Production of Formaldehyde from \(N^5\)-Methyltetrahydrofolate by Normal and Leukemic Leukocytes

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SUMMARY

Extracts of human normal and leukemic leukocytes contain an enzyme that catalyzes a transfer of labeled methyl carbon from \(N^5\)-[\(^{14}\)C]methyltetrahydrofolate to tryptamine. Evidence is presented that this reaction is not attributable to a methyltransferase but to the following reaction sequence: (a) an oxidation of \(N^5\)-[\(^{14}\)C]methyltetrahydrofolate to \(N^5\).\(N^10\)-[\(^{14}\)C]methylenetetrahydrofolate that is catalyzed by \(N^5\).\(N^10\)-methylenetetrahydrofolate reductase (EC 1.1.1.68); (b) spontaneous release of [\(^{14}\)C]formaldehyde from \(N^5\).\(N^10\)-[\(^{14}\)C]methylenetetrahydrofolate; and (c) nonenzymatic condensation of [\(^{14}\)C]formaldehyde with tryptamine to form a radioactive carbohylne derivative. The occurrence of this sequence in leukocytes is suggested by data that show that the enzyme reaction is strongly stimulated by addition of flavin adenine dinucleotide and that the final product is chromatographically identical to the adduct formed in the reaction of \([^{14}\)C]formaldehyde with tryptamine. In the absence of tryptamine, a product accumulates that can react with other HCHO acceptors, i.e., \(\beta\)-phenylethylamine and dimedone; another reaction product is tetrahydrofolate.

Production of formaldehyde is relatively more active in normal lymphocytes than in normal granulocytes, but it is even higher in lymphocytes of chronic lymphocytic leukemia. Activity in granulocytes from a subject with chronic myelocytic leukemia is also elevated but to a lesser extent than activity in lymphocytes of chronic lymphocytic leukemia. Formaldehyde production in leukocytes is only slightly active in normal lymphocytes than in normal granulocytes, but it is even higher in lymphocytes of chronic lymphocytic leukemia. Activity in granulocytes from a subject with chronic myelocytic leukemia is also elevated but to a lesser extent than activity in lymphocytes of chronic lymphocytic leukemia. We conclude that the system is cobalamin independent. Thus, there exists an active pathway from \(N^5\)-methyltetrahydrofolate to tetrahydrofolate other than the one catalyzed by cobalamin-dependent \(N^5\)-methyltetrahydrofolate-homocysteine methyltransferase.

INTRODUCTION

Transfer of the methyl carbon from \(CH_3\)-H\(_4\)folate to aromatic amines has been demonstrated in a variety of mammalian tissues (3, 14, 22, 25, 29, 46). It was first considered that the methyl group was transferred intact in analogy with the methyl transfer known to take place from AdoMet to various amines (2). This early conclusion rested on the assumption that radioactive reaction products were \(N\)-methyl derivatives of the amine substrates (14, 22). However, later studies identified the products as the corresponding carboxylines formed by spontaneous condensation of amines with HCHO (4, 17, 28, 29, 36, 42, 46). These data suggested that transfer of the methyl carbon from \(CH_3\)\(_4\)folate to an aromatic amine occurs in 2 steps: an enzymatic conversion of \(CH_3\)-H\(_4\)folate to HCHO, followed by a nonenzymatic reaction of HCHO with amine.

It was then shown that, in assays for \(N^4\).\(N^10\)-methylene-H\(_4\)folate reductase (EC 1.1.1.68) by the method of Kutzbach and Stokstad (20), tissue extracts catalyze a conversion of \(CH_3\)-H\(_4\)folate to HCHO; i.e., \(CH_3\)-H\(_4\)folate is oxidized in the presence of an electron acceptor to \(N^4\).\(N^10\)-methylene-H\(_4\)folate, which then dissociates to HCHO and H\(_4\)folate (9, 10, 20). This observation is of interest because transfer of the methyl carbon from \(CH_3\)-H\(_4\)folate to amines is stimulated by electron acceptors such as FAD or menadione (15, 31, 44).

