724. *Coenzyme A. Part VI.* The Identification of Pantothenic Acid-4′ and -2′ : 4′ Phosphates from a Hydrolysate.*

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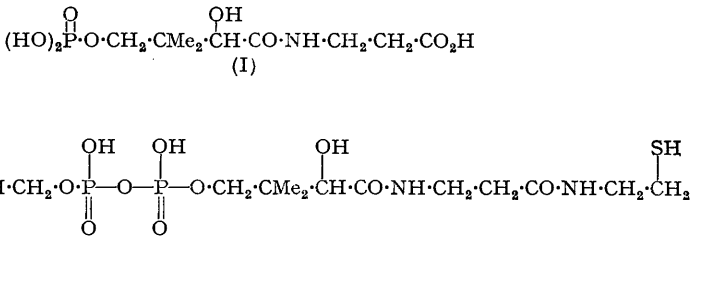
Cautious alkaline hydrolysis of coenzyme A gives, in addition to adenine nucleotides and 2-mercaptoethylamine, three phosphorylated derivatives of pantothenic acid. These have been isolated by the use of ion-exchange resins and chromatography on cellulose, and two of them identified as pantothenic acid-4′ and -2′ : 4′ phosphates; the third is probably the 2-mercaptoethylamide of the cyclic 2′ : 4′-phosphate. These findings support our earlier suggestions about the structure of the coenzyme.

In Part II of this series (Baddiley and Thain, *J.*, 1951, 2253) it was shown that among the products of acid or alkaline hydrolysis of coenzyme A was a substance indistinguishable on paper chromatography from pantothenic acid-4′ phosphate (I). The conclusion that the synthetic 4′-phosphate was, in fact, identical with the natural product, together with more indirect supporting evidence, led us to propose the tentative structure (II) for the coenzyme. As certain microbial growth experiments (see later) were not entirely explained by this formula we considered that the actual isolation and chemical study of the pantothenic acid phosphate fragment or fragments from coenzyme A should be attempted. However, only 10 mg. of coenzyme were available for our initial experiments. Through the courtesy of Dr. F. Lipmann a somewhat larger sample of impure coenzyme has now been hydrolysed and the products separated and isolated. Although this material was not more than about 25% pure, as shown by its capacity to effect acetylation of sulphanilamide under specified

* Part V, *J.*, 1952, 800.

conditions in the presence of a liver preparation, it sufficed for our purposes. Quite impure coenzyme can be used in such experiments as those described in this paper without serious risk of obtaining invalid results provided that attention is confined to the pantothenic acid moiety and not to other parts of the molecule. This follows from the observation that all, or nearly all, of the " bound " pantothenic acid occurring in Nature is present as coenzyme A (Novelli and Schmetz, *J. Biol. Chem.*, 1951, **192**, 181). Consequently, any pantothenic acid derivatives isolated from even crude coenzyme concentrates must have originated from coenzyme A itself by hydrolytic decomposition. It was considered most probable that the low activity in the acetylation test was the result of partial hydrolysis during preparation of this sample.



(I)

(II)

Alkaline hydrolysis was effected under conditions similar to those described in Part II but the length of the hydrolysis was reduced in order to demonstrate the production of unstable intermediates. Nucleotides, adenosine, amino-acids, and other substances containing basic groups were removed together with the alkali by passage through a column of the sulphonic acid type resin " Amberlite IR-120 " in its acidic state. Paper chromatography of the resulting eluate showed that, in addition to the spot corresponding to pantothenic acid-4′ phosphate, three other organic phosphate-containing spots were present; these four products we designate A, B, C, and D in order of decreasing R_F values. By methods recorded in the Experimental section, 300 mg. of crude coenzyme A yielded 11·3 mg. of B and 9·2 mg. of C.

