The Michaelis–Menten constant of α-oxoisovaleric acid oxidase with α-oxoisovalerate is $1.0 \cdot 10^{-4}$ M; the pH optimum for this enzyme is at pH 7.4.

In order to measure the enzyme activity, the ^{14}C-labelled α-oxoacids were incubated with the enzymes. The $^{14}CO_2$ liberated was absorbed on filter paper soaked with KOH, and then quantitatively estimated in the liquid scintillation spectrometer. The oxoacids which are not available were prepared by incubation of 1-^{14}C-labelled amino acids with amino acid oxidase from snake venom and isolated by thin-layer chromatographic techniques, and ion-exchange column chromatography, respectively. Exact details on the applied methods, determination of the kinetics of the reactions, purification of the enzymes, and the characterization of the α-oxo-β-methyl-valeric acid oxidase, will be published elsewhere[9].

Results on the investigation of five families in which diminished activity or total lack of α-oxoacid oxidase activity (these children died) were found, have been published[10,11]. In the liver of a child who died from this disease, there was a highly reduced activity of all the three oxoacid oxidases in comparison to a control.

Department of Biochemical Genetics,
Institute of Human Genetics and
Anthropology, University of Freiburg,
Freiburg i. Br. (Germany)

H. W. GOEDDE
M. HÜFNER
F. MÖHLENBECK
K. G. BLUME

1 J. MENKES, P. HURST AND J. CRAIG, *Pediatrics*, 14 (1954) 462.
2 K. TANAKA, M. A. BUDD, M. L. EFRON AND K. J. ISSELBACHER, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 236.
3 J. DANCIS, J. HUTZLER AND M. LEVITZ, *Biochim. Biophys. Acta*, 43 (1960) 342.
4 J. DANCIS, J. HUTZLER AND M. LEVITZ, *Biochim. Biophys. Acta*, 78 (1963) 85.
5 L. E. HOLT, JR., S. E. SNYDERMAN, J. DANCIS AND P. M. NORTON, *Federation Proc.*, 19 (1960) 10.
6 D. LONSDALE AND D. H. BARBER, *New Engl. J. Med.*, 271 (1964) 1338.
7 H. W. GOEDDE, E. RICHTER, C. STAHLMANN, B. SIXEL, *Klin. Wochschr.*, 41 (1963) 953.
8 H. W. GOEDDE, E. RICHTER, M. HÜFNER AND B. SIXEL, *Klin. Wochschr.*, 42 (1964) 818.
9 H. W. GOEDDE *Biochemical Genetics*, Plenum Publ. Corp., New York, N.Y., in preparation.
10 H. W. GOEDDE, E. RICHTER, M. HÜFNER AND A. V. Z. MÜHLEN, *Humangenetik*, 1 (1964) 163.
11 H. W. GOEDDE, F. MÖHLENBECK AND K. -G.BLUME, *Med. Klinik*, 61 (1966) 2063.

Received November 15th, 1966

Biochim. Biophys. Acta, 132 (1967) 524–525

BBA 61126

Cofactor requirements of thymine 7-hydroxylase

In the conversion of thymine to the pyrimidines of RNA by Neurospora, 5-hydroxymethyluracil appears to be an intermediate[1,2]. Cell-free extracts of this mold have been found to contain an enzyme, thymine 7-hydroxylase, which effects the formation of 5-hydroxymethyluracil by catalyzing the hydroxylation of the methyl group of thymine[3]. Thymine 7-hydroxylase activity was demonstrable in these extracts when they were assayed in the presence of NADPH and GSH. The loss of

activity resulting from the exclusion of air from the incubation mixture suggested that the enzyme is a mixed-function oxidase[4]. In this report we show that Fe^{2+}, ascorbate and α-ketoglutarate are requirements for thymine 7-hydroxylase activity. The inclusion of these compounds in the incubation mixture has resulted in the preparation of extracts with much higher activities.

A metal ion study was instigated by the finding that EDTA was a very potent inhibitor. As shown in Table I, when Fe^{2+} was included in the incubation mixture a marked stimulation of the conversion of thymine to 5-hydroxymethyluracil occurred. It can be seen that Fe^{3+} was stimulatory to a lesser extent.

