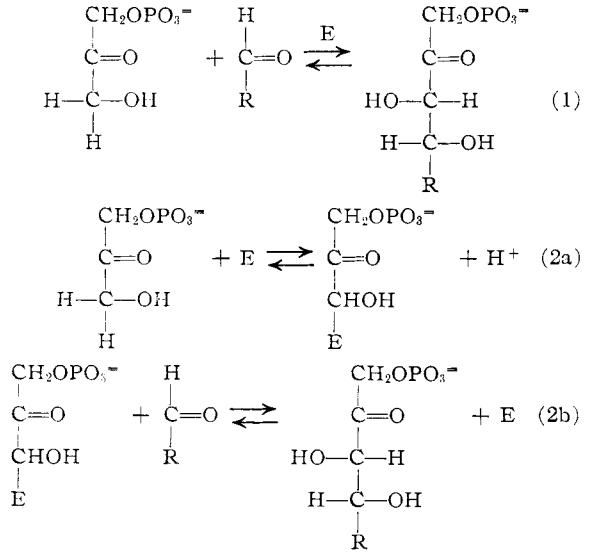

DHAP, HDP, or a mixture of DHAP and HDP was incubated with 6 times recrystallized rabbit muscle aldolase[3] (free of triose-phosphate isomerase) in the presence of tritiated water and the reaction was stopped by the addition of Ag^+ ions. The DHAP and HDP were isolated by use of Dowex-1 (Cl^-) ion exchange resin and counted in a liquid scintillation counter. Experiment 1 of Table I shows that the DHAP became radioactive upon incubation with aldolase, but that no isotope incorporation

TABLE I

THE INCORPORATION OF TRITIUM INTO DHAP BY ALDOLASE

Each incubation mixture contained, in addition to the noted components, an amount of aldolase which, in experiment 1, would have split 75 μmoles of HDP, and in Experiment 2, 100 μmoles of HDP at 30° in the 30 minutes of the incubations. The total radioactivity of TOH was 6.53×10^6 c.p.m./ml. The final volume was 1.0 ml. and the *p*H was 7.4 (by adjustment). The reaction was stopped by adding $AgNO_3$ to a final concentration of 2×10^{-5} M and the DHAP and HDP were isolated by ion exchange on Dowex-1 (Cl^-). The samples were concentrated by lyophilization and counted (in a liquid scintillation counter made by Technical Measurement Corp., New Haven, Conn.) in a final mixture containing 0.5 ml. water, 5.0 ml. absolute ethanol, and 10 ml. of phosphor-toluene solution.[4]

Exp.	Additions (μmoles)	Sample counted (μmoles)	C.p.m. above background	Hydrogen exchanged (μmoles/μmole sample counted)
1	DHAP (14.5) with heated enzyme	DHAP (3.0)	0	0
	DHAP (14.5)	DHAP (4.0)	309	1.3
2	DHAP (10) + $AgNO_3$ (0.08)	DHAP (6.0)	0	0
	DHAP (10)	DHAP (4.6)	256	0.95
	HDP (8)	HDP (1.6)	7	0.07
	DHAP (10) + HDP (8)	DHAP (3.5) HDP (3.4)	173 2	0.84 0.01

occurred with heat-inactivated enzyme. This result supports reaction 2a. In experiment 2 of Table I it is shown that, under conditions in which the DHAP became labeled, the HDP was non-isotopic either in the presence or the absence of DHAP. It is also shown that Ag^+ ion, which completely inhibits the over-all reaction starting with HDP,[5] also inhibited the exchange of tritium with DHAP. It may be concluded from the non-labeling of the HDP that the enzyme is able to distinguish between the two hydrogens on the carbinol carbon of DHAP. That the labeling of DHAP is in excess of 1 μmole of exchangeable hydrogen may be attributed to isotope enrichment or experimental error.

The presence of small amounts of contaminating aldehyde in the incubation mixture of DHAP and enzyme could be responsible for the labeling of DHAP according to reaction 1. If this were true the addition of a condensation product to the incubation mixture containing DHAP and enzyme would stimulate the exchange reaction. On the other hand, according to reaction 2, the addition of a condensation product should not stimulate but could decrease the rate at which DHAP becomes labeled. The extent of this decrease would depend upon the relative "affinities" of the DHAP and condensation product for the enzyme. In a separate experiment the rate of labeling of DHAP was found to be much reduced by the addition of HDP to the incubation mixture. This finding further supports reaction 2 as the mechanism of action of aldolase.

These data do not permit one to distinguish between labilization of the hydrogen atom by the enzyme or actual displacement of it to form an enzyme-DHAP complex. However, in either case, the activation of the hydrogen atom is a feature common to the enzymatic and the base-catalyzed aldol condensations. Further work is in progress to elucidate the mechanism of the aldolase-catalyzed reaction.

