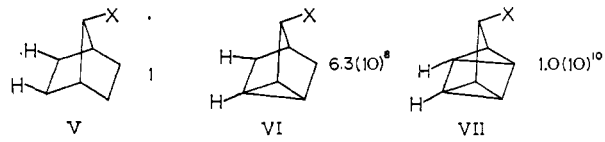
observed for a 7-norbornyl derivative V.[3,4] An explanation of the effect of introducing two cyclopropyl rings into a 7-norbornyl derivative must also accom-



V VI VII

modate the observation that most of this dramatic rate enhancement occurs[4,6] on introducing only one cyclopropyl ring to give the nortricyclyl system VI.

The low solvolytic reactivity of the 7-norbornyl system has been attributed to (1) the small C-1–C-7–C-4 angle, resulting in increased strain on going to the cation, (2) steric hindrance by the hydrogens on C-2 and C-3 to solvation of the cation, and (3), probably most important, unfavorable orientation of the C-1 and C-4 hydrogens for hyperconjugation.[7] In going from V to VI to VII, the orientation of the C-1 and C-4 hydrogens does not change, and though the C-1–C-7–C-4 angle probably increases slightly, only a minor rate increase could be attributed to this factor. Hindrance by the C-2 and C-3 hydrogens to solvent approach is diminished considerably in VI and somewhat more in VII; however, acetolyses of secondary arylsulfonates would not be expected[8] to be extremely sensitive to hindrance to solvation. Stabilization of carbonium ion formation by cyclopropyl rings is an attractive explanation for the large part of the rate enhancement not otherwise accounted for. The dilemma remains that, despite its similar geometric placement, the second cyclopropyl ring has much less effect than the first, contrary to experience in other systems.[9] The attachment of two cyclopropyl rings together or their face-to-face proximity may reduce their ability to delocalize positive charge.

The acetate[10] of I, b.p. 68–70° (2 mm.), m.p. 33–35°, was prepared by photolysis of the acetate[11] of IV using a procedure similar to that described for synthesis of quadricyclo[2.2.1.$0^{2,6}$.$0^{3,5}$]heptane (quadricyclane).[12] The acetate was saponified to I, b.p. 52° (2 mm.); *p*-nitrobenzoate, m.p. 147.5–149°; β-naphthalenesulfonate, m.p. 96–97.5°. The acetolysis rate was measured by a standard procedure[13] in acetic acid containing 1% of acetic anhydride and 0.1 *M* in potassium acetate. The solvolysis product was obtained by addition of water followed by extraction with carbon disulfide; its components were identified and their amounts estimated by their absorptions in the n.m.r. and infrared spectra.[14]

N.M.R. SPECTRA[a] IN CS_2

H-values at	I	Acetate	Naphthalenesulfonate
C-7	5.4 (1)	4.6 (1)	4.7 (1)
C-1–C-6	8.2–8.9 (6)	8.3–8.6 (6)	8.2–8.7 (6)
Other positions	7.3 (1)[b]	8.0 (3)[c]	1.6–2.6 (7)[d]

[a] Chemical shifts are in p.p.m. relative to tetramethylsilane as 10.0. The numbers in parentheses are approximate peak area ratios. Spectra were taken at 60 Mc. [b] Assigned to the hydroxyl H. [c] Assigned to the methyl H's. [d] Assigned to the naphthalene H's.

Acknowledgment.—The support of this work by the National Science Foundation is gratefully acknowledged.

(3) The acetolysis rate of the *p*-toluenesulfonate is 6.36 × 10^{-15} sec.$^{-1}$ extrapolated from data at higher temperatures (S. Winstein, M. Shatavsky, C. Norton, and R. B. Woodward, *J. Am. Chem. Soc.*, **77**, 4183 (1955)).

(4) Relative acetolysis rates of *p*-bromobenzenesulfonates, β-naphthalenesulfonates, and *p*-toluenesulfonates are taken as 9:4:3 (ref. 5 and R. A. Sneen, K. M. Lewandowski, I. A. I. Taha, and B. R. Smith, *J. Am. Chem. Soc.*, **83**, 4843 (1961)).

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(6) The acetolysis rate of the *p*-bromobenzenesulfonate is about 1.2 × 10^{-5} sec.$^{-1}$ (ref. 5 and S. Winstein, H. W. Walborsky, and K. Schreiber, *J. Am. Chem. Soc.*, **72**, 5795 (1950)).

