar amounts of folate and hydroxymethyluracil were produced.

Hydrogenation of II in glacial acetic acid over platinum oxide resulted in the uptake of 2 moles of hydrogen/mole of II and the product, 10-thyminyltetrahydrofolate (III), was isolated by chromatography on DEAE-cellulose. This material, however, was quite labile to air oxidation and could not be crystallized. Although the spectra of 10-thyminyltetrahydrofolate

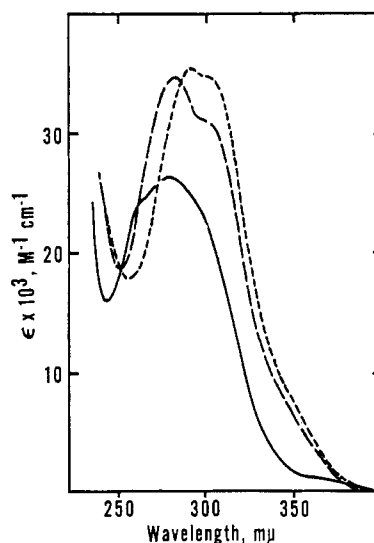


FIGURE 8: Spectra of 10-thyminyl-7,8-dihydrofolic acid.

(Figure 5) were somewhat similar to those of the 5-thyminy derivative (Figure 2), the two compounds could be readily distinguished by paper chromatography (R_F values of 0.55 and 0.60 for I and III, respectively, in 0.1 M phosphate buffer (pH 7.0), or by column chromatography on DEAE-cellulose (Figure 6). In the latter experiment, ^{14}C -labeled 5-thyminyltetrahydrofolate was admixed with the unlabeled 10-thyminy isomer before chromatography.

When treated further with chloromethyluracil, both I and III yielded the same product, 5,10-dithyminyltetrahydrofolate (IV), whose spectra are shown in Figure 7. The absence of appreciable side products in these reactions provides additional proof that alkylation of tetrahydrofolate occurs only at the N^5 and N^{10} positions, and that the 2-amino group and the N^8 position are inert toward chloromethyluracil under

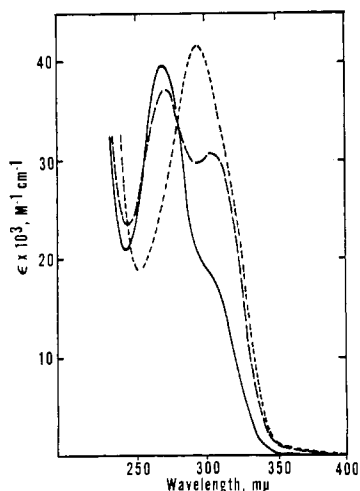


FIGURE 7: Spectra of 5,10-dithyminyltetrahydrofolic acid (IV).

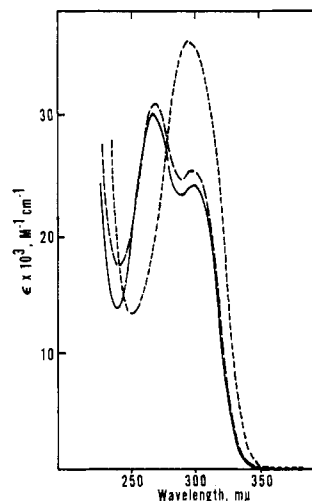


FIGURE 9: Spectra of 5-thyminyl-5,6-dihydrofolic acid.

these experimental conditions.

5- and 10-Thyminyll tetrahydrofolates were further distinguished by the properties of their corresponding dihydro derivatives. Oxidation of I and III with H_2O_2 in the presence of peroxidase yielded the two separate thyminyll derivatives of dihydrofolate. In the case of III, the oxidation product was presumably 10-thyminyll-7,8-dihydrofolate (V), as judged by the similarity of its spectra (Figure 8) to those of the product obtained by reducing 10-thyminyllfolate (II) with hydrosulfite. Conversely, oxidation of I yielded a product whose spectra (Figure 9) are similar to those of 5-methyl-5,6-dihydrofolate and it is, therefore, most probably 5-thyminyll-5,6-dihydrofolate (VI).

Experimental

Methods

Melting points were determined with a Fisher-Johns apparatus. Most of the compounds, however, melted over a broad range between 200 and 300°. Analyses were performed by Spang Microanalytical Laboratories, Ann Arbor, Mich., and by Analytical Corporation, N. Y. Absorbancy measurements at a single wavelength were determined with a Beckman spectrophotometer, Model DU. The Cary recording spectrophotometer, Model 14, was employed to measure complete spectra. Extinction coefficients were calculated on the basis of molecular weights established from analyses. The absorbancy of column effluents was monitored with a Vanguard automatic ultraviolet analyzer, Model 1056. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer using the method of Bray (1960). Ascending paper chromatography was carried out on Whatman No. 1 paper at 4°. Compounds were detected as "quenching" or fluorescent spots when viewed under a Mineralite ultraviolet light.

