L. BOSCH
H. BLOEMENDAL
M. SLUYSER*

Department of Biochemistry, Antoni van Leeuwenhoek-Huis, The Netherlands Cancer Institute, Amsterdam (The Netherlands)

[1] M. B. HOAGLAND, P. L. STEPHENSON, J. F. SCOTT, L. I. HECHT AND P. C. ZAMECNIK, *J. Biol. Chem.*, 231 (1958) 241.
[2] H. BLOEMENDAL AND L. BOSCH, *Biochim. Biophys. Acta*, in the press.
[3] J. W. LITTLEFIELD, E. B. KELLER, J. GROSS AND P. C. ZAMECNIK, *J. Biol. Chem.*, 217 (1955) 111.
[4] T. HULTIN AND A. VON DER DECKEN, *Exptl. Cell Research*, 16 (1959) 444.
[5] J. W. LITTLEFIELD AND E. B. KELLER, *J. Biol. Chem.*, 224 (1957) 13.

Received April 3rd, 1959

* Laboratory for Inorganic Chemistry of the University of Amsterdam, working under the auspices of The Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

Enzymic cleavage of phosphoramidic acid

The chemical hydrolysis of phosphoramidate has been thoroughly investigated by RATHLEV AND ROSENBERG[1]. Their results indicate that at pH values above 4 the rate of hydrolysis is proportional to the concentration of the anion. Phosphoramidate has been suggested as a metabolic intermediate both by RATHLEV AND ROSENBERG[1] and by SPECK[2], but no evidence for its participation in metabolic sequences has been presented. In view of its chemical reactivity[3] and the recent evidence for metabolic activity of adenylic-5'-phosphoramidate[4], a reinvestigation of possible enzymic reactions of phosphoramidate was undertaken.

Sonic extracts prepared from *Escherichia coli* (Crookes strain), grown on a mineral-salts medium with succinate as the sole carbon source, catalyzed a rapid evolution of NH_3 from phosphoramidate. Boiled extracts were without catalytic activity. Treatment of the *E. coli* extracts with protamine sulfate followed by fractionation with $(NH_4)_2SO_4$ and rigorous dialysis of the fractions revealed at least two separate enzyme systems capable of catalyzing the release of NH_3 from phosphoramidate. Fraction I, which was precipitated by $(NH_4)_2SO_4$ below 0.5 saturation, was shown to require a divalent metal (Mg^{++} or Mn^{++}) and a sulfhydryl compound (cysteine or glutathione) to achieve a maximum rate of NH_3 release (Table I). The optimum pH for activity of Fraction I was 7.4. Paper chromatography of a reaction mixture containing Fraction I, phosphoramidate, cysteine and Mg^{++} with *n*-propanol–NH_4OH–H_2O (6:3:1) as a developing solvent revealed inorganic phosphate and phosphoramidate as the only phosphate-containing compounds present. It is assumed from these results that the reaction catalyzed by Fraction I is the cleavage of phosphoramidate to phosphate and NH_3. Even with the mildest conditions phosphate and phosphoramidate cannot be differentiated colorimetrically since molybdate catalyzes a very rapid hydrolysis of the latter[1].

Another *E. coli* fraction, Fraction III, precipitated between 0.58 and 0.9 saturation with $(NH_4)_2SO_4$, was also shown to catalyze a rapid evolution of NH_3 from phosphoramidate, while the intermediate fraction (0.5 to 0.58 saturation) was low in catalytic activity. The rate of NH_3 release catalyzed by Fraction III was maximal at pH 5.1 and was not stimulated by the addition of either divalent metals or reducing

TABLE I

PHOSPHORAMIDATE CLEAVAGE BY *E. coli* ENZYMES

System	μmoles NH_3 formed in 10 min***	
	Fraction I*	Fraction III**
1. Complete	5.50	3.40
2. Minus phosphoramidate	0.90	0.25
3. Minus Mg^{++}	3.25	3.40
4. Minus Cysteine	5.20	3.45
5. Minus (Mg^{++} and Cysteine)	2.25	3.40
6. Minus Enzyme	0.70	0.80
7. Complete plus F^- (3 μmoles)	4.70	1.95
8. Complete plus F^- (10 μmoles)	1.50	1.00

* The complete system contained in 0.5 ml: tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.4, 100 μmoles; phosphoramidate, 10 μmoles; $MgCl_2$, 10 μmoles; cysteine, 5 μmoles; enzyme, about 0.9 mg protein (*E. coli*, $(NH_4)_2SO_4$ fraction precipitated below 0.50 satn.). Incubation at 37°.

** The complete system contained in 0.5 ml, acetate buffer, pH 5.5, 100 μmoles; phosphoramidate, 10 μmoles; $MgCl_2$, 10 μmoles; cysteine, 5 μmoles; enzyme, about 0.3 mg protein (*E. coli* $(NH_4)_2SO_4$ fraction precipitated between 0.58 and 0.9 satn.). Incubation at 37°.

