PTERINE REDUCTASE*

BARBARA E. WRIGHT AND MINNIE L. ANDERSON

Section on Enzymes of the Laboratory of Cellular Physiology, National Heart Institute, National Institutes of Health, Bethesda, Md.(U.S.A.)

INTRODUCTION

In a previous publication evidence has been presented for the reduction of teropterin or folic acid to their dihydro-derivatives, in the presence of extracts of *C. sticklandii*, CoASH** and a suitable electron donor[2]. Experiments concerning substrate specificity of the electron donor suggested that α-keto acids were the primary electron donors, and the enzyme system was consequently further purified using pyruvate as the electron donor. Using substrate levels of CoASH, pyruvate, and an oxidized pterine, stoichiometry for the following reaction has been obtained:

$$\text{Teropterin} + \text{pyruvate} + \text{CoASH} \longrightarrow \text{TerH}_2 + \text{AcSCoA} + \text{CO}_2$$

MATERIALS AND METHODS

Materials

The conditions for preparing the sonic extract from *C. sticklandii* have been described[2].

Teropterin (diglutamyl folic acid) and N^{10}-formyl-folic acid were gifts from Dr. H. P. BROQUIST of Lederle Laboratories. We are indebted to Dr. M. SILVERMAN for a sample of purified N^{10}-formyl-folic acid, and to Dr. E. R. STADTMAN for a preparation of phosphotransacetylase.

Methods

Protein concentration was determined on the basis of 280 mμ/260 mμ absorption[2].

The enzyme was purified using an assay system containing pyruvate, phosphate, phosphotransacetylase, and catalytic amounts of CoASH, since the latter is expensive. Experiments to be described, however, were carried out with substrate levels of CoASH in the absence of phosphate and phosphotransacetylase, unless specified otherwise.

Two assays for dihydropterines were used: (1) measurement at 420 mμ of a yellow degradation product formed upon the addition of acid and (2) decrease in absorption at 365 mμ at alkaline pH, where FA has a peak, but FAH_2 does not. The molecular extinction used was the difference between FA and FAH_2 at 365 mμ (8,000). Details of these two assays have been given previously[2].

Acetyl-S-CoA was determined by the hydroxamic acid method[4]; the acetate was identified by Duclaux distillation. Pyruvate was quantitatively estimated by following DPNH disappearance in the presence of lactic acid dehydrogenase[5]. $^{14}CO_2$ was trapped on the head of a pin which had been dipped in 1 *N* NaOH. The pin protruded about 5.0 mm from the tapered end of the rubber stopper placed in the top of the small tubes (10 mm diameter, filled with hydrogen) used for an experiment. Following incubation, the tubes were chilled, the stoppers removed, and a drop of 1 *N* HCl was allowed to roll down the side of the tube while the stopper was quickly replaced. The tubes were then incubated 10 more minutes to trap residual $^{14}CO_2$ released by the acid. The $Na_2{}^{14}CO_3$ was rinsed off the pin and an aliquot counted directly in a scintillation counter, or it was converted to Ba $^{14}CO_3$, dried, and counted in a gas-flow counter.

* A preliminary report of this work has been given[1].

** Abbreviations used in this paper: BAL, 2,3-dimercaptopropanol; CoASH, coenzyme A; FA, folic acid; FAH_2, dihydrofolic acid; $TerH_2$, dihydroteropterin; tris,tris(hydroxymethyl) aminomethane.

References p. 375.

EXPERIMENTAL

Enzyme purification

Data summarizing the various steps to be described are given in Table I. 100 ml of a *C. sticklandii* sonic extract diluted 1:1 with 0.01 *M* tris pH 8.5, were adjusted to this pH by the dropwise addition of *M* tris, pH 8.5. 15 ml of streptomycin sulfate (pure base, 150 mg/ml) were added to the enzyme at 0°. After chilling in an ice bath for 10 min. the enzyme was spun in the Servall super centrifuge at 20,000 × *g* for 15 min. and the precipitate discarded. Subsequent centrifugation was always done in a Servall at that speed.

