The reaction of methyllithium with vinyltriphenylphosphonium bromide in ether–tetrahydrofuran medium can be understood in the same manner, although the products observed account for only *ca.* 30% of the lithium reagent consumed. When the reaction mixture was quenched with cyclohexanone, benzene (17%), *n*-propylidenecyclohexane (13%), and methylenecyclohexane (6%) were obtained.

All reactions were carried out under an atmosphere of prepurified nitrogen. All new compounds reported had satisfactory analyses. Our work in this area is continuing.

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(17) (a) Alfred P. Sloan Research Fellow; (b) National Science Foundation Graduate Fellow, 1960–1963; National Institutes of Health Predoctoral Fellow, 1963–1964; (c) Fellow of the M.I.T. School for Advanced Study, 1961–1962.

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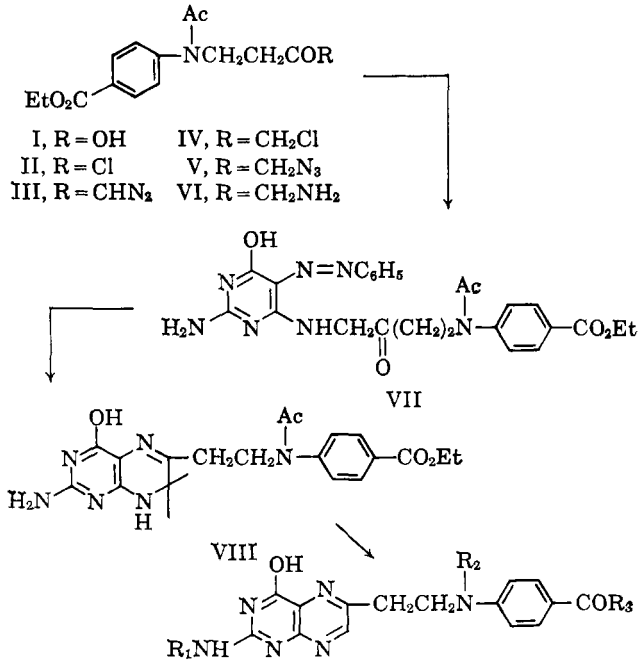
Tetrahydrohomofolate, a Specific Inhibitor of Thymidylate Synthetase

Sir:

This communication describes the synthesis and biological properties of homofolic acid (a trivial name for the compound possessing an additional methylene group between positions 9 and 10 of folic acid) and its reduced forms, dihydrohomofolate (homofolate-H_2) and tetrahydrohomofolate (homofolate-H_4).

The general approach of Boon and Leigh[1] for the synthesis of unambiguously 6-substituted 2-amino-4-hydroxypteridines was used to prepare XII. The N-phenyl-β-alanine derivative (I) was converted *via* II, III, IV, and V to the aminoketone (VI), the semicarbazone of which was condensed with 2-amino-4-hydroxy-5-phenylazo-6-chloropyrimidine to give the intermediate (VII), after hydrolysis of the semicarbazone function. Hydrogenation of VII resulted in spontaneous cyclization of the ketone carbonyl with the formed 5-amino group to give the 7,8-dihydropteridine (VIII), $\lambda_{max}^{pH\ 13}$ 234 mμ (ε 21,200), 277 (7780), 330 (5180), that was oxidized to the pteridine (IX). Saponification gave chromatographically homogeneous "homopteroic acid" (X), $\lambda_{max}^{pH\ 13}$ 256 mμ (ε 26,900), 277 (21,900), 365 (7625). *Anal.*[2] Found: C, 54.7, H, 4.62; N, 25.6. The blocked derivative (XI), after reaction with diethyl L-glutamate by the mixed anhydride method followed by saponification, gave XII, $\lambda_{max}^{pH\ 13}$ 255 mμ (ε 24,600), 281 (19,500), 365 (7880). *Anal.* Found (for the hemihydrate): C, 51.2; H, 5.09; N, 21.2. On paper chromatography in 0.1 *M* $(NH_4)HCO_3$, 99% of the material was present in a single spot with R_f 0.89. The method of synthesis precludes a 7-substituted compound and, considering the ultraviolet spectra that support the pteridine structure, the analytical data, and the chromatographic behavior, there can be little doubt that homofolic acid has structure XII.



IX, R_1 = H; R_2 = Ac; R_3 = OEt
X, R_1 = R_2 = H; R_3 = OH
XI, R_1 = Ac; R_2 = CF_3CO; R_3 = OH
XII, R_1 = R_2 = H; R_3 = $NHCH(CO_2H)CH_2CH_2CO_2H$

(1) W. R. Boon and T. Leigh, *J. Chem. Soc.*, 1497 (1951).

