TABLE II
ULTRAVIOLET SPECTRA AFTER STANDING FOR 90 MINUTES IN 0.1 N HCl
(N^5,N^{10}-METHENYLTETRAHYDROFOLATE)

Sample[a]	Max λ (nm)	Max ε (M^{-1} cm^{-1})	Min λ (nm)	Min ε (M^{-1} cm^{-1})	Ratio, max/min	Minimum purity estimated from ϵ_{252}[b] (%)
Leucovorin (Lederle)	352	23.9×10^3	302	10×10^3	2.39	90
Folinic acid (prepared above)	352	24.1×10^3	302	10×10^3	2.41	91

[a] See Table I.
[b] F. M. Huennekens, P. P. K. Ho, and K. G. Scrimgeour, Vol. VI [113].

then suspended in 5 ml of 1% mercaptoethanol solution; 1.0 N NaOH is added dropwise, just enough to dissolve the solid, and this solution is rechromatographed as described above. After lyophilization, treatment with acetone, washing with ether, and drying *in vacuo*, 35 mg of white powder are obtained.

Properties

To ascertain the purity of the above preparation some of its properties were compared with those of the calcium salt of "leucovorin" available from the Lederle division of American Cyanamid Company (Tables I and II).

On paper chromatography in 0.1 M phosphate buffer, pH 7, each compound appears as a single light-absorbing spot with an R_f: 0.73–0.74. In both preparations only traces of fluorescing materials are found.

[188] The Synthesis of N^5,N^{10}-Methenyltetrahydrofolic Acid

By PETER B. ROWE

N^5,N^{10}-Methenyltetrahydrofolic acid (anhydroleucovorin) is the specific formyl donor for the third step in purine biosynthesis, the introduction of the 8-carbon atom into the purine ring by formylation of glycinamide ribonucleotide to formylglycinamide ribonucleotide.[1,2]

[1] J. M. Buchanan and S. Hartman, *Advan. Enzymol.* **21**, 199 (1959).
[2] D. A. Goldthwait, R. A. Peabody, and G. R. Greenberg, *J. Am. Chem. Soc.* **76**, 5258 (1954).

This folic acid derivative is formed in acid solution (pH 1–2) from either N^5-formyl- or N^{10}-formyltetrahydrofolic acid by elimination of water[3]; neither of these is readily available.

An alternative procedure[4] consists in the platinum oxide-catalyzed reduction at pH 1–2 of N^{10}-formylfolic acid, produced by formylation of folic acid. A method has been developed for the direct synthesis, in high yield, of the pure N^5,N^{10}-methenyl derivative from commercial tetrahydrofolic acid.

Tetrahydrofolic acid (Sigma type III), 500 mg, is dissolved in 125 ml of 98% formic acid (Eastman Organic Chemicals) containing 2.0% (v/v) β-mercaptoethanol (Sigma) in a 500-ml round-bottom flask fitted with a reflux condenser. The apparatus is sealed from light with aluminum foil to minimize photodecomposition, and the flask is maintained at 60° for 3 hours in a heating mantle. Addition of thiol is required to prevent the oxidation of both the tetrahydrofolic acid and its N^{10}-formyl derivative, which is formed under these conditions. In the process, the N^{10}-formyl compound is dehydrated by the acid to form the N^5,N^{10}-methenyl derivative by a ring closure.

The cloudy yellow solution is filtered rapidly and lyophilized to dryness. The yield at this stage as calculated from an extinction coefficient of 2.5×10^4 (mole/liter)$^{-1}$ at 355 nm in 0.01 *M* HCl[5] is of the order of 80%.

Purification thereafter essentially follows that described by Huennekens.[6] A column 4 × 30 cm is packed under pressure with Whatman cellulose CF11 suspended in water. The column is washed with 1 liter of 0.1 *M* formic acid containing 0.01 *M* β-mercaptoethanol and is drained to near dryness. The lyophilized preparation, dissolved in 50 ml of the same acid–thiol solution, is adsorbed to the cellulose column. Elution is carried out with this acid–thiol solution. All effluent fractions exhibiting a value greater than 1.6 for the ratio of absorption at 355 nm to that at 280 nm (E_{355}:E_{280}) are pooled and lyophilized to dryness.