Substances A, B, and C were phosphates of pantothenic acid since they each gave β-alanine on acid hydrolysis. B was indistinguishable on paper chromatography in two different solvent systems from the synthetic 2′ : 4′ cyclic phosphate (III). Furthermore, like the synthetic substance it was relatively stable to acid and was hydrolysed by hot alkali to the 4′-phosphate (Part III, *J.*, 1951, 3421). Visual estimation of the relative intensities of the 2′ : 4′- and 4′-phosphate spots on paper after varying periods of hydrolysis with alkali indicated that opening of the cyclic structure occurred at the approximately same rate with both synthetic and natural cyclic phosphates. Conclusive evidence of identity of the two was obtained by a comparison of the rate of liberation of inorganic phosphate during acid hydrolysis and by infra-red spectroscopy. The curves obtained for the rate of phosphate liberation from both synthetic and natural substances were identical and differed markedly from those obtained for the 2′- and 4′-phosphates. First-order reaction kinetics were not observed. The cyclic phosphate was hydrolysed at a slower rate than was the 4′-phosphate, thereby suggesting that ring-opening and hydrolysis of the resulting phosphate occurred at comparable rates (cf. Fig. 1).

The infra-red absorption spectra of B and pantothenic acid-2′ : 4′ phosphate were indistinguishable (see Fig. 2). The small band at 13·13 μ is attributed to the presence of a slight impurity in the natural compound.

Although C was homogeneous with respect to organic phosphate it was probably impure since its infra-red absorption spectrum was ill-defined and unsuitable for comparison with that shown by the synthetic phosphates. However, it was identified as pantothenic acid-4′ phosphate from its behaviour on paper chromatography and by the fact that, like the synthetic compound, it was converted into pantothenic acid by prostate and intestinal phosphatase.

Whereas B and C were free from thiol-containing substances, A, the fastest-running product, gave a strong thiol-reaction on paper with the cyanide–nitroprusside spray. From its relatively high R_F, it seemed likely that this substance contained a di- or tri-substituted phosphate residue and this was confirmed by the observation that on further

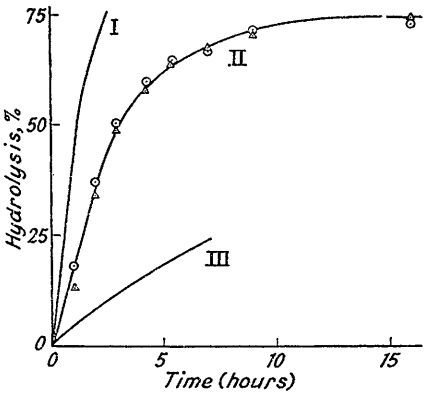


FIG. 1. *Acid hydrolysis of panothenic acid phosphates.*

I, Pantothenic acid-4′ phosphate.
II, Panothenic acid-2′ : 4′ phosphate.
⊙ Synthetic, △ From co-enzyme A.
III, Pantothenic acid-2′ phosphate.

cautious alkaline hydrolysis it was converted into pantothenic acid-2′ : 4′ phosphate together with a small amount of the 4′-phosphate. The estimated amounts present in the coenzyme A hydrolysate did not justify an attempt to isolate it in a pure state; consequently its structure could not be determined with certainty. However, on the evidence available

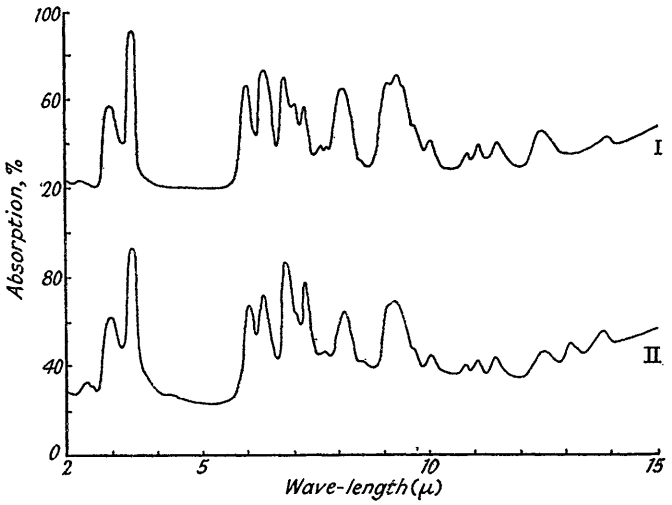
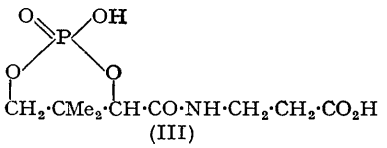