We have been prompted to study this reaction of \(CH_3\)-H\(_4\)folate in human hematologic tissues because of the particular importance of tissue folate metabolism and the controversies that have surrounded the distinctive cobalamin-dependent reaction, in which the methyl group of \(CH_3\)-H\(_4\)folate is transferred to homocysteine (28, 39). The purposes of this study are: (a) to determine whether a transfer of the methyl carbon of \(CH_3\)-H\(_4\)folate to tryptamine occurs in human leukocytes; (b) to characterize the nature of this reaction; (c) to compare levels of the reaction in leukocytes from normal individuals and subjects with leukemia and other disorders; and (d) to determine to what extent a cobalamin-independent conversion of \(CH_3\)-H\(_4\)folate to H\(_4\)folate can occur in these cells.

MATERIALS AND METHODS

Reagents. \([^{14}\)C]CH\(_3\)-H\(_4\)folate (54 \(\mu\)Ci/\(\mu\)mole), \([2-{^{14}\)C}]folic acid (58 \(\mu\)Ci/\(\mu\)mole), and \([^{14}\)C]formaldehyde (2 \(\mu\)Ci/\(\mu\)mole) were obtained from Amersham/Searle Corp, Arlington Heights, Ill. \([^{14}\)C]CH\(_3\)-H\(_4\)folate was stored under N\(_2\) at 20° as a 4.5 mM solution in 1 M mercaptoethanol at pH 7. 3°.5°-[\(^{14}\)H]folic acid (22 \(\mu\)Ci/\(\mu\)mole) was purchased from Schwarz/Mann, Orangeburg, N. Y.; \([^{14}\)C]formyl-H\(_4\)folate, calcium leucovorin, was obtained from Lederle Laboratories, Pearl River, N. Y.; tryptamine hydrochloride, \(N\)-methyltryptamine,
β-phenylethylamine, AdoMet, FAD, and folic acid were produced by Sigma Chemical Co., St. Louis, Mo. FMN, NADH, and NADPH were obtained from Calbiochem, La Jolla, Calif.; NAD and NADP were from Boehringer/Mannheim, Mannheim, West Germany; cyanocobalamin was from Schwarz/Mann. Silica gel and cellulose thin-layer chromatogram sheets were purchased from Eastman Kodak Co., Rochester, N. Y.

Methylcobalamin and propylcobalamin were synthesized by reduction of cyanocobalamin with sodium borohydride, followed by alkylation with methyl iodide and propyl bromide, respectively (32). Dihydrofolate and H4folate were prepared by reduction of folic acid with sodium hydrosulfite (11) or sodium borohydride (40), respectively. N4,N10-methenyl-H4folate was prepared by boiling H4folate in 80% formic acid:0.1 m mercaptoethanol for 5 min (37) or by treatment of N4-formyl-H4folate with HCl (33). N4,N10-methenyl-[3H]H4folate, prepared by reduction of [3H]folic acid with sodium borohydride (40) and boiling with formic acid (37), was purified by chromatography on cellulose thin-layer sheets in 1 n formic acid:0.2 m mercaptoethanol, followed by elution with 0.1 m mercaptoethanol. Unlabeled N4,N10-methenyl-H4folate (which gives a fluorescent spot at Rf 0.24) was used as reference standard. N4,N10-[14C]methylene-H4folate was prepared from [14C]CH3-H4folate and H4folate (18). CH3-[14C]H4folate was synthesized by reduction of [2-14C]folic acid with sodium borohydride and methylation with unlabeled HCHO in the presence of borohydride (6); samples were purified by chromatography on cellulose thin-layer sheets in 0.1 m potassium phosphate:0.2 m mercaptoethanol, eluted with 0.2 m mercaptoethanol, and evaporated to a smaller volume under N2. [14C]CH3-H4folate was used as reference standard.

Preparation of Lymphocytes and Granulocytes. Leukocytes were obtained from venous blood (10 to 60 ml) of ostensibly normal subjects and patients with the following various disorders: CLL, CML, unexplained monocytosis, and severe cobalamin deficiency (mean corpuscular volume, 117 cu μm; serum cobalamin, 20 μg/ml). Some of the leukemic subjects were receiving prednisone and cyclophosphamide (CLL) or busulfan (CML); none was receiving folic acid.

