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,i]	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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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 1000g for 5 min. The supernatant fluid then was centrifuged further at 20,000g 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).

(Table I). Furthermore, no reaction was observed when boiled enzyme was used, or when D-glucose-C^{14} or D-glucose 1-phosphate-C^{14} replaced guanosine diphosphate D-glucose. Incorporation of radioactivity into cellulose was proportional to time and enzyme concentration over a 30-min. period. Experiments indicated that particles from 3 to 4 day old mung bean seedlings had optimal activity.

TABLE I

SUBSTRATE SPECIFICITY FOR CELLULOSE SYNTHESIS[a]

C^{14}-Glucosyl donor	Product, c.p.m.
Guanosine diphosphate D-glucose	1165
Uridine diphosphate D-glucose	0
Thymidine diphosphate D-glucose	4
Adenosine diphosphate D-glucose	0
Cytidine diphosphate D-glucose	9
D-Glucose	0
D-Glucose 1-phosphate	0

[a] The complete incubation mixture contained the following in a total volume of 0.2 ml.: 0.5 μmole of 0.1 *M* Tris buffer (pH 7.6), 2×10^{-4} μmole (12,000 c.p.m.) of the indicated glucosyl donor (106 μc./μmole), and 0.1 ml. of enzyme representing about 2 g. wet weight of plant material (about 1.5 mg. of protein and 0.1 mg. of cellulose). The mixtures were incubated at 37° for 30 min. At the end of this time, 1 ml. of water was added, and the reactions were stopped by heating for 3 min. in a boiling water bath. One mg. of cellulose was added, and the precipitate was collected by centrifugation. The precipitate was again extracted with hot water and was then heated two times (100° for 5 min.) with 1 ml. portions of 2% NaOH. The precipitate was washed with 1 ml. of water, suspended in 0.2 ml. of water, and plated on planchets. Radioactivity in the samples was determined with a mica-window Geiger–Muller counter and conventional scaler.

When soluble or insoluble cellodextrins, cellobiose, D-glucose, or cellulose was added to the reaction mixture (with guanosine diphosphate D-glucose-C^{14} as described in Table I), no increased incorporation of radioactivity into the polymer was observed. The addition of yeast concentrate (Sigma Chemical Co.) to the reaction mixture caused a twofold stimulation in the incorporation of D-glucose into cellulose. This effect could not be duplicated by the separate addition of diphosphopyridine nucleotide, triphosphopyridine nucleotide, adenosine triphosphate, or Coenzyme A.

In order to isolate a larger amount of the product for characterization, the following reaction mixture was incubated at 37° for 30 min.: 10 μmoles of 0.1 *M* Tris buffer (pH 7.6), 3.2×10^{-3} μmole of guanosine diphosphate D-glucose (190,000 c.p.m.), 1 mg. of yeast concentrate, and 1 ml. of particulate enzyme (containing about 15 mg. of protein and 1 mg. of cellulose) in a total volume of 1.2 ml. After incubation, 5 mg. of cellulose was added, and the radioactive product was isolated as described in Table I. The alkali-insoluble material, containing 47,000 c.p.m. (24% of the total radioactivity), was soluble in 85% phosphoric acid and could be precipitated by dilution with water.[5] Partial acid hydrolysis in fuming HCl,[6] followed by circular chromatography in *n*-propyl alcohol–ethyl acetate–water (7:1:2), liberated a series of radioactive compounds indistinguishable from the authentic cellodextrin series.

The radioactive disaccharide isolated chromatographically from the partial acid hydrolysate was characterized as cellobiose on the basis of the following criteria.

(1) It was indistinguishable from authentic cellobiose upon descending chromatography in two solvent systems (*n*-propyl alcohol–ethyl acetate–water, 7:1:2; *n*-butyl alcohol–pyridine–0.1 *N* HCl, 5:3:2), and upon ionophoresis in 0.05 *M* borate buffer, pH 9.0. These systems readily separate the 1,4-linked glucosyl disaccharides from the other possible isomers.

(2) Treatment of the synthetic radioactive material with emulsin (β-glucosidase) liberated only radioactive glucose, indicating that the 1,4-glucosyl bond is in the β-configuration. Maltose, containing an α-linkage, was not hydrolyzed under the same conditions. Also, treatment with emulsin of radioactive cellotriose, isolated from the partial acid hydrolysate, liberated glucose and cellobiose, both of which were radioactive.

(3) The radioactive disaccharide was crystallized after the addition of 5 μmoles of authentic cellobiose.[7] The specific activity of the crystalline material (4 mg., m.p. 223–225°) remained constant during three recrystallizations (546, 493, and 517 c.p.m./mg.).

Further characterization of the radioactive cellulose was afforded by the following data.

(1) Radioactive cellotriose, obtained by partial acid hydrolysis, was subjected to lead tetraacetate oxidation.[8] Following acid hydrolysis with 1 *N* HCl at 100° for 30 min., one of the radioactive products had the same chromatographic mobility as a tetrose in *n*-propyl alcohol–ethyl acetate–water (threose and erythrose are inseparable in this solvent); the other radioactive products corresponded in position to glucose and cellobiose. A tetrose could only arise by lead tetraacetate oxidation of a 1,4-linked di- or trisaccharide.[8]

(2) The enzymatically formed cellulose was subjected to acetolysis at 50° for 7 days.[9] A radioactive product (cellobiose octaacetate) was isolated from the aqueous phase by extraction with chloroform. The dry chloroform extract was concentrated to a small volume and deacetylated with sodium methoxide. Following this treatment, 95% of the radioactivity could be extracted from the chloroform phase with water. The deacetylated product was identified as cellobiose by paper chromatography in *n*-propyl-alcohol–ethyl acetate–water.

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