The authors are grateful to Dr. Seymour Lipsky for the use of his scintillation counter, to Dr. E. Racker for a sample of α-glycerol phosphate dehydrogenase used in the assay of DHAP, to Dr. Melvin V. Simpson for a sample of triose phosphate dehydrogenase used in the assay of HDP, and to Mr. George Kalf for his help in the preparation of aldolase and DHAP.

(3) J. F. Taylor, A. A. Green and G. T. Cori, *J. Biol. Chem.*, **173**, 591 (1948).

(4) F. N. Hayes and R. G. Gould, *Science*, **117**, 480 (1953).

(5) D. Herbert, H. Gordon, V. Subramanya and D. E. Green, *Biochem. J.*, **34**, 1108 (1940).

DEPARTMENT OF BIOCHEMISTRY
YALE UNIVERSITY SCHOOL OF MEDICINE IRWIN A. ROSE
NEW HAVEN, CONN. SIDNEY V. RIEDER

RECEIVED SEPTEMBER 19, 1955

THE ENZYMATIC SYNTHESIS OF β-ALANYL COENZYME A

Sir:

In the course of studies to determine whether acrylyl Co A[1] is an intermediate in propionyl Co A oxidation by extracts of *Clostridium propionicum*, it was observed that a reaction is catalyzed between acrylyl Co A and ammonia to form β-alanyl Co A.

$$CH_2{=}CHCOS\ CoA + NH_3 \longrightarrow CH_2NH_2CH_2COS\ CoA$$

The enzyme catalyzing this reaction has been purified about 10-fold by means of protamine and ammonium sulfate precipitations. Since the enzyme

(1) CoA and Pa are abbreviations used for Coenzyme A and Pantotheine, respectively.

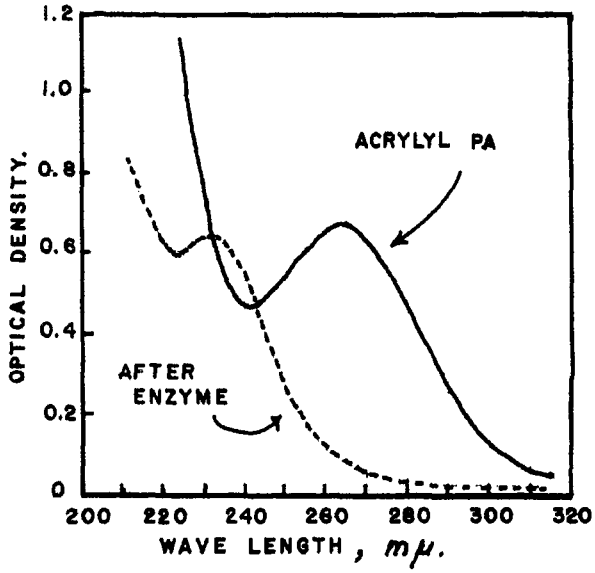

Fig. 1.—The reaction mixtures contained acrylyl Pa, 0.1 μM.; ammonium chloride, 50 μM.; potassium phosphate buffer, 50 μM. (pH 7.1); and 14 γ of enzyme where indicated in a total volume of 1.0 ml. The spectra were determined after incubation for two hours at 25°. The spectra of the sample with enzyme have been corrected for optical density of the enzyme alone.

also catalyzes the corresponding reaction between acrylyl Pa[2,2a] and ammonia to form β-alanyl Pa and since acrylyl Pa could be obtained in a relatively high state of purity, it was used in most of these studies. As shown in Fig. 1, acrylyl Pa has a strong light absorption band in the region of 240–300 mμ. Upon incubation with enzyme and ammonium salts, the absorption at 250–300 mμ disappears and a new substance is formed with an absorption maximum at 230–235 mμ. Accordingly, the reaction may be followed by measuring the decrease in optical density at 263–300 mμ. It can be seen from Table I that only in the presence of ammonium salts and enzyme is there a rapid disappearance of the acrylyl derivatives. The reaction product from experiment II, Table I, was isolated in good yield by means of paper chromatography in a solvent system composed of 20 parts water and 80 parts propanol (v./v.). It was identified as β-alanyl Pa by chromatographic and spectroscopic comparison with a synthetic sample of β-alanyl Pa.[4] The compound chromatographs on paper in the above solvent system with an R_f = 0.42 and shows

(2) Acrylyl CoA and acrylyl Pa were prepared by shaking an ether solution of the mixed anhydride of ethyl hydrogen carbonate[5] and acrylic acid with aqueous solutions of the respective mercaptans (CoA or Pa) in 0.15 M collidine buffer (pH 7.1). The acrylyl CoA which was obtained in only 8–10% yield was used without further purification. The acrylyl Pa was purified by ethylacetate extraction from neutral solution followed by chromatography on well-washed paper in a water-saturated butanol solvent.

