TETRAHYDROFOLATE DEHYDROGENASE FROM BAKERS' YEAST*

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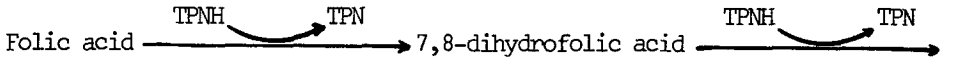
Folic reductase catalyzes the reduction of folic acid to tetrahydrofolic acid according to the following reactions (Zakrzewski, 1963):



(1)

Most systems require TPNH as the reducing agent although DPNH will serve to a limited extent. The reactions, which are catalyzed by a single enzyme (Zakrzewski and Nichol, 1960), are quite sensitive to folic acid antagonists and are irreversible (Blakley and McDougall, 1961; Futterman, 1957). The significance of the reactions is obvious since the cofactor form of the vitamin, which functions as a carrier of mobile metabolic one-carbon units, is tetrahydrofolic acid (Huennekens and Osborn, 1959). If folic acid, the vitamin form, has a metabolic function, it has never been defined. However, the presence of an enzyme which catalyzes the oxidation of tetrahydrofolic acid to folic acid has been observed in extracts of acetone dried bakers' yeast. The enzyme catalyzes the following reactions:

(2)

The present report describes the isolation and some properties of this enzyme.

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† This report is taken from the M.S. thesis of Maurice E. Bush, Howard University Medical School, 1965.

Tetrahydrofolate was prepared by catalytic reduction of folic acid (O'Dell et al., 1957). Dihydrofolate was prepared by reduction of folic acid with dithionate (Futterman, 1957). Bakers' yeast was a generous gift from the Wonder Bread Company of Washington, D. C.

Crude extracts of acetone dried yeast catalyzed the reduction of TPN and DPN in the absence of added substrate. Dialysis of these extracts was without significant effect on the rate of TPNH or DPNH formation, indicating that the endogenous substrate is protein bound. Addition of tetrahydrofolic acid to these extracts stimulates the rate of TPNH or DPNH formation.

The techniques for ridding the yeast extracts of endogenous activity varied from preparation to preparation. In some instances, aging with freezing and thawing 4 - 6 days was sufficient. With other preparations protamine sulfate treatment (1/3 volume of 2% solution), followed by $(NH_4)_2SO_4$ fractionation (40 - 80%), was required, and occasionally the $(NH_4)_2SO_4$ fraction had to be dialyzed and adsorbed on, and eluted from, calcium phosphate gel before the endogenous substrate was removed completely. Unless otherwise specified all experiments reported here were done with calcium phosphate gel eluate.

The partially purified enzyme was unstable at -4°. Loss of activity at this temperature was practically complete in 48 hours, and was not diminished by β-mercaptoethanol. The enzyme was stabilized (5 days) by 5% glycine.

The requirements for the oxidation of tetrahydrofolate are shown in Table I. While freshly prepared enzyme shows no requirement for mercaptoethanol, aged preparations (2 - 4 days) exhibit a definite requirement for -SH compounds. In the absence of TPN, tetrahydrofolate, or enzyme no TPNH is formed.

When DPN is used as the electron acceptor both the crude extracts and the gel fraction show a higher rate of absorbancy change than when TPN is the acceptor. This difference is probably due to the presence of large amounts of DPN-alcohol dehydrogenase in these extracts which have some activity for β-mercaptoethanol. In support of this is the observed DPNH formation in the absence of added tetrahydrofolate (Table I). In the presence of both tetrahydrofolate

and mercaptoethanol, DPNH formation is approximately two times greater than TPNH formation under similar conditions.

TABLE I

REQUIREMENTS FOR TETRAHYDROFOLATE OXIDATION

Omission	Δ O.D. 340/min	
	TPN	DPN
None	100	220
β-Mercaptoethanol	40	30
TPN or DPN	0	0
Tetrahydrofolate	0	125
Enzyme	0	0

The reaction mixture contained 400 μmoles Tris buffer, pH 7.8, 5 μmoles mercaptoethanol, 0.3 μmoles TPH or DPN, 0.5 μmoles dl,L-tetrahydrofolate and 400 μg enzyme protein $(NH_4)_2SO_4$ fraction, in a total volume of 3 ml.

The identity of the pteridine reaction product was determined as follows: Fifty mg of tetrahydrofolic acid in 5 ml of ascorbic acid (10 mg/ml, pH 7.2) was incubated with 60 mg of TPN, 5 ml of 0.5 M phosphate buffer, pH 7.8, 1 ml of 1% mercaptoethanol and 5 ml of enzyme (7.0 mg protein). Oxidation was followed to completion by observing the increase in absorbancy at 340 mμ. The reaction mixture was then adjusted to pH 1.5 with HCl to destroy the TPNH, readjusted to pH 8.0 with NaOH and chromatographed on DEAE-cellulose column (Keresztesy and Donaldson, 1961). Elution was effected with 10% Na_2HPO_4 and the effluent was collected in 10 ml portions with an automatic fraction collector. Eluate fractions 50 - 100, when examined spectrophotometrically, showed absorption maxima at 370 and 280 mμ, which are characteristic of folic acid. Comparison with authentic folic acid, similarly chromatographed, gave a similar spectrum (Fig. 1).