Materials

Chemicals were obtained from the following sources: folic acid, uracil, 5-hydroxymethyluracil, and thymine, California Corp. for Biochemical Research; 2-mercaptoethanol, thionyl chloride, naphthalene (crystallized from ethanol), Eastman Organic Chemicals; platinum oxide, Engelhard, N. J.; peroxidase, Worthington Biochemical Corp.; uracil-2- ^{14}C (sp act. 40 mc/mmole), Schwarz BioResearch Inc.; PPO¹ and dimethyl-POPOP, Packard Instrument Co., Inc.; DEAE-cellulose (exchange capacity = 0.88 mequiv/g; washed with 1 M K_2HPO_4 before use and stored in water), Schleicher & Schuell Inc.; Dowex 50 (200–400 mesh), Bio-Rad Laboratories, Richmond, Calif.; dimethylformamide, formaldehyde, glacial acetic acid, dioxane, and hydrogen peroxide, Matheson Coleman and Bell, Inc. All organic solvents were of reagent

grade and were used without further purification except for dioxane, which was distilled from sodium and stored frozen in brown bottles.

Tetrahydrofolic acid was prepared by the following modification of the procedure of Hatefi *et al.* (1960). Folic acid (4.0 g) was made into a slurry with 200 ml of glacial acetic acid and transferred to a hydrogenation flask. Platinum oxide (2 g, 94.5%) was added and the suspension was hydrogenated at 35 lb of pressure for 3 hr. At the end of this period most of the folic acid had dissolved, shaking was stopped, and the colorless suspension was allowed to stand for 4 hr in the hydrogen atmosphere. The catalyst was removed by filtration and tetrahydrofolate diacetate was isolated after lyophilization as a white fluffy powder (3.8 g).

5-Formyluracil was prepared by the application of a Reimer-Tiemann reaction to uracil using a modification of the procedure of Wiley and Yamamoto (1960). Uracil (0.3 g) was dissolved in 20 ml of 15% NaOH and mixed with 20 ml of chloroform in a 150-ml, two-necked, round-bottom flask equipped with a reflux condenser. The solution was heated to boiling and 30 ml of chloroform was added over a period of 1 hr. After the reaction mixture had been refluxed for 3.5 hr, the solution was concentrated to a thin paste at 50° using a water aspirator. The residue was dissolved in 30 ml of water and the solution was acidified to pH 1 with 5 N HCl. The pale yellow solution was chromatographed on a 3 × 30 cm column of Dowex 50 using water as the eluent. Fractions of 10 ml were collected and monitored for absorbancy at 260 mμ. There were two main peaks in the elution profile. The first (tubes 10–30) consisted largely of unreacted uracil characterized at pH 13 by its absorption maximum at 282 mμ and by paper chromatography (see below); the second (tubes 32–40) was characterized at pH 13 by absorption maxima at 298 and 250 mμ. Appropriate fractions in the second peak having an absorbancy ratio (298:250) of 1.4–1.6 were combined, concentrated to a turbid solution, and cooled overnight. 5-Formyluracil separated out as a yellow microcrystalline solid (186 mg); in various preparations the yield was 35–45%. The product (100 mg) was twice crystallized from hot water. The yellow rod-shaped crystals were washed with ethanol and ether and dried; yield, 80 mg; mp >300° dec; at pH 1, λ_{max} 275 mμ (ϵ 12.2 × 10³ M⁻¹ cm⁻¹) and 230 mμ (ϵ 10.4 × 10³ M⁻¹ cm⁻¹); at pH 13, λ_{max} 298 mμ (ϵ 14.8 × 10³ M⁻¹ cm⁻¹) and 250 mμ (ϵ 9.9 × 10³ M⁻¹ cm⁻¹) and a shoulder at 315 mμ (ϵ 3.0 × 10³ M⁻¹ cm⁻¹). *Anal.* Calcd for $\text{C}_5\text{H}_4\text{N}_2\text{O}_3$: C, 42.86; H, 2.87; N, 19.99. Found: C, 43.0; H, 2.90; N, 19.6.

Uracil and its 5-substituted derivative had the following R_F values in three solvent systems: (1) 1-butanol–water (86:14), (2) 1-butanol– NH_4OH –water (86:5:9), and (3) 1-propanol–5 N HCl (95:5); uracil, 0.35, 0.15, and 0.31; thymine (5-methyluracil), 0.51, 0.32, and 0.45; 5-formyluracil, 0.28, 0.10, and 0.28; and 5-hydroxymethyluracil, 0.23, 0.09, and 0.22. 5-Formyluracil reacted readily with 2,4-dinitrophenyl-

¹ Abbreviations used: PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

hydrazine to give the corresponding hydrazone (mp 296–298°). 5-Formyluracil-2-¹⁴C was synthesized in approximately the same yield using uracil-2-¹⁴C in the above procedure.