*** Reactions were stopped by the addition of 0.5 ml of absolute alcohol and an aliquot of the reaction mixture was placed in a Conway micro-diffusion dish; after sealing the Conway dish the sample and 1 ml 4 *M* K_2CO_3 were mixed in the outer chamber. NH_3 was distilled into 1 ml 0.05 *N* H_2SO_4 in the inner chamber at 50°. After a 1-h distillation period, NH_3 was determined by Nesslerization of an aliquot taken from the H_2SO_4 trap.

substances (Table I). Furthermore, dialysis of this fraction against 0.02 *M* ethylenediaminetetraacetate at pH 7.5 followed by dialysis against 0.02 *M* potassium phosphate (pH 7.5) neither inactivated the enzyme nor rendered it dependent on divalent metals. The hydrolysis of phosphoramidate catalyzed by both fractions was markedly inhibited by fluoride at low concentrations (Table I). Chelating agents were without effect on the rate of NH_3 release catalyzed by Fraction III at either pH 5.1 or 7.4. Paper chromatography of a reaction mixture containing Fraction III, phosphoramidate and acetate buffer (pH 5.5) revealed again phosphate and phosphoramidate as the only phosphate-containing compounds present.

Preliminary results indicate that the phosphoramidate hydrolase activity of Fraction III can be purified by diethylaminoethyl-cellulose chromatography about 70-fold. In this more purified state the hydrolase preparation does not catalyze the hydrolysis of pyrophosphate, glucose-6-phosphate, phosphoserine, α-glycerophosphate, glutamine, asparagine or adenylic-5'-phosphoramidate. Further purification of Fraction I has not yet been achieved.

Extracts of several micro-organisms including *E. coli*, *Acetobacter peroxydans*, *Fusarium moniliforme* and baker's yeast as well as beef-kidney, rabbit-muscle, and pea-seedling extracts have been shown to catalyze a rapid release of NH_3 from phosphoramidate. In view of the wide occurrence of phosphoramidate-cleavage enzymes, it seems likely that other metabolic functions of this compound may be revealed only after removal of these enzymes.

This investigation was supported in part by a grant from the Williams Waterman Fund of the Research Corporation.

Department of Chemistry, University of California at Los Angeles, Los Angeles, Calif. (U.S.A.) ROBERTS A. SMITH
D. J. BURROW

[1] T. RATHLEV AND T. ROSENBERG, *Arch. Biochem. Biophys.*, 65 (1956) 319.
[2] J. F. SPECK, *J. Biol. Chem.*, 179 (1949) 1405.
[3] R. W. CHAMBERS AND H. G. KHORANA, *Chem. and Ind.*, 1022 (1956).
[4] N. KATUMUMA, *Arch. Biochem. Biophys.*, 76 (1958) 547.

Received April 20th, 1959

Direct enzymic conversion of malonic semialdehyde to acetyl-coenzyme A

An enzyme obtained from a strain of *Pseudomonas fluorescens* has been reported[1] to catalyze the hydration of acetylenemonocarboxylic acid with the formation of malonic semialdehyde. The present communication is prompted by the discovery of a novel reaction for the further utilization of malonic semialdehyde. This involves a CoASH- and DPN-linked conversion to form acetyl-SCoA. The system is experimentally irreversible:

$$OHCCH_2COOH + DPN^+ + CoASH \rightleftharpoons CH_3COSCoA + CO_2 + DPNH + H^+ \qquad (1)$$

If the reaction proceeds by way of a CoASH-linked aldehyde dehydrogenase[2] followed by decarboxylation[3], malonyl-SCoA would be the expected first product. The system described is unique in that malonyl-SCoA has been ruled out as a free intermediate.

The product of reaction (1) has been shown to be a thiol ester which was identified as acetyl-SCoA by arsenolysis with phosphotransacetylase[4], and chromatography of the hydroxamate derivative formed from the isolated thiolester, with the solvent system of VAGELOS*. The last method definitively separates acetylhydroxamate (R_F, 0.62) from malonylmonohydroxamate (R_F, 0.14). The enzyme has been purified 200-fold by a combination of salt fractionation, elution from calcium phosphate gel and chromatography on N,N-diethylaminocellulose. Combination of eluate fractions produced only additive effects on activity; the system behaves as a single enzyme.

The stoichiometry of the reaction, detailed in Table I, is that expected for

TABLE I

STOICHIOMETRY OF THE REACTION

Incubations were carried out in the presence of the following, expressed as μmoles/ml: K-phosphate, pH 7.0, 50; mercaptoethanol, 5; an excess of the enzyme. Expts. 1 and 2 included CoASH, 0.3; malonic semialdehyde, 0.4; limiting quantities of DPN. When the reaction was completed as judged by following the formation of DPNH spectrophotometrically, aliquots were removed and assayed for aldehyde and thioester. Expts. 3 and 4 were conducted in manometric vessels at 30° and contained DPN, 4 and CoASH, 4. Expts. 3 and 4 included 4.0 and 4.2 μmoles, respectively, of malonic semialdehyde. After 20-min incubation, the reaction was terminated by the addition of 0.1 ml 1 *N* H_2SO_4 and the CO_2 evolved was measured. Aliquots were taken for aldehyde and hydroxamate determinations. All results are expressed in μmoles.

Expt.	*Malonic Semialdehyde*	*DPNH*	*Thiol ester*	CO_2
1	— 0.096	+ 0.089	+ 0.082	
2	— 0.150	+ 0.162	+ 0.156	
3	— 4.0			+ 3.6
4			+ 3.5	+ 3.8

Abbreviations: CoASH, coenzyme A; DPN+, diphosphopyridine nucleotide.
* Personal communication from Dr. P. R. VAGELOS.