Homofolate was converted to the dihydro derivative by dithionite reduction in 1 *M* 2-mercaptoethanol[3] and then was tested spectrophotometrically[4] as a substrate of dihydrofolate reductase from amethopterin-resistant mouse leukemia cells. Homofolate-H_2 was as effective a substrate as folate-H_2. The enzymatically formed homofolate-H_4 was not only completely inert as a participant in thymidylate synthesis but was a potent inhibitor of this enzyme. At 2.0×10^{-6} *M*, homofolate-H_4 caused a 50% inhibition of thymidylate synthetase from *E. coli*[5] in the presence of 80 times as much folate-H_4. Homofolate-H_4 analogs containing α-, β-, and γ-methyl glutamic acid moieties[6] were less inhibitory than homofolate-H_4.

Data obtained in a survey of the possible inhibitory action of homofolate-H_4 on a variety of tetrahydrofolate-requiring reactions indicate that thymidylate synthetase from *E. coli* appears to be the most sensitive of the enzymes tested (Table I).

In 0.006 *M* mercaptoethanol, homofolate-H_4 oxidized within 20 min., giving a compound with a spectrum closely resembling that of dihydrohomofolate. Addition of mercaptoethanol after oxidation did not reverse the reaction. Homofolate-H_4 bound formaldehyde mole for mole as does folate-H_4. In the presence of formaldehyde, homofolate-H_4 was stabilized over a period of 1 hr. at room temperature.

Homofolate is a poor growth inhibitor whereas homofolate-H_4 is a potent inhibitor of *Streptococcus faecalis* (ATCC 8043) and *Lactobacillus casei* (ATCC 7469, Table II). The inhibition is completely reversed for both organisms by thymidine (25 γ/ml.) even at levels of homofolate-H_4 140 times greater than that required for 50% inhibition. *Pediococcus cerevisiae* (ATCC 8081) is not affected by homofolate-H_4. This pattern of behavior is distinct from other antifolate agents.[7] Homofolate-H_2 surprisingly showed growth-factor activity for *S. faecalis*. It was about one-tenth as active as folate. This activity was not due to contamination with folate, as growth-promoting activ-

(2) Acceptable analytical data were obtained for all the solid substances encountered in the synthesis.

(3) M. Friedkin, E. J. Crawford, and D. Misra, *Federation Proc.*, **21**, 176 (1962).

(4) M. Friedkin, E. J. Crawford, S. R. Humphreys, and A. Goldin, *Cancer Res.*, **22**, 600 (1962).

(5) A. J. Wahba and M. Friedkin, *J. Biol. Chem.*, **237**, 3794 (1962).

(6) Substituted glutamates used in the synthesis of homofolate analogs were generously supplied by Dr. Alton Meister.

(7) R. L. Kisliuk, *Nature*, **188**, 584 (1960).

TABLE I

INHIBITION OF VARIOUS TETRAHYDROFOLATE-REQUIRING REACTIONS BY HOMOFOLATE-H_4

Enzyme preparaton	Source of enzyme and assay ref.	Folate-H_4[a] homofolate-H_4	Inhibition, %
Thymidylate synthetase	*E. coli*[b]	95	47
		38	61
		19	77
		10	81
Methionine synthetase	*E. coli*[c]	1	25
Formylglutamate formyl transferase	Hog liver[d]	15	10
		1	30
Formyltetrahydrofolate synthetase[e]	*Clostridium cylindrosporum*[f]	5	0
		1	0
Methylene tetrahydrofolate dehydrogenase	*E. coli*[g,h]	3	37
		2	43
		1	63
Purine synthetase	Pigeon liver[i]	4	0
		2	3
		1	39
L-Serine hydroxymethyl transferase	*E. coli*[g,j]	3	8
		2	17
		1	33
Deoxycytidylate hydroxymethylase	T_4 am82-phage-infected *E. coli*[k,l]	2	0

[a] Ratio of tetrahydrofolate concentration to tetrahydrohomofolate concentration in assay mixture. Homofolate-H_4 was prepared by dithionite reduction of homofolate at 75° exactly as described by Silverman and Noronha for the preparation of tetrahydrofolate[8] and was purified by gradient elution from DEAE-cellulose. It was stored frozen in a buffer mixture containing 0.005 *M* Tris (pH 7.0), 0.2 *M* mercaptoethanol, and 0.4 *M* NaCl. Homofolate-H_4 was also prepared by catalytic reduction with hydrogen and PtO_2 [R. L. Kisliuk, *J. Biol. Chem.*, **227**, 805 (1957)]. [b] See ref. 5. [c] R. L. Kisliuk, *J. Biol. Chem.*, **236**, 817 (1961). [d] M. Silverman, J. C. Keresztesy, G. J. Koval, and R. C. Gardiner, *ibid.*, **226**, 83 (1957). [e] A generous gift of crystalline enzyme from the laboratory of Dr. J. Rabinowitz. [f] J. C. Rabinowitz and W. E. Pricer, Jr., *ibid.*, **237**, 2898 (1962). [g] K. G. Scrimgeour and F. M. Huennekens, *Biochem. Biophys. Res. Commun.*, **2**, 230 (1960). [h] Method used is described in footnote *g*. TPN was used as cofactor. [i] D. A. Goldthwait and G. R. Greenberg, "Methods in Enzymology," Vol. II, S. P. Colowick and N. O. Kaplan, Ed., Academic Press, New York, N. Y., 1955, p. 504. [j] Same method was used as for methylene tetrahydrofolate dehydrogenase assay except serine was substituted for HCHO. The dehydrogenase activity was not limiting at concentrations of homofolate-H_4 that inhibited the transferase. [k] M. L. Dirksen, J. C. Hutson, and J. M. Buchanan, *Proc. Natl. Acad. Sci. U. S.*, **50**, 507 (1963). [l] Enzyme was generously supplied by Dr. J. M. Buchanan.