5-Chloromethyluracil was synthesized by the reductive chloromethylation of uracil (Skinner *et al.*, 1960; Burckhalter *et al.*, 1960) or by the direct chlorination of 5-hydroxymethyluracil using thionyl chloride in acetone (Farakas and Sorm, 1961). The former procedure was modified as follows for synthesis of chloromethyluracil-2-¹⁴C. Uracil-2-¹⁴C (700 μ C) and uracil (1.2 g) were dissolved with stirring in 7 ml of concentrated HCl and 1.2 ml of formaldehyde (37%) in a 30-ml, two-necked, round-bottom flask. Gaseous HCl was passed through the solution for 6 hr, after which it was concentrated over KOH pellets and P₂O₅ in a vacuum dessicator at room temperature until it became turbid. The dessicator was kept at 5° for 12 hr and the white crystalline solid which separated was filtered by suction and dried rapidly under high vacuum over P₂O₅. No attempt was made to purify this compound further because of its extreme lability. The dry product was stored in a brown bottle in a dessicator at room temperature; yield 470 mg, mp >300° dec, sp act. 1.4×10^5 dpm/ μ mole.

Attempted Reaction between 5-Formyluracil and Tetrahydrofolic Acid. Tetrahydrofolic acid was treated for 5–15 min with 5-formyluracil in molar ratios varying from 1:2 to 1:10 at pH 4.5 under anaerobic conditions at room temperature. Each solution was made 0.1 M with respect to mercaptoethanol, the pH was adjusted to 8.5, and chromatographed at 4° on a 3 \times 15 cm column of DEAE-cellulose. Columns were eluted with various buffers (0.1–0.4 M, pH 8.5) including ammonium acetate, Tris, and EDTA. No product resembling an aldehyde adduct of tetrahydrofolate was found in the effluent. The above reaction mixtures were treated with potassium borohydride (10–100-fold molar excess) under conditions similar to those used for synthesis of 5-methyltetrahydrofolic acid (Keresztesy and Donaldson, 1961), and chromatographed at neutral pH on columns of DEAE-cellulose at 4° using a variety of buffers; again no product resembling 5-thyminyltetrahydrofolate could be obtained. These experiments were also extended by the use of 5-formyluracil-2-¹⁴C; no radioactivity was associated with the pteridine compounds in the column effluents.

Interaction of 5-Chloromethyluracil with Tetrahydrofolic Acid. Tetrahydrofolic acid (30 mg) was dissolved by agitation in a mixture of 6 ml of 0.01 M phosphate buffer (pH 7.0) and 12 ml of dioxane that had been cooled previously to 4° and deaerated. 5-Chloromethyluracil (60 mg, sp act. 1.28×10^5 dpm/ μ mole) was added to the solution and the mixture was kept at 4° for 10 min under a continuous stream of nitrogen. The solution was diluted to 25 ml with cold water, 0.1 ml of mercaptoethanol was added, and the solution was chromatographed on a 3 \times 15 cm column of DEAE-cellulose. Gradient elution was performed using 1 l. of 0.05 M ammonium acetate (pH 7) in the mixing flask and 1 l. of 0.4 M ammonium acetate (pH

7) in the reservoir; both solutions also contained mercaptoethanol at a concentration of 0.05 M. Fractions of 10 ml were collected and monitored at 300 m μ . From every second tube in regions having ultra-violet-absorbing material, 0.1 ml was added to Bray's (1960) solvent and the radioactivity was determined. Figure 1 illustrates a typical elution profile showing both absorbancy at 300 m μ and radioactivity. The labeled component in tubes 8–22 was identified as 5-hydroxymethyluracil (λ_{\max} at 287 m μ at pH 13 and R_F 0.17 in 1-butanol–water (86:14)). The principal product in tubes 142–160, later identified as 5-thyminyltetrahydrofolate, was characterized by absorption maxima at 302 and 275 m μ ; the absorbancy ratio (302:275) was approximately 1.15. The contents of these tubes were pooled and lyophilized. Upon re-chromatography of this pooled fraction under the above conditions, a single symmetrical peak was observed at essentially the same position in the profile; the latter material also moved as a homogeneous quenching spot at R_F 0.55 on paper chromatograms using a 0.1 M phosphate buffer (pH 7) as the solvent system. Based upon an extinction coefficient of ϵ 28.5×10^3 M⁻¹ cm⁻¹ at pH 7.0, the yield of this product varied between 50 and 60% in different preparations.

Fractions 124–140 in Figure 1 showed absorbancy maxima at 300 m μ and a shoulder at 268 m μ ; the absorbancy ratio (300:268) varied between 1.0 and 1.1. From their spectra, these tubes appeared to contain a mixture of 10-thyminyltetrahydrofolate and 5-thyminyl-5,6-dihydrofolate, compounds whose characteristics are described below. Oxidation of the former to the dihydro compound could be largely prevented by increasing the concentration of mercaptoethanol to 0.1 M in the eluting buffers.

Large-Scale Synthesis of 5-Thyminyltetrahydrofolate (I). Tetrahydrofolate diacetate (3.6 g) was suspended in a mixture consisting of 60 ml of 0.5 M phosphate buffer (pH 7) and 120 ml of dioxane which had been cooled previously to 4° and deaerated with a continuous stream of nitrogen. The solid was dissolved by the addition of 5 M NaOH to adjust the pH to 8. Chloromethyluracil (3.6 g) was added, the pH was adjusted to 7.5, and the solution was lyophilized to dryness. The residue (12 g), a pale, cream-colored powder, was usually chromatographed immediately although it could be stored at –20° for several weeks.

