Studies on folic acid reduction

Previous investigations carried out in this laboratory have been concerned with the reduction of dihydrofolic acid by a sheep-liver enzyme[1,2]. The subject of the present communication is the reduction of folic acid, pteroylaspartic acid, and teropterin (diglutamyl folic acid) by a sheep-liver enzyme and the non-competitive inhibition of this enzyme by aminopterin.

The enzyme was prepared from an acetone powder of sheep liver by extraction with water and was purified about 3-fold by $(NH_4)_2SO_4$ fractionation and isoelectric precipitation of protein at pH 4.5. Most of the enzyme activity was in the fraction which precipitated between 30 and 50 % saturation with $(NH_4)_2SO_4$. The enzyme was stable at low pH values and remained in solution at pH 4.5. The reduction of folic acid was measured by following the non-enzymic decomposition of dihydrofolic acid as described previously[2]. The specific requirement for TPNH[3] and the low pH optimum (about pH 5) of folic acid reductase made it appear unlikely that folic acid was being reduced to the tetrahydro level by the relatively crude enzyme preparation. The enzyme preparation contained dihydrofolic acid reductase but this enzyme requires DPNH as an electron donor at pH 5[1,2], TPNH being only slightly effective. Folic acid reductase showed only slight activity when DPNH was used as the electron donor. Table I shows the substrate specificity of the enzyme.

TABLE I

SUBSTRATE SPECIFICITY OF FOLIC ACID REDUCTASE

Incubations were carried out at 37° for 30 min in the presence of 0.25 mg TPNH, 1.0 ml of enzyme preparation, and 1.0 ml 0.1 *M* sodium acetate buffer, pH 5.0, in a total vol. of 2.4 ml. The reactions were terminated and the mixtures deproteinized by the addition of 1.0 ml 15 % trichloroacetic acid. Aliquots of the deproteinized mixtures were assayed by diazotization as described elsewhere[2]. The concentration of substrate in each case (except aminopterin) was determined in a preliminary experiment to be sufficient to yield a zero-order reaction under the given conditions.

Substrate	*Substrate reduced (μmoles)*
Folic acid	1.34
Teropterin	1.34
Pteroylaspartic acid	0.23
Aminopterin	0.00

Aminopterin (4-aminofolic acid) at extremely low levels completely inhibited folic acid reductase. Folic acid was ineffective in reversing the inhibition but the degree of inhibition was found to be a function of the enzyme concentration, as has been reported in the case of irreversible or pseudo-irreversible inhibitions[4–6]. Thus it was found possible to titrate folic acid reductase with aminopterin (Fig. 1), the equivalence being $5.4 \cdot 10^{-4}$ μg ($\pm 0.3 \cdot 10^{-4}$ μg) aminopterin/unit of folic acid reductase. A unit of enzyme is defined here as that amount which will bring about the reduction of 1 mμmole folic acid in 30 min under the conditions given in Table I.

Abbreviations: TPNH, reduced triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

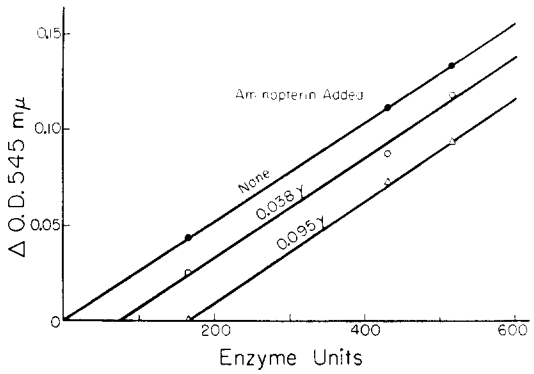

Fig. 1. Titration of folic acid reductase with aminopterin. The conditions were the same as those given in Table I except that the enzyme preparation was an aqueous extract of sheep-liver acetone powder and TPNH was generated *in situ* from 0.5 mg TPN and 10 μmoles sodium citrate. Extrapolation of the curves to zero Δ O.D. yield the number of enzyme units which would be completely inhibited by the given amount of aminopterin.

Aided by research grants from the National Cancer Institute (CY-3175), U.S. Public Health Service, and the American Cancer Society, California Division (151).

Department of Biochemistry, School of Medicine, University of California, San Francisco, Calif. (U.S.A.) JAMES M. PETERS
DAVID M. GREENBERG

[1] J. M. PETERS AND D. M. GREENBERG, *Nature*, 181 (1958) 1669.
[2] J. M. PETERS AND D. M. GREENBERG, *J. Am. Chem. Soc.*, in the press.
[3] S. F. ZAKRZEWSKI AND C. A. NICHOL, *Biochim. Biophys. Acta*, 27 (1958) 425.
[4] W. W. ACKERMANN AND V. R. POTTER, *Proc. Soc. Exptl. Biol. Med.*, 72 (1949) 1.
[5] O. H. LOWRY, O. A. BESSEY AND E. J. CRAWFORD, *J. Biol. Chem.*, 180 (1949) 399.
[6] E. C. SLATER, *Biochem. J.*, 45 (1949) 130.

Received August 18th, 1958

Nitrogen fixation by nodules formed on isolated bean roots*

It has been shown[1] that nodules will form on isolated bean roots inoculated with the appropriate rhizobia if a modified technique of aseptic root culture[2] is used. Furthermore, such nodules are histologically similar to those formed on the roots of intact plants. The present paper reports work undertaken to find whether such nodules also will function in nitrogen fixation.

Isolated roots of *Phaseolus vulgaris* L., var. "Pencil Pod" black wax bean, were grown and inoculated as described earlier[1], except that three roots, instead of one, were grown per Petri dish. Each dish contained 50 g of washed silica sand moistened with 10 ml of the inorganic salts of medium "O"[1]. The vials contained 10 % sucrose, and glycine, thiamin, niacin and pyridoxine at the levels indicated for medium "O" or

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Rockefeller Foundation.