After sedimentation of erythrocytes for 1 hr at 37° following the addition of 0.1 volume of 6% dextran (41), platelet-free leukocytes were isolated by centrifugation of the supernatant fraction at 140 × g for 10 min. Lymphocytes and granulocytes were purified from normal blood by fractionation of defibrinated blood on a Ficoll-Hypaque gradient (1, 12). Residual erythrocytes were removed from leukocyte preparations by hypotonic hemolysis (7, 32). Cells were washed 3 times in 0.85% NaCl solution:10 mM phosphate (pH 7.4), sonically disrupted in 20 mM potassium phosphate (pH 7.0) (32), and centrifuged at 27,000 × g for 30 min. The supernatant fraction was used in enzyme assays. Protein was assayed by the method of Lowry et al. (27) with bovine serum albumin as a standard.

Bone marrow obtained by aspiration was diluted in Ringer’s solution containing 0.2% sodium lactate and heparin (1 unit/ml). Cells were washed in 0.85% NaCl solution:10 mM phosphate, pH 7.4; residual erythrocytes were removed by hypotonic hemolysis (7). Remaining cells were sonically disrupted and centrifuged as described for blood leukocytes.

Assay of Formaldehyde Production from CH3-H4folate. Cell extracts were incubated with [14C]CH3-H4folate in the presence of a saturating concentration of tryptamine. During this incubation, enzymatically produced HCHO was converted to the corresponding carboline, which was extracted in toluene:isoamyl alcohol (pH 10) (14). Standard incubation mixtures contained (in a total volume of 0.25 ml): [14C]CH3-H4folate, 9 nmoles; tryptamine, 5 μmoles; FAD, 0.5 μmole; potassium phosphate (pH 6.5), 25 μmoles; and cell extract, 0 to 0.5 mg protein. After incubation for 30 min at 37° in the dark, 0.5 ml of 0.5 M sodium borate (pH 10) was added; tryptoline was extracted with 3.5 ml of toluene:isoamyl alcohol (97:3) (“toluene extract” and “toluene extractible” are used in subsequent discussion to refer to this extraction). After centrifugation for 5 min at 400 × g, aliquots of the toluene extract were evaporated to dryness and taken up in 1 ml isoamyl alcohol. Radioactivity was assayed in Aquasol liquid scintillation fluid in a Beckman LS-150 scintillation counter. Incubations lacking enzyme were used as controls. Enzyme activity was expressed as nmoles HCHO formed per hr per mg protein.

Chromatographic Characterization of Reaction Products. Chromatographic methods differed depending on whether substrates were [14C]CH3-H4folate or CH3-[2-14C]H4folate. When the substrate was [14C]CH3-H4folate in standard incubation mixtures, aliquots of toluene extracts were applied to a silica gel thin-layer chromatography sheet and developed in acetone:1 n ammonium hydroxide (10:3) (16). In a separate reference mixture, 0.05 ml of a 40 μM solution of [14C]formaldehyde was mixed with 0.2 ml of 125 mM tryptamine and 0.5 ml of 0.5 M sodium borate (pH 10) and extracted with 3.5 ml of toluene:isoamyl alcohol (97:3). Aliquots of this toluene extract were chromatographed on the same sheet. A reference sample of nonradioactive N-methyltryptamine was also developed on the same sheet. Sheets were dried, examined under UV, cut into portions, and assayed for radioactivity in Aquasol liquid scintillation fluid.

However, when the substrate was CH3-[2-14C]H4folate, before the addition of borate mixtures were boiled in 80% formic acid:0.1 M mercaptoethanol for 5 min. Aliquots were mixed with 24 nmoles of N4,N10-methenyl-[3H]H4folate, applied to a cellulose thin-layer sheet, and chromatographed in 1 mM formic acid:0.2 M mercaptoethanol. The sheet was dried, examined under UV, cut into portions, and eluted with water. Eluates were centrifuged at 400 × g for 5 min; supernatant fractions were assayed for radioactivity with appropriate corrections for quenching of radioactivity by water. The absorption spectra of eluates were determined with a Carey Model 15 recording spectrophotometer.

RESULTS

Identification of Reaction Products. Extracts of various types of leukocytes were found to catalyze formation of a radioactive product from [14C]CH3-H4folate and tryptamine, according to the procedure originally proposed as an assay for aromatic alkylamine N-methyltransferase (14). Before
attempting quantitative comparisons of various cell types, we undertook to document the nature of the observed reaction with extracts of CLL cells as a convenient enzyme source. The following experiments were intended to show that such extracts catalyze the conversion of [14C]CH3-H4folate to [14C]HCHO, which then condenses nonenzymatically with tryptamine to form a toluene-extractable adduct, [14C]tryptoline.