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(14) The acetates of I and IV were recovered unchanged from similar solutions.

(15) A portion of this work is contained in the Senior Thesis of N. C. B., the Pennsylvania State University, March, 1963.

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The Structure of Dihydrofolic Acid Prepared by Dithionite Reduction of Folic Acid

Sir:

The widespread use of reduced forms of pteridine derivatives as intermediates in a variety of enzymic and microbiological systems has recently focused attention on the exact location of the hydrogen atoms on the pyrazine ring of dihydrofolate (folate-H_2). Folate-H_2 rather than folate is the initial compound formed in the biosynthesis *de novo* of this class of pteridine derivatives.[1] It is the precursor of tetrahydrofolate (folate-H_4) and is formed in stoichiometric amounts during the biosynthesis of thymidylate.[2] The results of chemical, physical, and enzymatic procedures described in this communication have convinced us that the 7,8-dihydro structure, the one originally suggested by O'Dell, *et al.*,[3] is the correct one rather than the 5,8-dihydro alternative recently put forth by Zakrzewski[4] and by Huennekens' laboratory.[5] The 5,6-dihydro formulation has been excluded on the basis that essentially all of the folate-H_2 is enzymatically convertible to a single diastereoisomer of folate-H_4.[6]

Tritium Incorporation Experiments.—The possibility of a 5,8-dihydro structure was tested by isotopic experiments in T_2O (1) by enzymatic conversion of folate-H_2 to folate-H_4 and (2) by dithionite reduction of folate to folate-H_2.[7,8] In neither case could the 5,8-dihydro assignment be substantiated.

We had previously shown that in the enzymic reduction of folate-H_2 one nonexchangeable hydrogen was incorporated into folate-H_4 from TPNH.[9] The second hydrogen for the reduction must come from the medium and, assuming the 5,8-dihydro structure, would also be nonexchangeable (reaction 1).

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$$TPNH + H^+ + FH_2 \xrightarrow[\text{reductase}]{\text{dihydrofolate}} FH_4 + TPN^+ \quad (1)$$

When reaction 1 was carried out in T_2O (0.59 curie of T_2O per ml.), the folate-H_4 isolated by column chromatography on DEAE-cellulose[9] was unlabeled. The tritium from the medium must have been transferred to an exchangeable position, *i.e.*, N-5 or N-8. This excludes the 5,8-dihydro structure.

If dithionite reduction of folate resulted in the formation of 5,8-folate-H_2, hydrogen would be incorporated into exchangeable positions only, *i.e.*, N-5 and N-8. When the dithionite reduction of folate was carried out in T_2O (0.67 curie of T_2O per ml.), the dihydro derivative isolated by column chromatography contained tritium (specific activity of folate-H_2/specific activity of one tritium atom of T_2O, 0.064). Since the tritium had been incorporated into a nonexchangeable position, the results of this experiment provide additional evidence against the 5,8-dihydro formulation. Although the marked dilution might be attributed to an indirect incorporation of label, it is a frequent finding with tritium because of isotope discrimination. To distinguish between the two alternatives dithionite reduction of folate was repeated in 99.8% D_2O.

Deuterium Incorporation Experiments.—Two preparations of folic acid were reduced to folate-H_2 with dithionite and ascorbate in D_2O. The folate-H_2 obtained was dissolved and reprecipitated several times in H_2O, lyophilized, and dried to constant weight over P_2O_5 *in vacuo* at 40°. One preparation was lyophilized as the ammonium salt and the other as the free acid. In order to exclude the possibility of isotope exchange, folate-H_2 was prepared by reduction in H_2O and then equilibrated in either D_2O and ascorbate or in a mixture of D_2O, ascorbate, and dithionite. The compound was dissolved and precipitated several times from H_2O and dried over P_2O_5 *in vacuo* at 40°.

The data in Table I clearly indicate that the dithionite reduction of folate to folate-H_2 results in the incorporation of approximately one atom of deuterium (80–90% of theory) per molecule of folate-H_2. This high level of deuterium incorporation rules out the unlikely possibility that the deuterated folate-H_2 could have arisen indirectly by oxidation of deuterated folate-H_4. It is therefore concluded that the deuterium was *directly* incorporated into a nonexchangeable position during the conversion of folate to folate-H_2. This eliminates the 5,8-dihydro assignment.