TABLE I

EFFECT OF Fe^{2+} ON THYMINE 7-HYDROXYLASE ACTIVITY

Neurospora crassa 1A was grown, harvested and homogenized as previously described[1] with the exception that the buffer utilized for the extraction procedure was 0.05 M Tris–HCl (pH 8.0) which was 1.0 mM with respect to GSH. The extract was centrifuged at 12 000 × *g* for 60 min, and the supernatant fluid was incubated and assayed as previously described[4]. The complete incubation mixture contained (in 0.2 ml) 0.1 ml of the enzyme preparation; [2-^{14}C]thymine, (43 counts/min per mμmole) 100 mμmoles; Tris–HCl, (pH 8.0) 10 μmoles; GSH, 50 mμmoles; NADPH, 100 mμmoles; $FeSO_4$, 5.0 mμmoles; and where indicated $Fe_2(SO_4)_3$, 5.0 mμmoles.

Complete system		*5-Hydroxymethyluracil produced*
Omissions	*Additions*	*(mμmoles)*
None	None	7.8
Fe^{2+}	None	2.0
NADPH	None	0.8
Fe^{2+} and NADPH	None	1.1
Fe^{2+} and NADPH	Fe^{3+}	0.8
Fe^{2+}	Fe^{3+}	4.8

The presence of GSH was not required in either the extraction or assay procedure when Fe^{2+} was included in the incubation mixture. 2-Mercaptoethylamine, L-cysteine, and 2-mercaptoethanol were also found to have no stimulatory effect. However, *p*-hydroxymercuribenzoate at a concentration of 1.0 mM in the incubation mixture completely inhibited thymine 7-hydroxylase activity.

Passing the enzyme through a Sephadex G-50 column usually resulted in a complete loss of activity even when NADPH and Fe^{2+} were included in the incubation mixture. A similar inactivation occurred when the enzyme preparations were subjected to dialysis or various purification techniques. That this dramatic loss of activity was due to the removal of a cofactor rather than to denaturation was indicated by the occasional restoration of activity resulting from the addition to the incubation mixture of an aliquot of the elutant containing the compounds of smaller molecular weights separated from the enzyme by Sephadex G-50 chromatography. In order to identify this unknown cofactor the testing of a large number of substances was carried out which included vitamins (and related compounds such as tetrahydrofolic acid and 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine), metal ions, amino acids and nucleotides. Only with ascorbate were positive results obtained. Some of the extracts not treated with Sephadex showed an increase in activity when ascorbate was added to the standard incubation mixture containing Fe^{2+} and NADPH. Also, it was found that if the extraction procedure were carried out in the presence of ascorbate, a more

TABLE II

REQUIREMENTS FOR THE DEMONSTRATION OF THYMINE 7-HYDROXYLASE ACTIVITY IN A SEPHADEX G-50-TREATED EXTRACT

The enzyme extract was prepared and assayed as described in Table I except that the buffer utilized for extraction was 0.05 M Tris–HCl (pH 8.0) which was 1.0 mM with respect to ascorbate and the centrifugation was carried out at 100 000 × *g* for 60 min. 5.2 ml of supernatant fluid was applied to a column (1.5 cm × 30 cm, 0.2 ml/min flow rate) of Sephadex G-50 which had been equilibrated with 0.05 M Tris–HCl (pH 8.0) at 4°. Thymine 7-hydroxylase was eluted in the excluded high molecular weight fraction (as determined visually with Blue Dextran 2000). The complete system contained (in 0.2 ml) 0.1 ml of the Sephadex-treated enzyme preparation (19 mg of protein per ml as determined by the biuret method[6]); [2-^{14}C]thymine, 50 mμmoles; Tris–HCl, (pH 8.0) 10 μmoles; $FeSO_4$, 5.0 mμmoles; ascorbate, 50 μmmoles; α-ketoglutarate, 50 mμmoles; and where indicated the following: GSH, 50 mμmoles; NADPH, 100 mμmoles; 0.1 ml of the enzyme preparation (26 mg of protein per ml) prior to its being subjected to Sephadex chromatography.

