753. *Coenzyme A. Part III.* Synthesis of Pantothenic Acid-2′ : 4′ Phosphate and Further Structural Considerations.*

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The cyclic phosphate of pantothenic acid (II; R = R′ = H) has been synthesised by phosphorylation of benzyl pantothenate with phenyl dichlorophosphinate followed by hydrogenolysis. Its behaviour on paper chromatography before and after hydrolysis has been studied. From its inactivity in the *Acetobacter* test it is concluded that this substance is not identical with any known degradation product of coenzyme A.

The earlier suggestion that a phosphate group is situated at 2′ or 3′ in the adenosine moiety of coenzyme A is supported by its behaviour towards periodate.

IN Part II * it was shown that coenzyme A gave on mild acid or alkaline hydrolysis a substance indistinguishable on paper chromatography in two different solvent systems from pantothenic acid-4′ phosphate which had been synthesised earlier (Baddiley and Thain, *J.*, 1951, 246). A tentative formula (I) was advanced for the coenzyme. However, the inability of this synthetic phosphate to stimulate the growth of *Acetobacter suboxydans* was somewhat disturbing. It is known (Novelli, Flynn, and Lipmann, *J. Biol. Chem.*, 1949, **177**, 493) that certain enzymic fission products of coenzyme A, in which both phosphate and pantothenic acid groupings are present, exhibit activity in this test. A possible explanation was advanced at the time, namely, that activity in this test required the integrity of the 2-mercaptoethylamine residue normally present in coenzyme A. From a study of the enzymic degradation of coenzyme A, Novelli (private communication) has shown this to be incorrect, the 2-mercaptoethylamine residue not being an essential component of coenzyme fragments which show marked growth activity towards *A. suboxydans*. The 2′-phosphate and the 2′ : 4′-diphosphate of pantothenic acid were also without activity in this test and were in fact absent in coenzyme hydrolysates. There remained the possibility that a cyclic phosphate structure might be present in the coenzyme.

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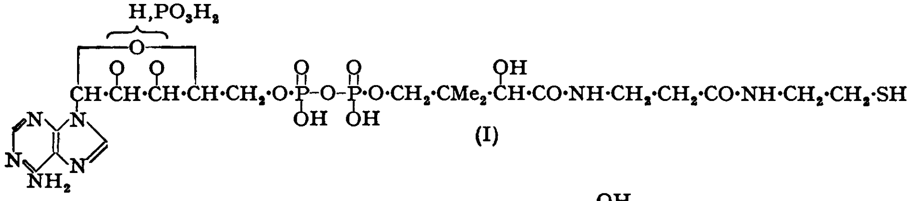
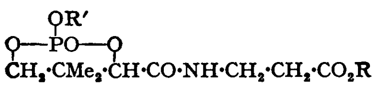
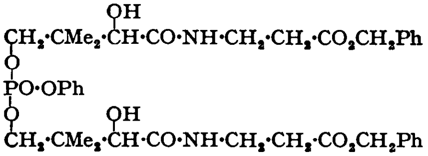
* Part II, *J.*, 1951, 2253.

Pantothenic acid-2′ : 4′ phosphate (II; $\mathrm{R} = \mathrm{R'} = \mathrm{H}$) was hitherto unknown and its behaviour on paper before and after hydrolysis could not be predicted. This cyclic phosphate has now been prepared and its stability towards acids and alkalis examined.

Benzyl pantothenate was phosphorylated with phenyl dichlorophosphinate in pyridine giving a syrupy product, presumably (II; $\mathrm{R} = \mathrm{Ph{\cdot}CH_2}$, $\mathrm{R'} = \mathrm{Ph}$). The formation of products from reaction between one molecule of phenyl dichlorophosphinate and two molecules of benzyl pantothenate, *e.g.*, (III) and its isomers, was prevented by effecting phosphorylation in dilute solution. The probable intermediate in this reaction, (IV) may react further in two possible ways. With a second molecule of benzyl pantothenate it would give (III) or an isomer in which the phosphate linkage involves the 2′-hydroxyl group. Cyclisation on the other hand would give (II; $\mathrm{R} = \mathrm{Ph{\cdot}CH_2}, \mathrm{R'} = \mathrm{Ph}$). The latter reaction, unlike the former, should be independent of concentration and so more likely to predominate in dilute solution. The syrupy product was hydrogenated with a palladium catalyst to remove the benzyl group, and the phenyl group was removed more slowly by hydrogenolysis with a platinum catalyst in acetic acid. Pantothenic acid-2′ : 4′ phosphate (II; $\mathrm{R} = \mathrm{R'} = \mathrm{H}$) was isolated as its barium salt and purified through its strychnine and brucine salts. Analysis of the reaction mixture before isolation showed that only traces of phosphorylated by-products were present.