TABLE I

ENZYME PURIFICATION

Step	*Units/ ml**	*Total Units*	*mg Protein/ ml*	$\frac{280\ m\mu}{260\ m\mu}$	*Units mg protein*
Crude sonicate	850	85,000	25.0	0.65	34.0
Streptomycin supernatant	742	85,100	19.5	0.85	38.0
Heat supernatant	592	68,200	11.5	0.98	51.0
Ammonium sulfate	3,950	63,300	28.1	1.16	140.0
Acid supernatant	2,300	55,020	14.9	1.40	153.0
Gel eluate	2,072	33,100	10.0	1,39	207.0

* One unit = the amount of enzyme required to produce an increase in optical density (upon acidification) of 0.01 at 420 mμ after 45 min. incubation at 38° in the presence of FA, 0.21 μmole; potassium phosphate, 30 μmoles at pH 6.5; phosphotransacetylase, 22 units[3]; CoASH, 0.04 μmole; BAL, 0.2 μmole; pyruvate, 1.0 μmole.

The supernatant solution was distributed in small tubes (about 5.0 ml/tube) and heated in a hot water bath 4.0 minutes at 50°. The tubes were chilled, spun, and the precipitate discarded. Solid ammonium sulfate was added to the supernatant solution at 0° and the protein precipitating between 55% and 85% saturation was taken up in 0.01 *M* tris, pH 8.5 and dialyzed for 3½ hours against *M*/50 tris, pH 7.4. To this solution (16.0 ml) were added 4.8 ml of 0.1 *M* Na acetate buffer, pH 4.0. After 10 min in the ice bath with periodic stirring, the solution was centrifuged and the precipitate discarded. The supernatant solution was treated with successive additions of Al $(OH)_3$ gel (25 mg)/ml); 4.65 ml and 7.02 ml respectively. The two gels were eluted together in a hand homogenizer with 0.1 *M* Na citrate pH 7.0. After centrifugation, the volume of the gel eluate was 16.0 ml. To this final enzyme preparation (6-fold purified) was added 0.05 ml of 0.1 *M* BAL. The enzyme is stable for about a month if it is not thawed and frozen excessively.

As reported earlier[2], enzyme preparations using serine as the electron donor in the assay mixture, could utilize a variety of other electron donors. Table II compares such an enzyme preparation with the one described above which was purified using pyruvate as the electron donor. It can be seen that virtually all the activity on substrates other than pyruvate and α-keto butyrate has been eliminated, and that activity on the latter substrate has been reduced.

References p. 375.

TABLE II

ELECTRON DONORS

Substrate	Δ 420 mμ	
	Enzyme purified with: Pyruvate	Serine
Pyruvate	0.560	0.560
α-Keto butyrate	0.310	0.445
Serine	0.009	0.373
Cysteine	0.072	0.273
Methionine	0.000	0.246
Threonine	0.006	0.037

Conditions: For the enzyme purified with pyruvate as electron donor: All substrates were present at a concentration of 1.0 μmole/0.4 ml total volume; samples were incubated 40 min at 38°. The concentration of components were folic acid, 0.5 μmoles; CoASH, 1.3 μmoles; β-mercaptoethanol, 2.0 μmoles; protein, 0.08 mg, potassium arsenite, 10^{-2} *M*, and 20.0 μmoles potassium succinate pH 6.3. Comparable activities for these substrates using an enzyme purified with serine as electron donor[2] are given for the sake of comparison. See text.

Requirements and stoichiometry of the reaction

Figure 1 shows that dihydropterine formation is linear over a wide range with increasing concentrations of enzyme, pterine, CoASH, and with increasing periods of incubation. Fig. 2 demonstrates that dihydropterine formation is also linear with pyruvate concentration, and that approximately 50% of the pyruvate is utilized for the reaction.

