SYNTHESIS OF A NEW FORM OF DIHYDROFOLATE*

Karin Smith, K. G. Scrimgeour, and F. M. Huennekens

Division of Biochemistry
Scripps Clinic and Research Foundation
La Jolla, California

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Dihydrofolate occurs as an intermediate in tetrahydrofolate biosynthesis, as a product of the thymidylate synthetase and phenylalanine hydroxylation reactions, or it can be prepared chemically from folate or tetrahydrofolate. The absolute structure of dihydrofolate in these reactions is difficult to establish because three tautomeric forms (5,6-, 5,8-, and 7,8-dihydrofolate) are possible. To date, only one form of dihydrofolate has been available as a stable, isolated compound. This material, defined as "standard" dihydrofolate (Mathews and Huennekens, 1963), is prepared by reduction of folate with hydrosulfite (Futterman, 1957; Blakley, 1960) or by catalytic hydrogenation of folate in an alkaline medium (O'Dell et al., 1947). On the basis of previous evidence, "standard" dihydrofolate was considered to be the 7,8-isomer, although the 5,8-configuration was not excluded (Huennekens, 1963).

The present communication describes the synthesis and properties of a new stable form of dihydrofolate. Evidence is presented that the new compound is 7,8-dihydrofolate, and that "standard" dihydrofolate is the 5,8-isomer.

The new form, 7,8-dihydrofolate, was prepared by reduction of folate with potassium borohydride at 37° C. Folic acid (190 mg.) was dissolved in 8 ml. of 0.1 N NaOH, diluted to 50 ml., and adjusted to pH 5.0 with 0.1 N HCl.

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The solution was heated to 37° C., 150 mg. of KBH_4 was added, and the solution was stirred for 10 minutes. After cooling the solution in an ice bath, the product was precipitated by careful adjustment of the pH to 2.8 with 1 N HCl. The solid was collected by centrifugation, redissolved in a small volume of water, adjusted to pH 6.0, and again precipitated with 1 N HCl. The precipitate was washed twice with cold 0.001 N HCl, suspended in 200 ml. of 0.001 N HCl, and lyophilized to dryness. The dry, orange powder was stored *in vacuo*.

The absorption spectra of 7,8-dihydrofolate (3×10^{-5} M) in acidic (pH 1), neutral (pH 7), and basic (pH 12) solutions are compared in Fig. 1. At neutral pH, the compound has its principal absorption maximum at 282 mμ and a second maximum at 350 mμ. By comparison, "standard" dihydrofolate at pH 7 has its maximum at 282 mμ with a shoulder at *ca.* 310 mμ (Osborn and Huennekens, 1958; Futterman, 1957; Blakley, 1960).

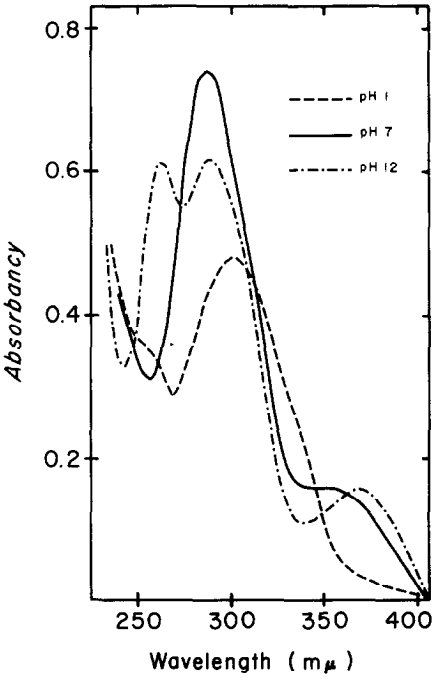

Fig. 1. Absorption spectra of 7,8-dihydrofolate

Catalytic hydrogenation of 1.7 mg. of 7,8-dihydrofolate in neutral solution over platinum oxide resulted in the consumption of 100 μliters of hydrogen in 2 hours. This corresponds to an uptake of 1.1 moles of hydrogen for each mole of dihydrofolate. 7,8-Dihydrofolate can also be reduced in an alkaline medium, in contrast to "standard" dihydrofolate which is relatively inert under these conditions (O'Dell, et al., 1947). The hydrogenated product of 7,8-dihydrofolate was established as tetrahydrofolate by: (a) absorption spectrum (λ max at 298 mμ); (b) reactivity with the formate-activating enzyme from chicken liver; and (c) reaction with formaldehyde.