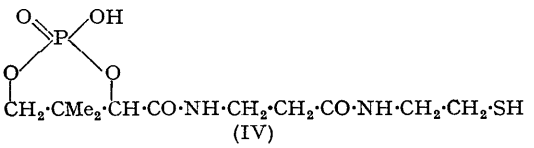
FIG. 2. *Infra-red spectra of pantothenic acid-2′ : 4′ phosphate.*

I, Synthetic.
II, From co-enzyme A.

it is probably the cyclic phosphate (IV) of pantotheine which would be expected as an early hydrolysis product of coenzyme A.



(III)



(IV)

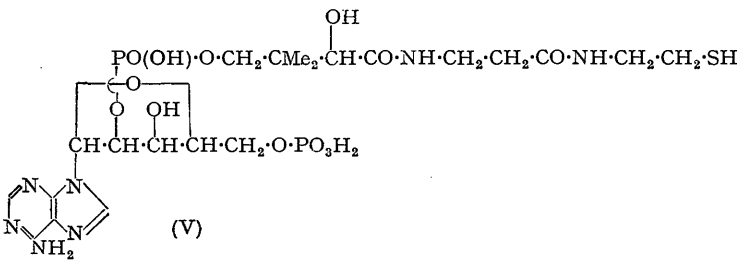
Fraction D has not yet been studied in detail. Although homogeneous with respect to phosphate its purity is not known. Whereas A, B, and C are pantothenic acid derivatives, giving β-alanine on acid hydrolysis, fraction D was free from β-alanine but in acid gave α-alanine, identified by chromatography on paper in two solvents. From the fact that it was not retained by the strongly acidic " Amberlite IR-120 " resin it is highly probable that the amino-group of the alanine residue is substituted by an acyl group. The only known

derivatives of α-alanine occurring in Nature as phosphoric esters are the uridine diphosphate nucleotides recently described by Park (*J. Biol. Chem.*, 1952, **194**, 897). The detailed structure of these substances has not yet been disclosed but it seems not unlikely that the α-alanine-containing fraction D may have arisen from similar substances accompanying coenzyme A in the earlier stages of purification.

The isolation of the 4′- and cyclic phosphates (I and III) from the coenzyme confirms several of the earlier conclusions about the structure of coenzyme A (cf. Parts II and III, *locc. cit.*) as will be seen from the following discussion. In the first place there can be no doubt that the coenzyme bears a phosphate substituent directly attached to the pantothenic acid moiety. Now the cyclic phosphate structure can not be present in the original coenzyme as was shown in Part III. The formation of a cyclic phosphate in alkali is entirely consistent with formula (II) for coenzyme A and may be visualised as having arisen through intramolecular phosphorylation. In this connection it is known that substituted pyrophosphates are powerful phosphorylating agents and a close analogy may be drawn between this example and the similar cyclisations which have been observed by the action of alkalis on other dinucleotides, *e.g.*, Forrest and Todd (*J.*, 1950, 3295) noted the formation of riboflavin-4′ : 5′ phosphate by the action of ammonia on flavin-adenine dinucleotide. Similarly, Leloir (*Arch. Biochem.*, 1951, **33**, 186) obtained a cyclic glucose phosphate from an alkali hydrolysate of uridine diphosphate-glucose.

A cyclic phosphate might arise from a structure such as (II) or from a similar structure in which the pyrophosphate linkage is attached at position 2′ (the secondary hydroxyl group) in the pantothenic acid moiety. A 2′-phosphate residue can not be present, however, since acid hydrolysis of the coenzyme gives pantothenic acid-4′ phosphate and no -2′ phosphate. It has been observed earlier (Part II, *loc. cit.*) that monophosphates of pantothenic acid show no tendency to isomerise under acid conditions and so it is concluded that the phosphate linkage in question in coenzyme A is situated at the 4′-position.