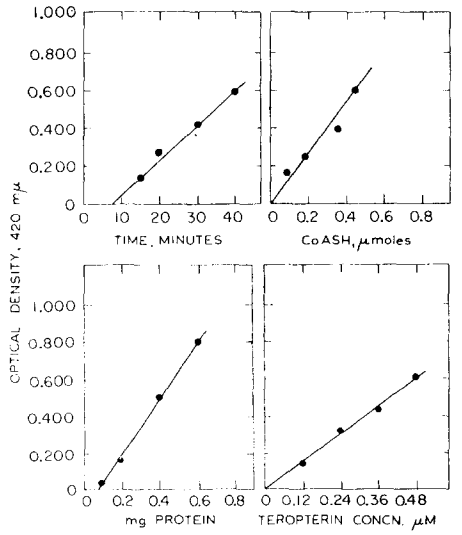

Fig. 1. Dihydropterine formation as a function of time and the concentration of pterine, CoASH and enzyme. Except where a particular component was varied, these experiments were all done under the following conditions: Samples were incubated 1 h at 38° with 20.0 μmoles potassium succinate, pH 6.3; also present were an oxidized pterine, 0.5 μmoles; potassium arsenite, 10^{-2} *M*; protein, 0.05 mg; β-mercaptoethanol, 2.0 μmoles; CoASH, 1.3 μmoles; and pyruvate, 1.0 μmoles in a total volume of 0.4 ml. In the case of the enzyme curve, each sample contained 0.05% albumin.

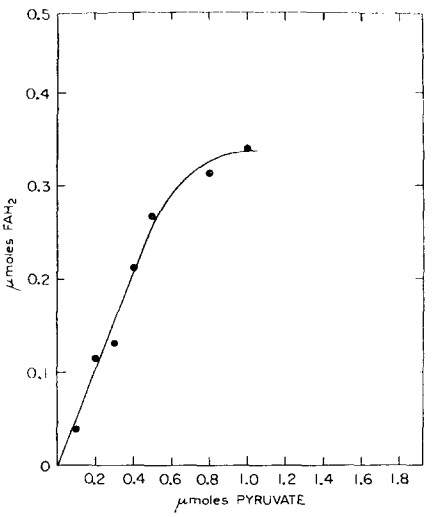

Fig. 2. Dihydropterine formation as a function of the concentration of pyruvate. Experimental conditions are similar to those for Fig. 1.

References p. 375.

In the presence of excess pyruvate, also, about 40–50% of the pyruvate disappearing is used for the formation of dihydropterine. This can be seen in Table III, which presents data for the stoichiometry of the reaction. It can be seen that the amount of dihydropterine formed is equivalent to the pterine-dependent utilization of pyruvate and the pterine-dependent formation of CO_2 and acetyl SCoA. The latter was identified by Duclaux distillation. The fact that this reaction involves the transfer of two electrons is additional evidence, (to that already presented[2]) for the formation of a pterine reduced to the dihydro-level.

TABLE III

STOICHIOMETRY

Compound, μmoles	Final concentration, μmoles		
	Presence of pterine	Absence of pterine	Δ due to presence of pterine
I. Ter H_2*	0.35	—	+ 0.35
Ac S CoA**	0.56	0.25	+ 0.35
residual pyruvate***	0.23	0.58	— 0.35
II. FAH_2,	0.24	—	+ 0.24
Ac S CoA	0.40	0.17	+ 0.23
residual pyruvate	0.40	0.63	— 0.23
III. FAH_2,	0.23	—	+ 0.23
Ac ^{2}S CoA	0.49	0.27	+ 0.22
$^{14}CO_2$	0.30	0.10	+ 0.20

Conditions: The same as for Fig. 1, (with 1 μmole pyruvate/0.4 ml) except that samples were incubated for 45 min in the presence of 0.08 mg protein. 1-^{14}C pyruvate was present in Expt. III.

* Determined by decrease in optical density at 365 mμ at pH 11.0.
** Determined by the hydroxamic acid method[4].
*** Determined in the presence of DPNH and lactic acid dehydrogenase[5].