It appeared that, if the reaction product were in fact [14C]HCHO, then omission of tryptamine from the incubation mixture would lead to accumulation of a product that would react with subsequently added tryptamine or with the HCHO acceptor, dimedone (24). Results of such an experiment, summarized in Table 1, show that a CLL cell extract did catalyze formation of a product that could react with tryptamine or dimedone. Dimedone was in fact a more efficient HCHO acceptor.

For determination of whether tryptoline formation is due to spontaneous condensation of the enzymatic reaction product with tryptamine, we chromatographed toluene extracts on silica gel thin-layer sheets to separate tryptoline from N-methyltryptamine, the product to be expected of a methyl transfer reaction (16). A radioactive peak was observed at the same position (Rf 0.92) as that of the product obtained when a mixture of [14C]HCHO and tryptamine was extracted with toluene in the presence of borate (pH 10) and similarly chromatographed. A simultaneously chromatographed reference sample of N-methyltryptamine had a mobility of Rf 0.83. Thus, the radioactive product appeared to result from a condensation of [14C]HCHO with tryptamine rather than from a methyl transfer reaction.

The reaction was shown to be dependent on tryptamine; activity was decreased by more than 90% in the absence of tryptamine. Similarly, production of [14C]tryptoline from [14C]HCHO incubated with tryptamine (in the absence of enzyme) was a function of tryptamine concentration, reaching a plateau at 20 to 30 mM at which 75% of the [14C]HCHO had reacted (Chart 1). For investigation of the acceptor specificity of the reaction of [14C]HCHO with tryptamine, [14C]HCHO was incubated with other potential acceptors. With β-phenylethylamine a toluene-extractable product was obtained, presumably methylene-β-phenylethylamine (17), the formation of which was also influenced by acceptor concentration; however, the saturating concentration was above 60 mM at which solubility became limiting. A reaction of [14C]HCHO with H4folate was suggested by a decrease in the radioactivity available to react with tryptamine. Formation of this product, presumably N9,N10-methenyl-H4folate, increased with the concentration of added H4folate; the concentration curve resembled that obtained when the acceptor was tryptamine (Chart 1).

For investigation of the fate of the folic acid moiety after release of the 1-carbon moiety, CLL cell extract was incubated in a mixture containing CH3-H4folate labeled in the pteridine ring (CH3-[2-14C]H4folate) as substrate. For protection of reduced folates from oxidation, incubations were conducted under N2. For determination of whether [14C]-H4folate had been released from CH3-[14C]H4folate, the incubation mixture was boiled in 80% formic acid, which converts H4folate to N9,N10-methenyl-H4folate (37). When chromatographed on cellulose thin-layer sheets in 1 N formic acid:0.2 M mercaptoethanol, reference preparations of N9,N10-methenyl-H4folate, which were synthesized from formic acid and H4folate or from N9-formyl-H4folate, gave a fluorescent spot at Rf 0.24, with the characteristic absorption maximum at 350 mm (37) when eluted with 0.1 M HCl. CH3-[14C]H4folate boiled in formic acid formed a radioactive peak at Rf 0.78. Other folate derivatives treated in this way did not migrate to Rf 0.24. N9,N10-[14C]Methylene-H4folate gave a radioactive peak at Rf 0.87; folic acid and dihydrofolate did not migrate to Rf 0.78.