Random samples from the eluate fractions 50 - 100 from the reaction mixture were assayed microbiologically for folic acid. The results showed that

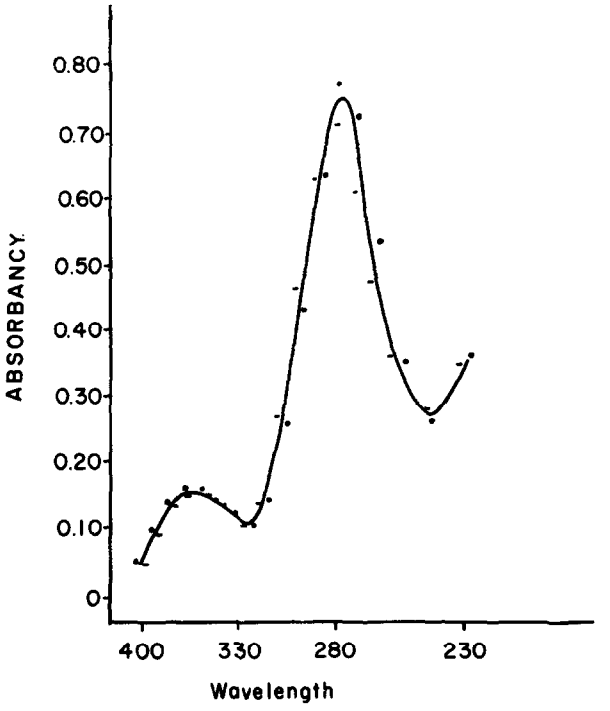

Fig. 1. - Spectral comparison of authentic and enzymatically synthesized folic acid. ●_____● synthesized; -_____- authentic folic acid.

the eluates stimulated the growth of Streptococcus faecalis R but not Pediococcus cerevisiae, indicating that the column eluate from the reaction mixture contained folic acid and no reduced pteridine (Bakerman, 1961).

The other reaction product was identified as TPNH by the fall in absorbancy at 340 mμ, upon the addition of α-ketoglutaric acid, NH_4Cl and glutamic acid dehydrogenase to the reaction mixture after equilibrium was reached (Fig. 2A).

The complete oxidation of 1 mole of tetrahydrofolic acid to folic acid should require 2 moles of TPN (Reaction 2). To determine the stoichiometry of this reaction, tetrahydrofolic acid in ascorbic acid (7 mg/ml) was added to the yeast tetrahydrofolic acid dehydrogenase systems and to the Escherichia coli - methylenetetrahydrofolic acid dehydrogenase system (Scott and Donaldson, 1964). Both reactions were allowed to proceed to completion and then α-ketoglutaric acid,

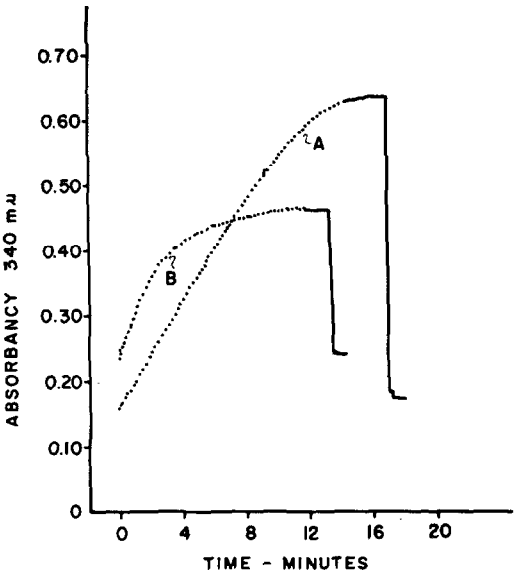

Fig. 2. - Qualitative and quantative identification of TPNH production by tetrahydrofolate dehydrogenase, Curve A, and methylenetetrahydrofolate dehydrogenase, Curve B. The reaction mixture, Curve A, was like that of Table I except that it contained 0.27 μmoles of tetrahydrofolate in ascorbic acid and 100 μg of enzyme, gel fraction; Curve B,400 μmoles Tris buffer, pH 8.0, 11 μmoles ascorbic acid, pH 7.5, 3μ moles formaldehyde, 0.3 μmoles TPN, 0.27 μmoles tetrahydrofolate and 5 μg methylenetetrahydrofolate dehydrogenase in a volume of 3 ml.

NH_4Cl and glutamic acid dehydrogenase were added to each cuvette. The immediate decrease in absorbancy at 340 mμ as shown in Fig. 2 supports the view that the tetrahydrofolic acid dehydrogenase system produced twice as much TPNH as the methylenetetrahydrofolic acid dehydrogenase system. The latter system has been established as a two electron transfer reaction (Ramasastri and Blakley, 1962). This experiment also furnished evidence for the stereospecificity of the tetrahydrofolic acid dehydrogenase for one isomer of tetrahydrofolic acid. Since the methylenetetrahydrofolic acid system has been shown to be stereospecific for 1,L-diastereoisomer of methylenetetrahydrofolic acid (Scott and Donaldson, 1964; Ramasastri and Blakley, 1962). If both isomers of tetrahydrofolic acid were active in the tetrahydrofolic acid dehydrogenase system, the amount of TPNH produced would have been twice that observed.

The mechanism whereby tetrahydrofolic acid is oxidized to folic acid is unknown at this time. 7,8-Dihydrofolic acid is not an intermediate in this conversion. Added dihydrofolate will not serve as substrate in place of tetrahydrofolate. Preliminary experiments indicate that the reaction is relatively insensitive to aminopterin. The 34% inhibition observed with 4×10^{-4} M aminopterin is reversed by added tetrahydrofolic acid, indicating that the aminopterin inhibition is of a competitive type.

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