The N^5,N^{10}-methenyl derivative is crystallized from 0.1 *M* HCl–0.1 *M* β-mercaptoethanol according to Huennekens,[6] washed with absolute alcohol and ether, and stored in a vacuum desiccator at −20°.

This preparation, when examined by descending chromatography on

[3] M. T. May, J. Bardos, F. L. Barger, M. Lansford, J. M. Ravel, G. L. Sutherland, and W. Shive, *J. Am. Chem. Soc.* **73,** 3067 (1951).

[4] L. D. Kay, M. J. Osborn, Y. Hatefi, and F. M. Huennekens, *J. Biol. Chem.* **235,** 195 (1960).

[5] J. C. Rabinowitz, *in* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 2, p. 185. Academic Press, New York, 1960.

[6] F. M. Huennekens, P. P. K. Ho, and K. G. Scrimgeour, Vol. VI [114].

Whatman No. 1 paper with a 1.0 *M* formic acid–0.01 *M* β-mercaptoethanol solvent system, demonstrates a single white fluorescent spot (R_f = 0.50) under ultraviolet light. The classical absorption spectrum is also seen with the absorption maximum further into the visible region than that of any of the other formyl derivatives.[7] In 1.0 *M* HCl, the absorption ratio at 348:305 nm ($E_{248}:E_{305}$) is 2.40, approaching the value of 2.46 quoted for the pure material.[6] The overall recovery, based on an extinction coefficient of 2.65×10^4 (mole/liter)$^{-1}$ at 348 nm in 1.0 *M* HCl, is of the order of 50%. The compound stoichiometrically formylates glycinamide ribonucleotide in the assay system[8] for formyl glycinamide ribonucleotide synthetase.

N^{10}-Formyltetrahydrofolic acid can be formed from the N^5,N^{10}-methenyl compound by allowing the latter to stand for 3 hours at 25° in the presence of 0.1 *M* Tris-Cl buffer, pH 8.0, containing 0.01 *M* β-mercaptoethanol. The N^{10}-formyl derivative is the formyl donor for the formylation of 5-amino-4-imidazole carboxamide ribotide.[9] Both the N^5,N^{10}-methenyl and N^{10}-formyl derivatives of tetrahydrofolic acid have been shown to be absolutely required for the biosynthesis of purines de novo in a partially purified enzyme system from pigeon liver.[10]

[7] E. L. R. Stokstad and J. Koch, *Physiol Rev.* **47,** 88 (1967).
[8] L. Warren and J. M. Buchanan, *J. Biol. Chem.* **229,** 613 (1957).
[9] J. G. Flaks, M. J. Erwin, and J. M. Buchanan, *J. Biol. Chem.* **229,** 603 (1957).
[10] P. B. Rowe, unpublished data (1968).

[189] Preparation and Properties of Antigenic Vitamin and Coenzyme Derivatives

By JEAN-CLAUDE JATON and HANNA UNGAR-WARON

The primary biochemical role of folic acid (pteroylmonoglutamic acid, vitamin B_c) appears to be involved in the synthesis of nucleoproteins.[1] Although folic acid is not active as such in the mammalian organism, it is the precursor of the various coenzyme forms of the vitamin which participate in single-carbon transfer reactions. Its tetrahydro derivative, for example, serves as an acceptor of hydroxymethyl and formyl groups involved in the synthesis of compounds such as purines, pyrimidines, and certain amino acids like serine.[1] Because of the importance of folate co-

[1] E. L. R. Stokstad, *in* "The Vitamins" (W. H. Sebrell, Jr. and R. S. Harris, eds.), Vol. III, p. 89. Academic Press, New York, 1954.