Nuclear Magnetic Resonance Studies.—An unambiguous demonstration of the fact that one of the hydrogens added in the reduction of folate to folate-H_2 is attached to carbon is possible by n.m.r. The n.m.r. spectrum of folate in D_2O consists of five groups of peaks (I) a single proton singlet at −1.82 p.p.m. (with respect to benzene as an external standard) which can be assigned to the H-7 of pteridine; (II) an A_2B_2 quartet from the *p*-aminobenzoic acid moiety centered at −0.47 p.p.m.; (III) a single proton triplet from the α-CH group of glutamic acid at +2.3 p.p.m.; (IV) a two-proton singlet at +2.6 p.p.m. attributable to the bridge CH_2 groups; and (V) a group of poorly resolved lines from the ABCD system of the two methylenes of glutamic acid at about +4.45 p.p.m. The assignments are based on the known spectra of glutamic acid[10] and *p*-aminobenzoic acid[11] and are unequivocal. In the spectrum of folate-H_2 the single proton singlet (I) is replaced by a *two-proton* singlet (VI) at +3.05 p.p.m., other peaks remaining the same. Thus, the two protons (the original H-7 and the added proton) are (1) equivalent since a single resonance line is observed and (2) attached to a fully saturated carbon since no resonance absorption from unsaturated systems is observed in this region of the spectrum. This rules out both a 5,8- and a 5,6-dihydro structure. The former would have given rise to a spectrum with the same number of protons as folate, the H-7 singlet appearing in the unsaturated region, −0.4 to +1.5 p.p.m. The spectrum of the latter would have shown a doublet for H-7 in the unsaturated region and a more complex pattern (ABX, AB_2, or ABC) for the C-6H, C-9H_2 system. The chemical shift of the new peak (VI) corresponds closely to the shift of the ring methylene in isosepiapterin, allowing for differences in substituents, solvent, and references.[12] The only reasonable interpretation is that (VI) represents a methylene group in position 7 on the pteridine ring.

Fu and Chinoperos have independently concluded that the 7,8-dihydro structure is the correct one based on ultraviolet spectral evidence.[13]

TABLE I
INCORPORATION OF DEUTERIUM INTO DIHYDROFOLATE PREPARED BY DITHIONITE REDUCTION OF FOLATE

Sample	Description	Atom % excess Found	Atom % excess Theory
1	FH_2[a] (diammonium salt)	3.00	2.86[e]
2	FH_2[b] (free acid)	2.97	3.84[f]
3	FH_2[c] (free acid)	0.30	None
4	FH_2[d] (free acid)	0.45	None

[a] Folate-H_2 was reduced according to the procedure of Blakley[8] in 99.8% D_2O, redissolved, and reprecipitated from H_2O–ascorbate and then washed three times with 0.001 *N* HCl. The compound was dissolved in a few milliliters of H_2O by cautious addition of solid NH_4HCO_3, lyophilized, and dried to constant weight *in vacuo* at 40° over P_2O_5. [b] Folate-H_2 was prepared as in *a*, dissolved, and reprecipitated once from ascorbate–D_2O and twice from ascorbate–H_2O. The sample was lyophilized as the free acid and dried to constant weight *in vacuo* at 40° over P_2O_5. [c] Folate-H_2 was prepared in H_2O,[8] dissolved, and precipitated twice from ascorbate–D_2O, twice from ascorbate–H_2O, washed three times with 0.001 *N* HCl, lyophilized, and dried to constant weight as in *b*. [d] Folate-H_2 was prepared in H_2O,[8] dissolved, and precipitated once from ascorbate–dithionite–D_2O, once from ascorbate–D_2O, and twice from ascorbate–H_2O. The sample was washed three times with 0.001 *N* HCl, lyophilized, and dried to constant weight as in *b*. [e] Based on $C_{19}H_{27}O_6N_9 \cdot 2.5\ H_2O$. [f] Based on $C_{19}H_{21}O_6N_7 \cdot 2.5\ H_2O$.

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The Stereochemistry of Ion Pair Return Associated with Solvolysis of *p*-Chlorobenzhydryl *p*-Nitrobenzoate[1]

Sir:

We have shown recently that ion pair return associated with solvolysis (alkyl–oxygen cleavage) of O^{18}-labeled benzhydryl[2] and allylic[3] *p*-nitrobenzoates

(1) A preliminary report of this work was presented at the 141st National Meeting of the American Chemical Society, Washington, D. C., March, 1962.

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