7,8-Dihydrofolate can be reduced by hydrosulfite at room temperature, but it is inert to further reduction with borohydride. "Standard" dihydrofolate, on the other hand, is unaffected by hydrosulfite but is reduced by borohydride. In contrast to the compounds believed to be 5,6-dihydrofolate and N^5-methyl-5,6-dihydrofolate, both of which are reduced to the tetrahydro level by thiols (Kaufman, 1961; Donaldson and Keresztesy, 1962), neither 7,8-dihydrofolate nor "standard" dihydrofolate is reduced by thiols.

The inability of 7,8-dihydrofolate to accept a hydride ion, presumably at C^6, is also reflected by its unreactivity with other nucleophilic agents. In contrast, "standard" dihydrofolate interacts readily with bisulfite and other nucleophiles (cyanide, hydroxylamine, acetone, etc.) to form equilibrium adducts having similar spectra (λ max at 300 mμ) to that of tetrahydrofolate (Smith and Scrimgeour, 1963). With "standard" dihydrofolate the nucleophile apparently adds at C^7 and the accompanying proton at C^6. When folate is treated with nucleophilic agents, products are obtained having spectra similar to 7,8-dihydrofolate. In this instance, the nucleophile is believed to add at C^7 and the proton at N^8. Mechanisms for these reactions have been discussed elsewhere (Huennekens and Scrimgeour, 1962).

Reaction of folate compounds with formaldehyde or other aldehydes provides a simple diagnostic test for the absence of a double bond involving N^5. Both N^5 and N^{10} must contain a replaceable hydrogen in order for the folate compound to form a stable complex with the aldehyde. Glyoxylate is especially

useful in this connection since the resulting N^5,N^{10}-carboxymethylene compound is easily oxidized to the corresponding N^5,N^{10}-carboxymethenyl derivative (Ho, et al., 1960). At pH 4.5 "standard" dihydrofolate reacts with glyoxylate to yield N^5,N^{10}-carboxymethylene dihydrofolate, which is converted spontaneously to N^5,N^{10}-carboxymethenyl dihydrofolate. 7,8-Dihydrofolate is unreactive under these conditions because it contains no replaceable hydrogen at N^5.

"Standard" dihydrofolate and 7,8-dihydrofolate have been compared as substrates for the purified dihydrofolic reductase from chicken liver*. At neutral pH values, the former compound is reduced rapidly and completely (Osborn and Huennekens, 1958; Mathews and Huennekens, 1963), whereas 7,8-dihydrofolate is not reduced and, in fact, is an inhibitor of the enzyme. Thus, the double bond at N^5-C^6 is refractory toward both chemical and enzymatic reduction.

If the reduction of folate to dihydrofolate is visualized in terms of a hydride ion mechanism (Huennekens and Scrimgeour, 1962), either 5,6- or 7,8-dihydrofolate would be the expected product when borohydride is used as the reductant. 5,6-Dihydrofolate has been excluded by the present experiments with glyoxylate; previous data (Kaufman, 1961) suggested that the 5,6-isomer is labile toward isomerization to "standard" dihydrofolate.

Reduction of folate by hydrosulfite, alternatively, might be expected to produce an isomer with the 5,8-structure. Both DPNH and $FADH_2$, which are prepared by reduction of DPN and FAD with hydrosulfite, have structures in which the double bond system is analogous to 5,8-dihydrofolate. Like folate, both DPN and FAD are reduced much more rapidly by hydrosulfite than by borohydride. Hydrosulfite reductions, moreover, are known to proceed via a mechanism not involving hydride ions (Albert, 1959).

Previous evidence has shown that "standard" dihydrofolate cannot be the 5,6-isomer and must, therefore, be either the 5,8- or the 7,8-isomer. If the present characterization of the new form of dihydrofolate as the 7,8-isomer is correct, "standard" dihydrofolate must be the 5,8-isomer; this is in accord

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with experimental observations reported in this paper. The above conclusion is also in agreement with the preliminary report of Zakrzewski (1963), who has presented tracer data indicating that "standard" dihydrofolate is the 5,8-isomer.

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