The above material (12 g) was dissolved with stirring in 800 ml of cold water containing mercaptoethanol at a concentration of 0.05 M, the pH was adjusted to 7.0, and the solution was filtered. The clear yellow solution was passed through a 3.8 \times 42 cm column of DEAE-cellulose which had been washed previously and equilibrated with 0.05 M mercaptoethanol at 4°. The column was eluted with an ammonium acetate gradient (0.1–0.4 M), as described in the preceding section. The first 1300 ml of eluate was collected in bulk and discarded. The column was then transferred to an automatic fraction collector and 10-ml fractions were collected. Spectra were taken of every tenth tube after appropriate dilution with the eluting solvent.

10-Thyminyltetrahydrofolate, accompanied by a small amount of 5-thyminyltetrahydrofolate, was eluted in the first 30 tubes. These fractions were combined, lyophilized, and processed separately for recovery of the former material by rechromatography on DEAE-cellulose. The next 100–150 tubes were collected as the gradient elution was completed and followed thereafter by 2 l. of 0.4 M ammonium acetate, containing 0.05 M mercaptoethanol. The contents of these latter tubes showed the correct spectral characteristics for 5-thyminyltetrahydrofolate (i.e., λ_{\max} at 302 m μ with a shoulder at 275 m μ). After pooling, these fractions were lyophilized to yield crude 5-thyminyltetrahydrofolate as a white or slightly yellow fluffy powder (1.25 g).

Crystallization of 5-Thyminyltetrahydrofolate as the Barium Salt. For further purification, 2.4 g (combined product from two runs) of 5-thyminyltetrahydrofolate was dissolved in 50 ml of deaerated water and the pH was adjusted to 7.0 with 1 N NaOH. Mercaptoethanol was added to a concentration of 0.05 M and the solution was chromatographed on a 3.8×40 cm column of DEAE-cellulose, as described above. The eluate (1400 ml) containing the desired material (302:275 ratio between 1.2 and 1.3) was lyophilized to yield a white powder (1.95 g). This was dissolved in 30 ml of deaerated water containing 500 mg of NaCl and, after adjustment to pH 7.5 with 1 N NaOH, the solution was filtered. BaCl₂ (2 g) and mercaptoethanol (0.2 ml) were added to the filtrate, and the barium salt was precipitated by the addition of 100 ml of absolute ethanol. After standing overnight at 4°, the barium salt was filtered, washed, and dried under high vacuum to yield 1.9 g of product. The ultraviolet absorption spectrum showed a purity of 95%. An analytically pure sample was obtained by dissolving 1.5 g of the barium salt in 250 ml of deaerated water by warming. This solution was concentrated on a rotary evaporator under high vacuum until it became turbid and then kept overnight at 4°. The product was filtered, washed with four 5-ml portions each of cold water, methanol–water (1:1), and methanol, and dried overnight at 25° (0.94 g). This crystallization procedure was repeated to yield the barium salt of 5-thyminyltetrahydrofolate as microcrystalline rosettes. This material could be stored for several months in brown bottles at room temperature. *Anal.* Calcd for C₂₄H₂₅N₉O₈Ba·5H₂O: C, 36.25; H, 4.43; Ba, 17.27; N, 15.86. Found: C, 36.0; H, 4.3; Ba, 16.8; N, 16.5.

Crystallization of 5-Thyminyltetrahydrofolate as the Free Acid. 5-Thyminyltetrahydrofolate (1.5 g), obtained as described above, was dissolved in 150 ml of deaerated water and the solution was filtered. The filtrate was adjusted to pH 3.5 with 1 N acetic acid, and the solution was cooled slowly to 4°. After standing for several hours at this temperature, the precipitate was filtered and washed with two 5-ml portions of cold water and 3-ml portions each of cold water–methanol (1:1) and methanol. The cream-colored microcrystalline material was recrystallized as described above and dried quickly under high vacuum at 30° to yield 0.6

g of 5-thyminyltetrahydrofolate dihydrate. The dried material was stable under anhydrous conditions at room temperature; R_F 0.55 in 0.1 M phosphate buffer, pH 7.0; as shown in Figure 2, at pH 7.0, λ_{\max} at 302 m μ (ϵ 29.2×10^3 M⁻¹ cm⁻¹) with a shoulder at 275 m μ (ϵ 24.9×10^3 M⁻¹ cm⁻¹). *Anal.* Calcd for C₂₄H₂₇N₉O₈·2H₂O (sample dried at 30° to constant weight): C, 47.59; H, 5.15; N, 20.82. Found: C, 48.2; H, 5.3; N, 20.4.