Table 1
Dem  

Postincubation additions

<table>
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<tr>
<th>Duration of incubation (min)</th>
<th>Incubation mixture</th>
<th>Sodium borate</th>
<th>Sodium borate and tryptamine</th>
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<td>15 Complete</td>
<td>654</td>
<td>21,826</td>
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<tr>
<td>30 Complete</td>
<td>1,074</td>
<td>41,730</td>
<td>57,453</td>
<td></td>
</tr>
</tbody>
</table>

Chart 1. Reaction of various acceptors with [14C]HCHO. A sample of [14C]HCHO (20 nmoles) was incubated with 25 μmoles potassium phosphate buffer (pH 6.5) in a volume of 0.25 ml at 37°. Varying amounts (1.2 to 19 μmoles) of tryptamine (17) or β-phenylethylamine (17) were added. After incubation for 30 min, 0.5 ml of 0.5 M sodium borate (pH 10) and 3.5 ml of toluene:isoamyl alcohol (97:3) were added; extracting and assaying for radioactivity as described in "Materials and Methods"; or (b) adding 0.2 M mercaptoethanol, reference preparations of N9,N10-methenyl-H4folate, which were synthesized from formic acid and H4folate or from N9-formyl-H4folate, gave a fluorescent spot at Rf 0.24, with the characteristic absorption maximum at 350 mm (37) when eluted with 0.1 M HCl. CH3-[14C]H4folate boiled in formic acid formed a radioactive peak at Rf 0.78. Other folate derivatives treated in this way did not migrate to Rf 0.24. N9,N10-[14C]Methylene-H4folate gave a radioactive peak at Rf 0.87; folic acid and dihydrofolate did not migrate to Rf 0.78.
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The '4C radioactivity formed a peak corresponding to unreacted CH₃-[14C]H₄folate with a trace at Rₚ 0.24. After incubation with the cell extract, 14C radioactivity increased at Rₚ 0.24. In all cases no radioactivity was found at the origin.

These results show that the appearance of methenyl-[14C]H₄folate was due to an enzyme-dependent formation of H₄folate from CH₃-H₄folate. The initially added 14C radioactivity was recovered quantitatively (>95% of it as CH₃-[14C]H₄folate). However, recovery of methenyl-[3H]H₄folate at Rₚ 0.24 was only partial and amounted to only 43% of the amount added initially. This reflects total loss of radioactivity plus some degradation to a faster-moving compound.

After correction for incomplete recovery of methenyl-[3H]H₄folate, it is possible to estimate that about 1 n mole of H₄folate had been produced from CH₃-H₄folate for 2.6 n moles of [14C]HCHO produced from [14C]CH₃-H₄folate. It was also observed that, after [3H]H₄folate had reacted with formic acid, only about one-half of the radioactivity was recovered before chromatography. This correction brings the observed molar ratio closer to 1:1.

Requirements and Kinetic Properties of Enzyme Reaction

The foregoing experiments showed that [14C]CH₃-H₄folate is converted to [14C]HCHO and H₄folate. Such a reaction would require an oxidative step; therefore, we investigated the effects of various electron acceptors and other cofactors on the production of [14C]HCHO from [14C]CH₃-H₄folate by leukocyte preparations. The results, summarized in Table 2, show that added FAD increased activity 6-fold. Added FMN, menadione, NADP, methylcobalamin, and propylcobalamin were stimulatory only to a minor extent. Cyanobalamin had no significant effect. In the presence of methylcobalamin or menadione, the stimulatory effect of FAD was lessened considerably. NADPH and metabisulfite were inhibitory; NAD, NADH, AdoMet, anaerobiosis, and addition of a boiled extract of mixed normal leukocytes were without significant effects.

Dependence of the enzyme reaction on an electron acceptor such as FAD suggests that in leukocytes [14C]HCHO

![Chart 2](chart2.png)

**Effect of added cofactors on enzyme activity**

Enzyme activity was assayed in a CLL extract that had been dialyzed for 6 hr (with 2 changes) against 100 volumes of 20 mM potassium phosphate, pH 7. Activity is expressed as a percentage of activity observed in a control incubation lacking added cofactors. When a sequence of figures is reported, each figure represents relative activity at the concentration denoted by the corresponding figure in the left column. Actual activity in the control was 0.43 n mole HCHO per hr per mg protein.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentrations (mM)</th>
<th>Relative activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None, control</td>
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<tr>
<td>Cyanocobalamin</td>
<td>0.1, 0.4</td>
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<tr>
<td>Methylcobalamin</td>
<td>0.1, 0.4</td>
<td>221, 185</td>
</tr>
<tr>
<td>Propylcobalamin</td>
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<td>154, 182</td>
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<tr>
<td>NAD</td>
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<td>122</td>
</tr>
<tr>
<td>NADH</td>
<td>0.2</td>
<td>123</td>
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<tr>
<td>NADP</td>
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<td>132</td>
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<td>NADPH</td>
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</tr>
<tr>
<td>FMN</td>
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<tr>
<td>AdoMet</td>
<td>0.05, 0.2</td>
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</tr>
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<td>Potassium metabisulfite</td>
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</tr>
<tr>
<td>EDTA</td>
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<td>100</td>
</tr>
<tr>
<td>Menadione</td>
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<tr>
<td>Potassium metabisulfite</td>
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<tr>
<td>Plus EDTA</td>
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<tr>
<td>Methylcobalamin</td>
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<tr>
<td>Plus FAD</td>
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<tr>
<td>Menadione</td>
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<td>173</td>
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<tr>
<td>Plus FAD</td>
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<td></td>
</tr>
<tr>
<td>Boiled extract of normal granulocytes</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>Anaerobiosis</td>
<td>101</td>
<td></td>
</tr>
</tbody>
</table>