Cofactors involved

Lipoic acid and thiamine pyrophosphate are frequently involved in pyruvate oxidation[6]. It was, therefore, pertinent to examine their possible role in the system described here where a pterine can act as the electron acceptor. Arsenite sensitivity is characteristic of systems dependent upon lipoic acid[6], and the effect of this inhibitor was consequently tested in the present pterine-linked system. Arsenite ($10^{-2}M$) has no effect on the pterine-dependent decomposition of pyruvate. It does, however, inhibit pyruvate disappearance and acetyl-S-CoA appearance in the absence of pterine.

It can be seen from Fig. 2 and Table III that about half of the pyruvate disappearing is involved in (a) non-pterine-dependent reaction(s). This is also true of the acetyl-S-CoA formed. If arsenite is omitted, however, about 80% of the pyruvate utilized is involved in a non-pterine-dependent reaction(s). Arsenite did not inhibit the pterine-dependent reaction. These results suggest that lipoic acid is not involved in the oxidation of pyruvate when pterine is the electron acceptor.

No requirement for thiamine pyrophosphate could be detected, although it is possible that the procedures employed did not resolve the enzyme for this cofactor. Subsequent to a heat step and an acid step (see purification), the enzyme withstood dialysis for 20 h against 0.02 *M* sodium pyrophosphate (pH 8.5) followed by dialysis

References p. 375.

for 5 h against 0.01 *M* phosphate, pH 7.0. A similar treatment resolved another bacterial enzyme completely for thiamine pyrophosphate [7]. Attempts to resolve this co-enzyme with a more strenuous acid step[8] than that employed in the purification (pH 4.0) resulted in inactivation of the enzyme.

Specificity of pterine requirement

In order to determine whether a substituted pterine could also be reduced to the dihydro-derivative, N^{10}-formyl-folic acid and N^{10}-formyl-diglutamyl-folic acid[9] were investigated. N^{10}-formyl-dihydrofolic acid has been described by JAENICKE AND GREENBERG[10] as a breakdown product of N^{10}-formyl-tetrahydrofolic acid. They describe the formylated dihydro-compound as having one absorption maximum at 260 mμ at pH 7.0 and another low maximum between 335 mμ and 340 mμ, with a 260 mμ/340 mμ ratio of about 3.4–3.6. They report the R_F in 0.5 *M* formic acid to be 0.37 and in 0.1 *M* K_2HPO_4 to be 0.58 [10].

Fig. 3 gives spectra at neutral and alkaline pH for N^{10}-formyl-folic acid and its reduction product, which in all probability, is N^{10}-formyl-dihydrolfolic acid. The 260 mμ/340 mμ ratio of the reduced compound is between 3.4 and 3.6 — in this experiment it is 3.6. The maxima are at 260 mμ and between 335 mμ and 340 mμ, and the R_F values in the above solvent systems are 0.40 and 0.51 respectively. The molecular extinction for the reduced compound at 260 mμ, pH 7.0, is about 19,000. The maxima (pH 7.0) at 245 mμ, for the oxidized pterine and 233 mμ for the reduced compound