10-Thyminylfolate (II). Folic acid (100 mg) was dissolved in 2 ml of 0.5 M Na₂CO₃ and the solution was diluted with 2 ml of dimethylformamide. The reaction mixture was cooled in ice bath to 5° and chloromethyluracil-2-¹⁴C (100 mg, sp act. 1.67×10^5 dpm/ μ mole) was added. The color of the solution changed instantaneously from orange-yellow to reddish-brown. After being stirred for 45 min, the reaction mixture was diluted with 10 ml of water and lyophilized to dryness. The lyophilized material was dissolved in 25 ml of water, the pH was adjusted to 6.9, and the solution was chromatographed on a 3×19 cm column of DEAE-cellulose at room temperature. After adding the solution to be chromatographed, the column was washed with 700–800 ml of water to remove hydroxymethyluracil, and then eluted with a gradient of ammonium acetate (0.1–0.6 M, pH 7). The column effluent (11.5 ml/tube) was monitored for absorbancy at 275 m μ . Fractions 88–112 contained most of the radioactive and ultraviolet-absorbing material; the contents of every alternate tube in this region was scanned spectrophotometrically after appropriate dilution with 0.1 M phosphate buffer (pH 7.0) and those having an absorption maximum at 275 m μ with a shoulder at 300 m μ were combined and lyophilized. The pale yellow powder was dissolved in 150 ml of water containing 1.0 g of NaCl, the pH was adjusted to 7.5 with 1 N NaOH, and the solution was filtered. The filtrate was adjusted to pH 2.9 with 1 N HCl and cooled overnight. 10-Thyminylfolic acid separated out as a yellow microcrystalline solid (45 mg). The product was recrystallized as described above, filtered with suction and, after drying under high vacuum at room temperature, weighed 40 mg. The material moved as a single quenching spot (R_F 0.45 in 0.1 M phosphate buffer, pH 7.0). The specific activity of the crystalline material (1.68×10^5 dpm/ μ mole) was the same as that of the starting chloromethyluracil.

Large-Scale Preparation of 10-Thyminylfolate. Folic acid (10 g) was dissolved with stirring in 200 ml of 0.5 M Na₂CO₃. Dimethylformamide (200 ml) was added and the solution was cooled to 3–5°. Solid chloromethyluracil (10.0 g) was added and the suspension was stirred vigorously for 45 min at 5°. The suspension was diluted with 750 ml of water, the pH was adjusted to 7.5, and the solution was lyophilized to dryness. The reddish-brown powder was dissolved in 1400 ml of water containing 5 g of NaCl and brought to pH 7.0 by the addition of 5 N NaOH. The solution was filtered, the pH was adjusted to 2.9, cooled overnight, and filtered. The filtrate was slightly reddish

colored whereas the crude product was light brown. The precipitate was washed exhaustively with cold water until the washings were almost clear, then with methanol-water (1:1) and finally with absolute methanol. The product was dried rapidly *in vacuo* over CaCl_2 . The absorption spectrum indicated that the yield was about 70% and the purity at this stage varied between 70 and 80%.

Further purification of the crude product was accomplished by chromatography on a DEAE-cellulose column by a slight modification of the procedure described above. Crude thyminylic acid (1 g) was suspended in 100 ml of water, dissolved by adjusting the pH to 7.0 with 5 N KOH, and chromatographed on a 3×40 cm column of DEAE-cellulose. The column was eluted first with a gradient of ammonium acetate (0.1–0.4 M, pH 7, 1 l. each) followed by 0.4–0.6 M, pH 7, 1 l. each. Three distinct bands were visible on the column. Most of the impurities were eluted by the first gradient which was collected in bulk and discarded. Fractions of 20 ml from the second gradient were collected automatically. After appropriate dilution with 0.1 M phosphate buffer (pH 7.0), every tenth fraction was scanned spectrophotometrically; those having the correct spectral characteristics were combined, lyophilized, and crystallized as described above (yield, 375 mg). A small sample of the product was recrystallized twice to yield 10-thyminylic acid as pale yellow, microcrystalline flakes; mp 260–290°; at pH 7.0, λ_{max} at 277 m μ (ϵ $27.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) with a shoulder at 300 m μ (ϵ $24.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). *Anal.* Calcd for $\text{C}_{24}\text{H}_{23}\text{N}_9\text{O}_8 \cdot \text{H}_2\text{O}$ (sample dried at 120° for 6 hr): C, 49.49; H, 4.31; N, 21.59; H_2O , 3.08. Found: C, 49.1; H, 4.52; N, 21.0; H_2O , 2.98. Calcd for $\text{C}_{24}\text{H}_{23}\text{N}_9\text{O}_8 \cdot 2\text{H}_2\text{O}$ (sample dried at 30° to constant weight): C, 47.91; H, 4.52; N, 20.96; H_2O , 6.16. Found: C, 48.0; H, 4.58; N, 20.8; H_2O , 6.67. The product was stored in brown bottles under anhydrous conditions for several months without any decomposition, but in solution it deteriorated rapidly below pH 5.0 and above pH 10.0.