Now whereas the experiments recorded in this paper are in good agreement with the pyrophosphate formulation the alternative formula (V) or its adenosine-3′ substituted



(V)

isomer already discussed in Part II can not be ruled out entirely. It will be seen that (V) contains the disubstituted monophosphate grouping in which an unsubstituted hydroxyl, the 2′-hydroxyl group in the pantothenic acid residue, may be available for accepting a phosphate group. This structure is somewhat analogous to the ribose phosphate residues in ribonucleic acid where it has been suggested that cyclisation may precede degradation during alkaline hydrolysis (Brown and Todd, *J.*, 1952, 52). On the other hand, such a structure is unlikely in view of the very great lability of coenzyme A to alkali and acids, which is more in keeping with a pyrophosphate than a disubstituted monophosphate of this type. Contrary to earlier statements (King and Strong, *J. Biol. Chem.*, 1951, **189**, 325) Novelli has confirmed (*Phosphorus Metabolism*, 1951, **1**, 414) that coenzyme A is split by a dinucleotidase, thus proving that the coenzyme is a dinucleotide.

Pantothenic acid-4′ phosphate shows no growth promoting action towards *A. suboxydans* whereas an enzymic degradation product of coenzyme A which was stated to be free from both nucleotides and sulphur-containing substances was effective. In Part III (*loc. cit.*) we suggested that this effect might be explained by the presence in the molecule of a substituent which was essential for biological acitivity but which had so far escaped detection. If this were the correct explanation then the most probable position for this substituent

would be 2′ in the pantothenic acid residue. The ready formation of a cyclic phosphate by the action of alkali on the coenzyme, however, implies that this group must be very labile to alkali. Drs. Novelli and Lipmann have informed us, however, that their degradation product may contain sulphur and consequently it is no longer necessary to postulate the presence in the coenzyme of a substituent at the 2′ position.

Experimental

The coenzyme A used in these experiments had an activity of 110 units/mg. when assayed by the liver-enzyme method (Kaplan and Lipmann, *J. Biol. Chem.*, 1948, **174**, 37) or 43 units/mg. by bacterial arsenolysis (Stadtman, Novelli and Lipmann, *ibid.*, 1951, **191**, 365).

Hydrolysis of Coenzyme A.—Coenzyme A (300 mg.) was heated with N-sodium hydroxide (3 c.c.) at 100° for 30 minutes in a platinum tube. The resulting brown solution was heated with charcoal (*ca.* 0·1 g.) for a few minutes and filtered through kieselguhr which was then washed thoroughly with water. Sodium and basic materials were removed from the bulked filtrate and washings by passage through a column (10 × 0·5 cm.) of "Amberlite IR −120" which had been activated by repeated washing with hydrochloric acid (2N), followed by water until the eluate was free from chloride ion. The acid eluate from the column was brought to pH 10 with barium hydroxide solution and the barium phosphate removed by centrifugation. The clear supernatant liquid was freed from barium by means of another column of IR-120, and the eluate neutralised with aqueous ammonia and dried from the frozen state.

Separation of Hydrolysis Products.—The dried ammonium salts were dissolved in water (0·3 c.c.) and to the solution was added ammonia (0·9 c.c.; *d* 0·88) and *n*-propyl alcohol (1·8 c.c.), the resulting yellow solution was passed through a previously washed column of Whatman's cellulose powder (60 × 1·8 cm.) and the chromatogram developed with *n*-propyl alcohol–ammonia–water (6 : 3 : 1). Fractions (*ca.* 3 ml.) were collected and those containing phosphate were run on paper chromatograms with the same solvent, with the following results :

Tube no.	R_F		Tube no.	R_F	
24—33	0·65	A	45—56	0·23	C
34—38	0·55	B	57—80	0·1—0·05	D

R_F of pantothenic acid-2′ : 4′ phosphate, 0·55. R_F of pantothenic acid-4′ phosphate, 0·23.