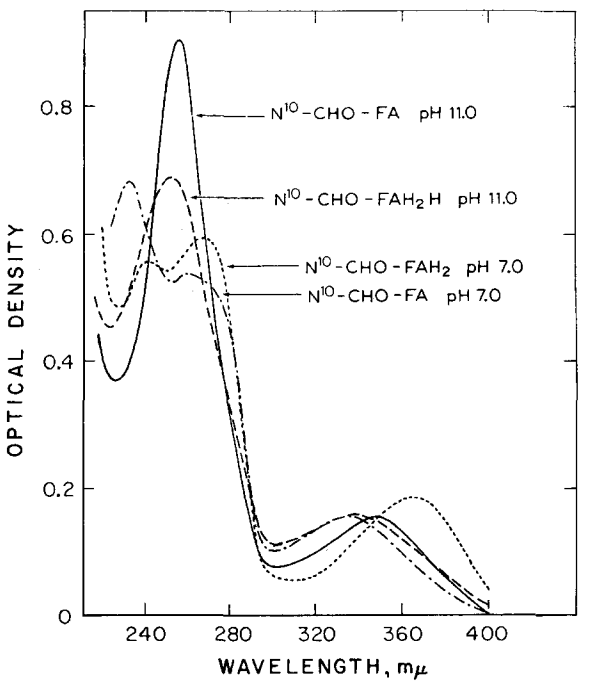

Fig. 3. The enzymic formation of N^{10}-formyl-dihydrofolic acid. Experimental conditions: pyruvate, 4.0 μmoles; CoASH, 0.04 μmole; BAL, 0.2 μmole; phosphotranacetylase, 22 units; enzyme, 0.1 mg; N^{10}-formyl-folic acid, 0.3 μmole, and 30 μmoles potassium phosphate at pH 6.5. The samples were incubated 2 h, in order to assure complete disappearance of the oxidized pterine.

References p. 375.

have not been reported previously, but we think they are genuine, and probably do not represent an impurity. They exist in the N^{10}-formyl-folic acid sample supplied by Lederle, as well as in two samples purified from the Lederle product by Dr. M. SILVERMAN and by ourselves. They also exist in N^{10}-formyl-diglutamyl-folic acid which was isolated from *C. cylindrosporum*[9], and which also undergoes reduction to the dihydro-derivative in this system.

DISCUSSION

A system which may be similar to this one, in extracts of *Clostridium butyricum* has been described by O'KANE, *et al.*[11]. This pyruvate oxidation does not involve lipoic acid, and riboflavin can act as the electron acceptor. A preliminary experiment with riboflavin in the present system suggests that it is reduced, but further work is required to determine the specificity of the enzyme system reducing pterines with respect to other possible electron acceptors. Since 50% of the pyruvate is oxidized by a non-pterine-dependent reaction, it is not yet possible to say whether the same enzyme system is responsible for the reduction of both the pterine and the flavin.

Aminopterin does not inhibit pterine reductase. This is unexpected in that all previously described systems in which folic acid is reduced to the tetrahydro-level are affected by this inhibitor [12,13].

SUMMARY

An enzyme system in extracts of a *Clostridium* has been described which catalyzes the following reaction:

$$\text{Teropterin} + \text{pyruvate} + \text{CoASH} \longrightarrow \text{AcS CoA} + CO_2 + \text{Ter } H_2$$

The enzyme acts upon monoglutamyl and triglutamyl pterines, as well as on their N^{10}-formyl derivatives.

REFERENCES

1 B. E. WRIGHT AND M. L. ANDERSON, *J. Am. Chem. Soc.*, 79 (1957) 2027.
2 B. E. WRIGHT, M. L. ANDERSON AND E. C. HERMAN, *J. Biol. Chem.*, 230 (1958) 271.
3 E. R. STADTMAN, G. D. NOVELLI AND F. LIPMANN, *J. Biol. Chem.*, 191 (1951) 365.
4 F. LIPMANN AND L. C. TUTTLE, *J. Biol. Chem.*, 158 (1945) 505.
5 A. MEISTER, *J. Biol. Chem.*, 184 (1950) 117.
6 I. C. GUNSALUS, *The Mechanism of Enzyme Action*, The Johns Hopkins Press, Baltimore, 1954, p. 545.
7 C. F. GUNSALUS, R. Y. STANIER AND I. C. GUNSALES, *J. Bacteriol.*, 66 (1953) 548.
8 B. L. HORECKER, P. Z. SMYRNIOTIS AND H. KLENOW, *J. Biol. Chem.*, 205 (1953) 661.
9 M. SILVERMAN AND B. E. WRIGHT, *J. Bacteriol.*, 72 (1956) 373.
10 L. JAENICKE AND G. R. GREENBERG, *Biochem. Preparations*, in the press.
11 R. S. WOLFE AND D. J. O'KANE, *J. Biol. Chem.*, 205 (1953) 755.
12 C. A. NICHOL AND A. D. WELCH, *Proc. Soc. Expt. Biol. Med.*, 74 (1950) 403.
13 S. FUTTERMAN AND M. SILVERMAN, *J. Biol. Chem.*, 224 (1947) 31.

Received October 28th, 1957