Conversion of 10-Thyminylic Acid to Folic Acid. 10-Thyminylic acid (50 mg) was dissolved in 60 ml of water, and, after adjustment of the pH to 7.0, the solution was heated at 100° under nitrogen for 30 min. The solution was then cooled, diluted with 6 ml of water, adjusted to pH 3.0, refrigerated overnight, and filtered. The product, after drying over CaCl_2 , was a pale yellow powder (40 mg) which moved as a single quenching spot with R_F 0.19 in 0.1 M phosphate buffer (pH 7.0) and had an absorption spectrum identical with that of folic acid. From the above supernatant solution, 5-hydroxymethyluracil was isolated and identified by its absorption spectrum (at pH 13 λ_{max} 287 m μ) and by paper chromatography using the solvent systems described earlier for pyrimidine compounds.

Attempted Reaction of 10-Thyminylic Acid with Formic Acid. 10-Thyminylic acid (25 mg) was added to 2 ml of formic acid (97–98%) and kept at 50° for 60 min. Formic acid was removed under

reduced pressure, the residue was dissolved in 75 ml of water by the addition of a few drops of NaOH, and the solution was filtered and adjusted to pH 2.9. After standing overnight at 4°, the product was filtered and dried (20 mg). The absorption spectrum and R_F values on paper chromatograms were identical with those of the starting material.

10-Thyminylic-5,6,7,8-tetrahydrofolic acid (III). 10-Thyminylic acid monohydrate (51.4 μmoles , 32 mg) was hydrogenated in 6.0 ml of glacial acetic acid containing 60 mg of prerduced platinum oxide in a microhydrogenation unit (Ogg and Cooper, 1949) at room temperature. The volume of hydrogen absorbed was 2.82 ml at standard, which corresponds to 2 moles/mole of starting material. The catalyst was removed by filtration and the solution was lyophilized. 10-Thyminylic-5,6,7,8-tetrahydrofolic acid was obtained as a white fluffy powder (30 mg); R_F 0.60 (quenching spot) in 0.1 M phosphate buffer, pH 7.0; at pH 7.0, λ_{max} 300 m μ with a shoulder at 262 m μ . Because of its lability to oxygen, the solid product was stored in vacuum-sealed ampoules, or solutions containing mercaptoethanol at a concentration of 0.1 M were stored frozen. After oxidation of this material by H_2O_2 -peroxidase (see below), the absorption spectrum of the product showed an absorption maximum at 282 m μ with a shoulder at 300 m μ ; this is identical with the spectrum of 10-thyminylic-7,8-dihydrofolic acid.

^{14}C -Labeled 10-Thyminylic-5,6,7,8-tetrahydrofolic acid. When 10-thyminylic- ^{14}C -folic acid was used in the above procedure, 10-thyminylic- ^{14}C -5,6,7,8-tetrahydrofolic acid was obtained. Its absorption spectra, R_F value on paper chromatograms and position on the DEAE-cellulose column were identical with those of the unlabeled material as described above. The specific activity of the product ($1.67 \times 10^5 \text{ dpm}/\mu\text{mole}$) was the same as that of the precursor.

5,10-Dithyminylic-5,6,7,8-tetrahydrofolic acid (IV). 10-Thyminylic-tetrahydrofolic acid diacetate (500 mg, 710 μmoles) or 450 mg of 5-thyminylic-tetrahydrofolic acid trihydrate (720 μmoles) was suspended in a mixture consisting of 25 ml of 0.5 M deaerated phosphate buffer (pH 7.0) and 50 ml of purified dioxane that had been cooled to 4°. Chloromethyluracil (500 mg) was added and the suspension was maintained at this temperature for 10 min. The reaction mixture was then diluted with 125 ml of water and, after addition of mercaptoethanol to 0.05 M, was lyophilized. The light yellow powder was dissolved in 400 ml of water containing mercaptoethanol (0.05 M), the pH was adjusted to 7.0, and the solution was passed through a 3.8×40 cm column of DEAE-cellulose that had been washed previously and equilibrated with 2 l. of 0.05 M ammonium acetate containing 0.05 M mercaptoethanol. The column was eluted with a gradient of ammonium acetate (0.1–0.4 M, 1 l. each) containing 0.05 M mercaptoethanol. After 1500 ml of the effluent had been discarded, 15-ml fractions were collected automatically. Unreacted 10-thyminylic-tetrahydrofolic acid or 5-thyminylic-tetrahydrofolic acid was found in fractions 30–45 or 50–90, respectively. As described previously, this elution pattern was