* Boiled 27,000 × g supernatant fraction (0.3 mg).
* Incubated under N₂.
production from \([^{14}C]CH_3-H_4\)folate involves a reductase-catalyzed oxidation of \([^{14}C]CH_3-H_4\)folate to \(N^5,N^{10}\)-\([^{14}C]\)methylene-\(H_4\)folate. This spontaneous dissociation of \(N^5,N^{10}-\[^{14}C]\)methylene-\(H_4\)folate to \([^{14}C]HCHO\) and \(H_4\)folate presumably follows.

The effect of FAD concentration was further studied in extracts from CLL lymphocytes (Chart 3A). Activity was strongly stimulated by FAD addition. The degree of stimulation increased with the concentration of added cofactor and reached a plateau at concentrations of 0.8 to 2.0 mM. Similar results were obtained with extracts of other types of leukocytes.

Enzyme activity in CLL cell extracts, assayed in the presence of 1.5 mM FAD, was found to be influenced by the concentration of added CH3-H4folate with an activity maximum at 30 to 40 \(\mu\)M (Chart 3B). By Lineweaver-Burk plot (26), the \(K_m\) for \(CH_3-H_4\)folate was \(1.1 \times 10^{-5}\) M. The rate of tryptolene formation was a function of tryptamine concentration with an activity maximum at 10 to 20 mM (Chart 3C). In the absence of added tryptamine, activity was less than 10% of that observed with 10 mM tryptamine.

A pH-activity curve (Chart 4A) revealed maximal activity at pH 6.5. HCHO production was also proportional to incubation time between 0 and 60 min (Chart 4B) and to the amount of extract added to the assay mixture between 0.1 and 0.5 mg protein (Chart 4C). When sonic extracts were centrifuged at 27,000 \(\times\) g, 95% of the activity was recovered in the supernatant fraction. Activity was unaffected by storage at \(-50^\circ\) for at least 30 days or by dialysis [against 20 mM potassium phosphate (pH 7)]. Similar conclusions were reached when CLL extracts were assayed in the absence of added FAD. When 20 mM \(\beta\)-phenylethylamine was used as HCHO acceptor in place of tryptamine, activity was consistently lower, but kinetic properties were unaltered.

**Comparison of Enzyme Activity in Leukocytes of Various Types.** Table 3 summarizes a comparative study of enzyme activity (expressed as nmoles HCHO formed per hr per mg protein) as assayed under standard conditions. Assays were also performed in which addition of tryptamine was deferred until after the end of the incubation and addition of borate. All activity was about 3 times higher when tryptamine was added after the incubation rather than at the beginning. This appears to indicate that tryptamine exerts an inhibitory effect on HCHO production even though it serves as an HCHO acceptor. The enhancement caused by late tryptamine addition was similar for all cell types studied.

The results in Table 3 show that mean activity in leukocytes from CLL patients was considerably higher than that found in mixed leukocytes from normal subjects and was more than twice that found in purified normal lymphocytes. Activity in granulocytes was relatively low. These differences are somewhat diminished if activity is expressed per cell instead of per mg protein, because protein content is higher in granulocytes than in lymphocytes (5). Experiments in which extracts of normal lymphocytes and granulocytes were mixed together gave no evidence of the presence of enzyme activators or inhibitors.