TABLE II

INHIBITION OF MICROBIAL GROWTH BY HOMOFOLATE AND REDUCED DERIVATIVES

Compound[a]	Concn. required for 0.5 maximal inhbition (mμg./ml.) *S. faecalis*[b]	*L. casei*[b]	*P. cerevisiae*[c]
Homofolate	1000	100	>10,000
Dihydrohomofolate	Supports growth	50	>500
Tetrahydrohomofolate	0.7	6	>1000

[a] All compounds were diluted in potassium ascorbate, 6 mg./ml. (pH 6.0), and added to growth media aseptically after autoclaving. Final assay contains 0.6 mg. of potassium ascorbate/ml. to stabilize reduced derivatives [H. A. Bakerman, *Anal. Biochem.*, **2**, 558 (1961)]. [b] Folate, 1.0 mμg./ml. [c] Calcium *dl*-L-5-formyltetrahydrofolate, 1 mμg./ml.

ity corresponded exactly to the eluted homofolate-H_2 obtained by gradient elution from DEAE-cellulose.

An outstanding biochemical alteration associated with the development of resistance to amethopterin in mouse leukemia is the pronounced increase of dihydrofolate reductase.[4,9] To take advantage of this augmented activity, we have been seeking folate analogs that can be readily reduced to tetrahydro products with marked inhibitory effect on thymidylate synthetase. Homofolate-H_2 uniquely fulfills these requirements.

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(8) M. Silverman and J. M. Noronha, *Biochem. Biophys. Res. Commun.*, **4**, 180 (1961).

(9) D. K. Misra, S. R. Humphreys, M. Friedkin, A. Goldin, and E. J. Crawford, *Nature*, **189**, 39 (1961).

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The Synthesis of Cellulose by an Enzyme System from a Higher Plant

Sir:

This report describes the synthesis of cellulose by a particulate enzyme preparation from mung beans (*Phaseolus aureus*); the glucosyl donor in this reaction is guanosine diphosphate D-glucose, which has been shown to be enzymatically formed from guanosine triphosphate and α-D-glucose 1-phosphate.[1] Although the cell-free synthesis of cellulose has previously been demonstrated with extracts of the bacterium, *Acetobacter xylinum*, *via* the glucosyl donor, uridine diphosphate D-glucose,[2] the synthesis of this important polysaccharide has never before been shown by enzyme isolated from higher plants.

Mung bean seedlings were grown in a moist atmosphere in the dark. One hundred grams (wet weight) of hypocotyls and roots were homogenized in 1.0 *M* tris(hydroxymethyl)aminomethane–HCl buffer (Tris), pH 7.6, and the homogenate was filtered through cheesecloth and centrifuged at 1000*g* for 5 min. The supernatant fluid then was centrifuged further at 20,000*g* for 20 min. The precipitated material was suspended in 3 ml. of 0.1 *M* Tris buffer, pH 7.6, and used as the source of enzyme. This preparation contained about 53% protein and 4% cellulose. Washing of the particulate material resulted in a substantial loss of activity.

All sugar nucleotides were labeled uniformly with C^{14} in the glucosyl moiety (106 μc./μmole). Guanosine diphosphate D-glucose was prepared enzymatically.[1] Uridine diphosphate D-glucose and thymidine diphosphate D-glucose were also prepared enzymatically.[3] Adenosine diphosphate D-glucose and cytidine diphosphate D-glucose were chemically synthesized.[4]

As shown in Table I, incubation of guanosine diphosphate D-glucose with the particles led to the incorporation of radioactivity into a material which was insoluble in hot water and hot alkali. None of the other C^{14}-glucose nucleotides was active in this system

(1) G. A. Barber and W. Z. Hassid, unpublished data.
(2) L. Glaser, *J. Biol. Chem.*, **232**, 627 (1957).
(3) G. A. Barber, *Biochemistry*, **1**, 463 (1962).
(4) S. Roseman, J. J. Distler, J. G. Moffatt, and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 659 (1961).