completed with 2 l. of 0.4 M ammonium acetate. The desired product, 5,10-dithyminyltetrahydrofolate, was located in fractions 115–165. The contents of these latter tubes were characterized by absorption maxima at 302 and 268 $m\mu$ (absorbancy ratio 268:302 greater than 1.16). These fractions were combined and lyophilized. The white powder (350 mg) was dissolved in 50 ml of water containing NaCl (150 mg), adjusted to pH 7.0, and filtered. $BaCl_2$ (1 ml of 3%) was added and the barium salt was obtained as a white flocculent precipitate by the addition of 160 ml of absolute ethanol. After cooling overnight, the barium salt was filtered, washed with cold ethanol (50 ml), and dried (265 mg). The barium salt was twice recrystallized by warming in deaerated water and diluting slowly with ethanol to produce very small crystals, largely in the form of rosettes; R_F 0.43 in 0.1 M phosphate buffer, pH 7.0; at pH 7.0, λ_{max} 270 $m\mu$ (ϵ $37.5 \times 10^3 M^{-1} cm^{-1}$) and 303 $m\mu$ (ϵ $30.8 \times 10^3 M^{-1} cm^{-1}$). *Anal.* Calcd for $C_{29}H_{29}N_{11}O_{10} \cdot Ba \cdot 3.5H_2O$: C, 39.04; H, 4.06; Ba, 15.39; N, 17.27. Found: C, 39.1; H, 4.07; Ba, 14.9; N, 17.1.

¹⁴C-Labeled 5-Formyl-10-thyminy-5,6,7,8-tetrahydrofolate. 5-Formyltetrahydrofolic acid (35.3 mg, 75 μ moles) was treated with labeled chloromethyluracil (48.2 mg, 300 μ moles, sp act. 1.28×10^5 dpm/ μ mole) under identical conditions as those described for the preparation of 5-thyminyltetrahydrofolate. The reaction mixture was chromatographed at room temperature on a 3×17 cm column of DEAE-cellulose and eluted with a gradient of ammonium acetate (0.1–0.4 M, 1 l. each). Fractions of 7 ml were collected and monitored for absorbancy at 290 $m\mu$. The principal ultraviolet-absorbing and radioactive peak was located in tubes 120–160. At pH 7.0 the material in these tubes was characterized by an absorption maximum at 290 $m\mu$. The main product in tubes 120–140 was characterized by absorption maxima at 292 $m\mu$ at pH 7.0. The contents of these tubes were pooled and lyophilized. The specific activity (0.75×10^5 dpm/ μ mole) of the crystalline material was the same as that of the starting chloromethyluracil. The product moved as a single quenching spot (R_F 0.53 in 0.1 M phosphate buffer, pH 7.0). Upon rechromatography, the product eluted as a single, symmetrical peak at essentially the same position in the profile; at pH 7.0, λ_{max} 290 $m\mu$ (ϵ $26.8 \times 10^3 M^{-1} cm^{-1}$); at pH 13, λ_{max} 286 with a shoulder at 305 $m\mu$ (ϵ 28.9×10^3 and $24.7 \times 10^3 M^{-1} cm^{-1}$); and at pH 1, λ_{max} 289 $m\mu$ (ϵ $26.1 \times 10^3 M^{-1} cm^{-1}$). The material was stable to oxidation and could be stored without decomposition under anhydrous conditions in brown bottles. When 5 mg of this material was dissolved in 10 ml of water at pH 7.0 and a 0.5-ml aliquot of this solution was treated with 2.5 ml of 1 N HCl for 1 hr, there was no increase in absorbancy at 355 $m\mu$.

If the molal ratio of chloromethyluracil to folinic acid in the original reaction was increased to ten, another product, in addition to 5-formyl-10-thyminyltetrahydrofolate was also formed in an over-all yield of 10–15%. Most of this second product was located in tubes 145–155 in the elution profile and was characterized in 0.1 M phosphate buffer (pH 7.0) by absorption maxima at

267 and 296 $m\mu$. Upon rechromatography this product was isolated as a single peak at essentially the same position. The rechromatographed material was pooled and lyophilized. The ultraviolet absorption spectra of this material at pH 1, 7, and 13 showed maxima at 265 and 300, 267 and 296, and 292 $m\mu$, respectively. The specific activity (1.53×10^5 dpm/ μ mole, assuming ϵ $27 \times 10^3 M^{-1} cm^{-1}$ at 296 $m\mu$) was double that of the chloromethyluracil used. The amount of label indicates that this compound is a dithyminy-5-formyltetrahydrofolate. This material was not characterized further.