The results in Table 3 also show that activity in CML leukocytes was higher than that in isolated normal granulocytes. However, extracts prepared from the bone marrow of a CML patient had lower activity. Activity in mononuclear cells isolated on a Ficoll-Hypaque gradient from the blood of a patient with monocytosis (75%) approximated that found in normal lymphocytes. Activity in the mixed leuko-
lymphocytes of a severely cobalamin-deficient patient was not notably different from that of a normal patient.

No activity was found in plasma from heparinized normal blood. RBC disrupted by sonic oscillation showed some activity (10 pmol/min/ml of RBC) only after dialysis. This value is insignificantly low when related to cellular protein.

**DISCUSSION**

The results are of interest from several points of view. First, they demonstrate that leukocytes contain an enzyme that causes the conversion of CH$_3$-H$_4$folate to HCHO and H$_4$folate. Activity levels in leukocytes from normal blood are similar to values observed in some mammalian tissues, e.g., brain (44), kidney (23), and platelets (42), but are lower than those in liver (20, 21) and various cultured mammalian cells (35, 43). The data show that in leukocytes, as in other tissues, transfer of methyl carbon from CH$_3$-H$_4$folate to various amines is not due to a methyltransferase reaction. It appears rather to be due to an enzymatic oxidation of CH$_3$-H$_4$folate to N$^0$,N$^{14}$-methylene-H$_4$folate that is catalyzed by N$^0$,N$^{14}$-methylene-H$_4$folate reductase. The immediate oxidation product dissociates to H$_4$folate and HCHO, although the equilibrium constant is not in favor of dissociation in the absence of a HCHO acceptor (19). Liberated HCHO then reacts nonenzymatically with an acceptor such as tryptamine to form an adduct. We have shown in leukocytes that free HCHO arises from CH$_3$-H$_4$folate in a reaction sequence that is stimulated by FAD and, hence, must include an oxidative step. Other electron acceptors, i.e., FMN, menadione, and NADP, are less stimulatory. It is unclear why stimulation by FAD is mitigated by the simultaneous addition of NADPH. The reducing cofactor, NADPH, is inhibitory. Presumably, such inhibition is due to the ability of NADPH to force the equilibrium of the N$^0$,N$^{14}$-methylene-H$_4$folate reductase reaction in the reductive direction (21, 31). In contrast, NADP is slightly stimulatory. Because enzyme activity is not depressed by NADH, leukocyte enzyme presumably has a higher affinity for NADPH, as is the case with pig liver N$^0$,N$^{14}$-methylene-H$_4$folate reductase (21).

An inhibitory effect of AdoMet on reductase activity has been reported in liver (20, 31), but AdoMet had no effect on HCHO production by leukocyte extracts. The extent of inhibition by this compound may depend on the method of enzyme preparation, as has been shown in liver extracts (20). The noteworthy enhancement in activity by late addition of tryptamine resembles the observations of Stebbins et al. (42) in platelets. We cannot account for the inhibitory effect of tryptamine during the incubation.

The results furnish another example of an enzyme that is relatively more active in lymphocytes than in granulocytes and that is higher in CLL lymphocytes than in normal lymphocytes. Indeed, the comparative activity pattern closely resembles that described for CH$_3$-H$_4$folate-homocysteine methyltransferase (32). Having earlier concluded that activity levels of the latter enzyme can be correlated with the proliferative potential of a particular cell line (32, 39), we are led to a similar conclusion for the HCHO-producing enzyme system in leukocytes. It is of interest to compare the activity levels of the 2 enzymes in various leukocyte types. When activities of HCHO-producing enzyme, i.e., N$^0$,N$^{14}$-methylene-H$_4$folate reductase, and CH$_3$-H$_4$folate-homocysteine methyltransferase (32) are both expressed in terms of nmoles of CH$_3$-H$_4$folate utilized per hr per mg, respective levels are: in CLL lymphocytes, 2.10 and 2.15; in normal lymphocytes 0.96 and 0.95; in normal granulocytes, 0.35 and 0.15. It may be inferred that the enzymatic machinery of folate metabolism is elevated in toto in CLL lymphocytes and other cells with high proliferative potential.