Tubes whose contents possessed the same R_F value were bulked and evaporated to dryness.

Properties of Fraction A.—The residue was dissolved in water (*ca.* 0·5 c.c.), and small portions (equivalent to 0·5 mg.) were removed for the following qualitative experiments : (*a*) The solution was heated with 6N-hydrochloric acid (0·2 c.c.) at 100° (sealed tube) for 3 hours, the hydrolysate was evaporated to dryness, and the residue run on an ascending paper chromatogram with the organic phase of *n*-butyl alcohol–acetic acid–water (4 : 1 : 5). After drying, the paper was sprayed with ninhydrin solution and heated at 90° for 10 minutes. Only one spot was formed, of R_F 0·3, identical with that produced from a standard β-alanine solution. (*b*) A drop of the solution was put on filter paper and after drying sprayed with cyanide–nitroprusside. A strong pink colour was produced indicating the presence of a thiol group. (*c*) The solution was heated with 0·3N-barium hydroxide (0·2 c.c.) at 100° for 1 hour, excess of barium precipitated by carbon dioxide, the barium carbonate removed by centrifugation, barium removed from soluble barium salts by treatment with a small amount of ammonium sulphate, barium sulphate removed by centrifugation, and the clear solution evaporated to dryness. The residue was run on a paper chromatogram with *n*-propyl alcohol–ammonia and developed by the usual method. Three spots were observed: (1) unchanged fraction A, R_F 0·65, (2) a spot identical with that of pantothenic acid-2′ : 4′ phosphate, R_F 0·55, and (3) one identical with that of pantothenic acid-4′ phosphate, R_F 0·23.

Further Alkaline Hydrolysis of Fraction A.—The remainder of fraction A was heated with N-sodium hydroxide (3 c.c.) at 100° for 1 hour. Sodium ions were removed as before on Amberlite IR-120, and the acid eluate neutralised with ammonia and evaporated to dryness. The ammonium salts were separated as above on a cellulose powder column (60 × 1·1 cm.) with *n*-propyl alcohol–ammonia, fractions of 3 c.c. being collected.

Tube no.	R_F
17	0·65, unchanged fast-moving material thiol-test positive.
19—26	0·55, pantothenic acid-2′ : 4′ phosphate.
28—35	0·25, pantothenic acid-4′ phosphate.

The contents of tubes 19—26 and 28—35 were evaporated to dryness and the residues bulked with fractions B and C respectively which were further purified as below.

Purification of Fraction B.—The ammonium salt was again passed through a cellulose powder column with *n*-propyl alcohol–ammonia, and fractions (3 c.c.) were collected. Those containing substance B (as determined by a paper chromatogram) were bulked and evaporated to dryness. The residue in water (1 c.c.) was run on to a column (15 × 0·5 cm.) of Amberlite IR-4B in its acetate form. After the column had been washed with acetic acid (75 c.c., 10%) the phosphate was eluted with 3N-ammonia, fractions (3 c.c.) being collected. Those containing phosphate were bulked and evaporated to remove excess of ammonia, ammonia from ammonium salts removed by passing the solution through Amberlite IR-120 in its acid form, the acid solution evaporated to dryness four times with water to remove acetic acid, and the residual acid neutralised with barium hydroxide. The solution was heated to boiling, carbon dioxide passed to precipitate excess of barium, the slight precipitate removed by centrifugation, and the clear solution dried from the frozen state. The solid was dissolved in water (0·5 c.c.), a slight insoluble fraction removed by centrifugation, and the clear solution again dried from the frozen state, yielding the barium salt of fraction B (11·3 mg.). This fraction had the same R_F value as the synthetic pantothenic acid-2′ : 4′ phosphate in *n*-propyl alcohol–ammonia-water (R_F 0·25) and *iso*butyric acid–ammonia–water (R_F 0·50).