5-Thyminy-5,6-dihydrofolate (VI). Crystalline 5-thyminyltetrahydrofolate trihydrate (140 mg, 100 μ moles) was suspended in 30 ml of deaerated water, and the pH was adjusted to 8.0 by the addition of 1 N NaOH. A freshly prepared solution of H_2O_2 (2.5 ml) (8%) and peroxidase (25 mg) was added. After standing in the dark for 10 min, the reaction mixture was applied to a 3×40 cm column of DEAE-cellulose at 4°. The column was eluted with an ammonium acetate gradient (0.1–0.4 M, pH 7, 1 l. each). After 750 ml of eluting buffer had passed through the column and was discarded, the column was transferred to an automatic fraction collector. Fractions of 10 ml were collected thereafter and every alternate tube was monitored for absorbancy at 265 $m\mu$. Following the elution of three minor components, the last of which was intensely yellow, most of the desired product was collected in tubes 40–80. Later fractions appeared to be contaminated with small amounts of unreacted 5-thyminyltetrahydrofolate and were discarded. The contents of tubes 40–80 were pooled and lyophilized. The light yellow, fluffy powder (200 mg) was dissolved in 30 ml of water containing 100 mg of NaCl, adjusted to pH 7.0 with 1 N NaOH, and filtered. $BaCl_2$ (200 mg) was added to the filtrate and the barium salt was precipitated by the addition of 100 ml of absolute ethanol. After cooling, filtering, washing with ethanol, and drying at 25°, 175 mg of a nearly white product was obtained. The above purification was repeated to yield a barium salt which was crystallized from water-methanol as microcrystalline platelets; R_F 0.74 in 0.1 M phosphate buffer, pH 7.0; at pH 7.0, λ_{max} at 268 $m\mu$ (ϵ $30.7 \times 10^3 M^{-1} cm^{-1}$) and at 300 $m\mu$ (ϵ $25.3 \times 10^3 M^{-1} cm^{-1}$); at pH 13.0, λ_{max} 294 $m\mu$ (ϵ $36.2 \times 10^3 M^{-1} cm^{-1}$); and at pH 1.0, λ_{max} 266 $m\mu$ (ϵ $30.0 \times 10^3 M^{-1} cm^{-1}$) and at 300 $m\mu$ (ϵ $24.1 \times 10^3 M^{-1} cm^{-1}$). *Anal.* Calcd for $C_{24}H_{23}N_9O_8Ba \cdot 4 H_2O$: C, 37.19; H, 4.03; Ba, 17.73; N, 16.25. Found: C, 37.6; H, 3.83; Ba, 17.2; N, 16.5.

The barium salt of 5-thyminy-5,6-dihydrofolate was quite stable and could be stored without decomposition for several months at room temperature under anhydrous conditions. The material was not reduced by mercaptoethanol but upon treatment with excess KBH_4 at 37° for 5 min yielded 5-thyminyltetrahydrofolate.

10-Thyminy-7,8-dihydrofolate (V). A solution containing 120 mg of 10-thyminyfolate in 5 ml of 0.2 M NaOH was added to a solution of sodium ascorbate (2.5 g in 25 ml of water) at 37°. After 10 min, sodium hydrosulfite (1.2 g) was added to the reaction mixture

which was then stirred for 7 min. The solution was cooled rapidly to 5°, 100 mg of NaCl was added, and the pH of the solution was slowly brought to 2.8 by the addition of cold 1 N HCl over a period of 20–30 min. The precipitate was recovered by centrifugation and washed four times with 10-ml portions of 10^{-3} M HCl, followed by two washings with 20-ml portions of methanol–water (1:1), and finally with 20 ml of absolute methanol. The product, 10-thyminy-7,8-dihydrofolate, was obtained as light yellow powder (70 mg). At pH 7.0, λ_{\max} 282 m μ with a shoulder at 300 m μ ; the absorbancy ratio (282:300) of 1.05–1.10 indicated a purity of 85–95% for various preparations. A compound having the same spectral properties was also obtained by treating 10-thyminytetrahydrofolate with H_2O_2 –peroxidase according to the procedure described in the preceding section.

The above product was dissolved in 25 ml of water, using a few drops of NaOH to adjust the pH to 7, and chromatographed on a 3×18 cm column of DEAE-cellulose. The column was washed with 200 ml of water and eluted with a gradient of ammonium acetate (0.1–0.6 M, pH 7, 1 l. each). The column effluent was monitored for absorbancy at 282 m μ . 10-Thyminy-7,8-dihydrofolate, eluted in fractions 132–155, was characterized by an absorption maximum at 282 m μ with a shoulder at 300 m μ . These fractions were pooled and lyophilized (34 mg). During lyophilization, the flask was covered with aluminum foil to avoid photochemical decomposition. The products from three such columns (80 mg) were dissolved in 60 ml of water, using 1 N NaOH to adjust the pH to 7.0, filtered, cooled, and the pH was adjusted very slowly to 2.9 as described above. A small sample was twice recrystallized in this manner to yield the product as a microcrystalline yellow powder which was filtered and dried quickly under high vacuum over P_2O_5 ; R_F 0.20 (fluorescent spot) in 0.1 M phosphate buffer, pH 7.0; at pH 7.0, λ_{\max} 282 m μ (ϵ 35.3×10^3 M $^{-1}$ cm $^{-1}$) with a shoulder at 300 m μ (ϵ 30×10^3 M $^{-1}$ cm $^{-1}$). *Anal.* Calcd for $\text{C}_{24}\text{H}_{25}\text{N}_5\text{O}_8 \cdot 1.5\text{H}_2\text{O}$: C, 48.48; H, 4.74; N, 21.20. Found: C, 48.8; H, 4.83; N, 19.8. The solid product was quite stable and could be stored

without decomposition at -20° for several months. In solution, it deteriorated slowly at 4° but quite rapidly at 30° . In the latter instance, the absorption maximum shifted to longer wavelengths (at pH 13, λ_{\max} 300 m μ); this decomposition product appeared to be identical with 10-thyminyfolate.

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