Finally, the results are of interest in the context of a longstanding controversy over the validity of the so-called "methylfolate trap" theory of vitamin B$_12$ action in animal cells (13, 30); i.e., cobalamin participates in the biosynthesis of DNA by promotion of activity of the CH$_3$-H$_4$folate-homocysteine methyltransferase reaction. This has turned permitted conversion of CH$_3$-H$_4$folate to H$_4$folate, which can serve as a direct precursor of N$^0$,N$^{14}$-methylene-H$_4$folate, a coenzyme of thymidylate synthetase. In this view methylcobalamin mediates "the escape" of CH$_3$-H$_4$folate from the "trap" in which it is said to be sequestered. An essential prerequisite to this theory has been the assumption that no cobalamin-independent avenue exists in man for the conversion of CH$_3$-H$_4$folate to H$_4$folate. Although other evidence has placed the methylfolate trap theory in question (6), we consider that the present data, obtained in hematopoietic tissues,
establish that H4folate can arise freely from CH3-H4folate without the intervention of a cobalamin-dependent methyltransferase. Similar observations have been reported in platelets (42). Further, we find that HCHO production by leukocytes is close to normal in a vitamin B12-deficient patient and that addition of various cobalamins in vitro has little influence on the reaction.

These results are also of interest in light of recent evidence that shows diverse effects of vitamin B12 deficiency on the activities of other folate-linked enzymes. It has been found, for example, that conversion in vivo of [14C]serine is depressed in vitamin B12-deficient leukocytes (34) and that thymidylate synthetase activity rises in extracts of vitamin B12-deficient bone marrow (38). Presumably, these effects are related in some way to repression or derepression of various enzymes in the pathway of DNA synthesis, as an indirect result of vitamin B12 deficiency.

The physiological role of N5,N10-methylene-H4folate reductase, as it expresses itself when assayed with CH3-H4folate and tryptamine, was originally believed related to the metabolism of aromatic amines, particularly in brain tissue (3, 14, 22). However, little is known of the natural occurrence or function of tryptophanases or other HCHO-aromatic amine condensation products. The nonspecificity of HCHO binding and the wide variety of possible HCHO acceptors make it difficult to ascertain the actual in vivo role of the HCHO-producing reaction in leukocytes. We are intrigued by evidence suggesting that enzymatically produced HCHO can react with H4folate itself. The extent to which such a train of events may participate in folate metabolism in vivo remains to be explored.

REFERENCES

8. Blair, J. A., and Saunders, K. J. A Convenient Method for the Preparation of dl-5-Methyltetrahydrofolic Acid (dl-5-Methyl-6,7,8-trihydropropyrol-
J. Thorndike and W. S. Beck

41. Skoog, W. A., and Beck, W. S. Studies on the Fibrinogen, Dextran and
Phytohemagglutinin Methods of Isolating Leukocytes. Blood, 11: 436-
454, 1956.
42. Stebbins, R. D., Meller, E., Rosengarten, H., Friedhoff, A., and Silber, R.
Identification of N\textsuperscript{a},N\textsuperscript{b}-Methylene Tetrahydrofolate Reductase as the
Enzyme Involved in the 5-Methyl Tetrahydrofolate-Dependent Formation
of a \(\beta\)-Carboline Derivation of 5-Hydroxytryptamine in Human Platelets.
43. Taylor, R. T., and Hanna, M. L. Folate-Dependent Enzymes in Cultured
Chinese Hamster Ovary Cells: Induction of 5-Methyltetrahydrofolate Ho-
mocysteine Cobalamin Methyltransferase by Folate and Methionine.
44. Taylor, R. T., and Hanna, M. L. 5-Methyltetrahydrofolate Aromatic Alkyl-
amine N-Methyltransferase: An Artefact of 5,10-Methylenetetrahydrofol-
45. Taylor, R. T., Hanna, M. L., and Hutton, J. J. 5-Methyltetrahydrofolate
Homocysteine Cobalamin Methyltransferase in Human Bone Marrow and
Its Relationship to Pernicious Anemia. Arch. Biochem. Biophys., 165:
46. Wyatt, R. J., Erdelyi, E., DoAmorai, J. R., Elliott, G. R., Renson, J., and
Barchas, J. D. Tryptoline Formation by a Preparation from Brain with 5-
Methyldihydrofolic Acid and Tryptamine. Science, 187: 853-855,
1975.
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