Identification of β-*Alanine.*—Fraction B (1 mg.) was heated with 6N-hydrochloric acid (0·2 ml.) for 3 hours at 100°, the hydrolysate was evaporated, barium removed by the addition of a little ammonium sulphate, and the residue run on a paper chromatogram in *n*-butanol–acetic acid–water, and developed with ninhydrin. A spot corresponding to β-alanine was produced (R_F 0·30).

Hydrolysis of Fraction A to Pantothenic Acid-4′ Phosphate.—Samples of pantothenic acid-2′ : 4′ phosphate and fraction B (0·5 mg.) were separately hydrolysed in 0·3N-barium hydroxide (0·2 c.c.) at 100° for 1 and 2 hours. The hydrolysates were worked up in the usual way and the products run on a paper chromatogram in *n*-propyl alcohol–ammonia. After 1 hour the cyclic phosphate and fraction A (R_F 0·50) were approximately half-converted into pantothenic acid-4′ phosphate (R_F 0·25) whilst after 2 hours this change was practically complete.

Determination of Rate of Hydrolysis of Fraction B and Pantothenic Acid-2′ : 4′ Phosphate.—The barium salts of the two phosphates (5 mg.) were separately dissolved in 2N-hydrochloric acid (10 c.c.), and aliquots (1·0 c.c.) of the solutions sealed in glass tubes. These were placed in a water-bath at 100° and individuals removed at intervals and stored at −10° until analysed. It was shown that no phosphorus was released from the glass during 24 hours' heating at 100° by this strength of acid and that the cyclic phosphate was not measurably hydrolysed at −10° over 48 hours. Phosphorus was measured colorimetrically by Allen's method (*Biochem. J.*, 1940, **34**, 858).

	Hydrolysis, %			Hydrolysis, %	
Time (hours)	Synthetic cyclic	Fraction B	Time (hours)	Synthetic cyclic	Fraction B
1	14·5	18	5·5	63·5	64·5
2	34	37	7	67	66·5
3	49	50	9	70·0	71·0
4·25	57·5	59·5	16	75·5	73·5

The greatest divergence between the figures for the synthetic cyclic phosphate and fraction B corresponds to an error of 1 μg. in the determination of phosphorus.

Purification and Properties of Fraction C.—Crude fraction C, as obtained from the cellulose column, was purified as described above for the fraction B. There was obtained a barium salt (9·2 mg.) possessing the same R_F values as pantothenic acid-4′ phosphate, in *n*-propyl alcohol–ammonia (R_F 0·25) and in *iso*butyric acid–ammonia (R_F 0·42). β-Alanine was detected in this fraction by the method given above for fraction B.

Purification and Properties of Fraction D.—The ammonium salt of fraction D was passed through a powdered cellulose column (50 × 1·5 cm.) with *n*-propyl alcohol–ammonia, fractions of 3 c.c. being collected. The fractions containing phosphorus were found by paper chromatography to contain a slow-moving organic phosphate of R_F 0·12 (same solvent); it was converted into the barium salt (20·7 mg.) by the usual method. A sample heated in 0·3N-barium hydroxide (0·2 c.c.) at 100° for 2 hours possessed unchanged R_F value in *n*-propyl alcohol–ammonia.

Acid hydrolysis of Fraction D for Amino-acids.—The barium salt of fraction D (1 mg.) was heated with 6N-hydrochloric acid (0·2 c.c.) at 100° for 3 hours, the hydrolysate evaporated to dryness, and barium removed as before. One-half of the solution was run on an ascending chromatogram in *n*-butyl alcohol–acetic acid–water along with a selection of amino-acids. On development with ninhydrin, fraction D gave a spot of R_F 0·22 corresponding to α-alanine

(R_F 0·22). The remaining half was run on a descending chromatogram in phenol saturated with water; again, fraction D gave a spot, 20 cm. from the base line, corresponding to α-alanine.

We are indebted to Dr. R. N. Haszeldine for the infra-red measurements and to the Department of Scientific and Industrial Research for a Special Research Grant.

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[*Received, May 14th*, 1952.]