(2a) We are indebted to Dr. O. D. Bird of Parke, Davis and Co. for the sample of pantotheine for these experiments.

(3) The mixed anhydride of ethylhydrogen carbonate and acrylic acid and the β-alanyl chloride·hydrochloride were prepared by Dr. H. T. Miles.

(4) β-Alanyl pantotheine was prepared by adding solid β-alanyl chloride·hydrochloride[3] to an aqueous solution of pantotheine in 0.1 M triethanolamine buffer (pH 7.4), and was purified by paper chromatography in a propanol (80%)-water (20%) solvent system.

TABLE I

THE ENZYMATIC CONVERSION OF ACRYLYL PA AND ACRYLYL CoA TO β-ALANYL DERIVATIVES

	Expt. I	Expt. II		Expt. III
	ΔAcrylyl-Pa[b]	ΔAcrylyl-Pa[b]	Δβ-alanyl-Pa	ΔAcrylyl-Coa[c]
Complete system[a]	−2.03	−2.02	+2.11,[d] 1.99[e]	−0.60
−Enzyme	−0.03	0	+0.12[e]	0
−NH_4Cl	−0.36			−0.09

[a] The complete systems for the experiments were as follows: (I) 2.06 μM. of acrylyl-Pa, 100 μM. of ammonium chloride, 50 μM. of potassium phosphate buffer (pH 7.8), and 9 γ of enzyme in a final volume of 0.7 ml.; (II) 2.6 μM. of acrylyl-Pa, 100 μM. of NH_4Cl, 50 μM. of potassium phosphate buffer (pH 7.4) and 70 γ of enzyme in a final volume of 0.55 ml.; (III) 0.6 μM. of acrylyl Co A, 400 μM. of NH_4Cl, 300 μM. of potassium phosphate buffer (pH 7.1) and 4.5 γ of enzyme in a volume of 3.0 ml. Samples were incubated at 25° for 12 minutes. [b] The Δ acrylyl Pa was calculated from the change in optical density at 263 mμ. [c] Since the ϵ_{max} of the acrylyl thiolester group coincides with the ϵ_{max} of the adenine moiety of Co A, the acrylyl CoA was calculated from the change in optical density at 295 mμ. [d] Determined as β-alanine by the ninhydrin method after hydrolysis in 0.1 N KOH, 15 min., 25°. [e] Determined by optical density measurements at 232 mμ.

a sharp absorption maximum at 232 mμ characteristic of thiolesters of saturated fatty acids. It gives a yellow spot on paper chromatograms when treated directly with ninhydrin, but after hydrolysis with 0.1 N KOH for 5 min. at 25° it gives a blue color reaction with ninhydrin due to the release of β-alanine; β-alanine was identified as a product of alkaline hydrolysis by paper chromatography in a variety of solvent systems. The β-alanyl Pa reacts with hydroxylamine to give a hydroxamic acid which moves on paper chromatograms in a water-butanol (18:100 v./v.) solvent system with an R_f of 0.052.

The β-alanyl Co A which was formed in experiment III, Table I, was partially purified by paper chromatography in a solvent system composed of equal volumes of ethanol and 0.1 N sodium acetate buffer (pH 4.5), and was characterized on the basis of reactivity with ninhydrin, hydroxylamine and dilute alkali; β-alanine was identified as a product of mild alkaline hydrolysis.

So far all attempts to show reversibility of these reactions have failed. Neither synthetic β-alanyl-Pa nor the isolated enzymatic product will produce acrylyl Pa when incubated with the enzyme in the absence of ammonium ions. It is not yet known what role β-alanyl Co A has in the metabolism of *C. propionicum*. The oxidation of propionate by dried cell suspensions of this organism leads to the accumulation of β-alanine. In light of the above experiment, this formation of β-alanine is presumptive evidence that acrylyl Co A is an intermediate in propionate oxidation. It is significant that β-alanyl Co A represents the first biologically produced Co A derivative of an amino acid. Such derivatives might play a role as precursors in the biosynthesis of β-alanyl peptides such as anserine and carnosine.

NATIONAL HEART INSTITUTE
NATIONAL INSTITUTES OF HEALTH
PUBLIC HEALTH SERVICE E. R. STADTMAN
DEPARTMENT OF HEALTH, EDUCATION AND WELFARE
BETHESDA 14, MARYLAND

RECEIVED MAY 27, 1955