Complete system		*5-Hydroxymethyluracil produced (mμmoles)*
Omissions	*Additions*	
None	None	26.7
Sephadex-treated enzyme	Enzyme prior to Sephadex treatment	29.1
Sephadex-treated enzyme	None	0
α-Ketoglutarate	None	0
α-Ketoglutarate	NADPH	0
Fe^{2+}	None	0
Ascorbate	None	0
None	NADPH	25.0
None	GSH	33.2

active enzyme preparation was usually obtained. However, as seen in Table II, the addition of ascorbate to the Sephadex-treated enzyme did not restore activity.

The testing of α-ketoglutarate was prompted by the stimulating effect of this compound on collagen proline hydroxylase[5]. Table II evidences that the presence of Fe^{2+}, ascorbate and α-ketoglutarate in the incubation mixture does restore activity to the Sephadex-treated enzyme. The addition of these compounds permitted the recovery of the total activity of enzyme preparations which had been subjected to either Sephadex G-50 chromatography or dialysis. NADPH under these conditions is no longer stimulatory and will not replace ascorbate, Fe^{2+} or α-ketoglutarate. Possibly the reason the less purified enzyme system was stimulated by the presence of NADPH was that it effected the formation of one of these cofactors. While neither fumarate nor succinate were able to replace α-ketoglutarate, oxaloacetate, isocitrate or glutamate did to a small extent when tested with several enzyme preparations. Comparative kinetic studies and the fact that these compounds were completely ineffectual in replacing α-ketoglutarate when tested with several very active enzyme preparations suggest that these compounds appear stimulatory because they are initially converted to α-ketoglutarate which is the true requirement.

Preliminary studies indicate that substantial purification of thymine 7-hydroxylase has been carried out and that these purified preparations are also dependent on the inclusion of Fe^{2+}, ascorbate and α-ketoglutarate in the incubation mixture.

This investigation was supported by grant AM09314-02 from the National

Institutes of Health, U. S. Public Health Service. One of us (T.S.P.) was supported as an NSF Undergraduate Research Participant. The signal encouragement and advice of Dr. ROBERT M. FINK is gratefully acknowledged.

Department of Chemistry,
San Diego State College,
San Diego, Calif. (U.S.A.)

M. T. ABBOTT
E. K. SCHANDL
R. F. LEE
T. S. PARKER
R. J. MIDGETT

1 R. M. FINK AND K. FINK, *Federation Proc.*, 21 (1962) 337.
2 R. M. FINK AND K. FINK, *J. Biol. Chem.*, 237 (1962) 2289.
3 M. T. ABBOTT AND R. M. FINK, *Federation Proc.*, 21 (1962) 377.
4 M. T. ABBOTT, R. J. KADNER AND R. M. FINK, *J. Biol. Chem.*, 239 (1964) 156.
5 J. J. HUTTON, JR., A. L. TAPPEL AND S. UDENFRIEND, *Biochem. Biophys. Res. Commun.*, 24 (1966) 179.
6 E. LAYNE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957. p. 450.

Received October 19th, 1966

Biochim. Biophys. Acta, 132 (1967) 525–528

BBA 61125

A kinetic study of the mechanism of action of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase in *Escherichia coli* K 12

The first step in the biosynthesis of aromatic amino acids in microorganisms is the formation of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) from erythrose 4-phosphate and phosphoenolpyruvate (ref. 1). Three distinct isoenzymes of DAHP synthase (7-phospho-2-oxo-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate phosphorylating), EC 4.1.2.15) which catalyze the formation of DAHP were detected in *Escherichia coli* K 12 and W (refs. 2–4). All these DAHP synthases catalyze the formation of DAHP, but the activity of isoenzyme 1a is inhibited allosterically by phenylalanine. Isoenzyme 1b is inhibited by tyrosine and isoenzyme 1c is not inhibited by any of the aromatic end-products.

The present paper describes kinetic studies on the mechanism of action of purified phenylalanine-sensitive DAHP synthases and the results obtained are compatible with the mechanism termed "Ping-Pong"[5].

Wild-type cells of *E. coli* K 12 were grown on mineral salts medium A containing 0.2% glucose[6]. Cells harvested from logarithmic-phase culture were washed twice in 0.1 M potassium phosphate buffer (pH 7.4), disrupted by sonic treatment and the extract was centrifuged. From the supernatant the phenylalanine-sensitive DAHP synthase was purified 48-fold in comparison to the crude extract by ammonium sulfate

Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; E4P, erythrose 4-phosphate; PEP, phosphoenolpyruvate.