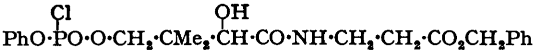

(I)


(II)



$$\begin{array}{l}\mathrm{CH_2{\cdot}CMe_2{\cdot}}\overset{\mathrm{OH}}{\mathrm{CH}}\mathrm{{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2CH_2Ph}\\ \mathrm{O}\\ \mathrm{PO{\cdot}OPh}\\ \mathrm{O}\\ \mathrm{CH_2{\cdot}CMe_2{\cdot}}\overset{\mathrm{OH}}{\mathrm{CH}}\mathrm{{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2CH_2Ph}\end{array}$$

(III)



$$\mathrm{PhO{\cdot}}\overset{\mathrm{Cl}}{\mathrm{P}}\mathrm{O{\cdot}O{\cdot}CH_2{\cdot}CMe_2{\cdot}}\overset{\mathrm{OH}}{\mathrm{CH}}\mathrm{{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2CH_2Ph}$$

(IV)

Paper chromatography provided a ready means of distinguishing between the cyclic phosphate and other phosphates of pantothenic acid. In *n*-propyl alcohol–ammonia and in *iso*butyric acid–ammonium *iso*butyrate–water it moved more rapidly than the other phosphates.

It is clear then that the substance obtained from hydrolysis of coenzyme A is not pantothenic acid-2′ : 4′ phosphate. The possibility that the coenzyme might contain a similar cyclic structure was not ruled out by this observation, however, since such a phosphate ring may have been opened during hydrolysis. In support of this argument it was shown that the 2′ : 4′-phosphate was hydrolysed very readily in alkali. Hydrolysis was complete after one hour at 100° in 0·35N-barium hydroxide solution, conditions similar to those employed in the hydrolysis of coenzyme A. The product was shown by paper chromatography to be pantothenic acid-4′ phosphate. No trace of the 2′-phosphate could be detected. The cyclic phosphate was largely unchanged by N-sulphuric acid at 100° during 20 minutes. Novelli has shown that coenzyme A hydrolysates retain their activity in the *Acetobacter* test after such treatment. Although these observations are consistent with the formulation of the coenzyme as a derivative of pantothenic acid-2′ : 4′ phosphate such a structure cannot be correct since the (±)-cyclic phosphate was quite inactive in stimulating the growth of *A. suboxydans.* Furthermore, it was not hydrolysed by acid or alkali phosphatase whereas the active fragments from coenzyme A were readily dephosphorylated by such treatment.

Forrest and Todd (*J.*, 1950, 3295) observed the formation of a cyclic phosphate of riboflavin during the hydrolysis of flavin–adenine dinucleotide with ammonia. Similarly, the isomerisation of adenosine phosphates during acid hydrolysis probably occurs through cyclic intermediates (Brown, Haynes, and Todd, *ibid.*, p. 2299). In the pantothenic acid phosphates neither cyclisation nor the formation of isomers during hydrolysis has been observed so far.

In view of the inability of all the synthetic pantothenic acid phosphates to stimulate the growth of *A. suboxydans* it would seem likely that coenzyme A bears an as yet unidentified residue attached to the pantothenic acid moiety. On the basis of present evidence it is not possible to state whether this group is situated at the 2′-position in a pantothenic acid-4′ phosphate structure or whether it is interposed between phosphate and pantothenic acid. However, we have shown that the phosphate group in adenosine-5′ phosphate and adenylic acids a and b are hydrolysed completely in 24 hours at 100° in 0·5N-alkali whereas the substance obtained by alkali hydrolysis of coenzyme A, like pantothenic acid-4′ phosphate, is quite stable under these conditions. This relative lability of derivatives of ribose phosphate suggests that if the unidentified residue in coenzyme A is interposed between the pyrophosphate linkage and pantothenic acid then it is most unlikely to be ribose.

It is now established that coenzyme A contains three phosphate groups (Novelli, Gregory, Flynn, and Schnetz, *Fed. Proc.*, 1951, **10**, 229). Two of these are very probably involved in pyrophosphate linkages as shown in (I) while the third, which from enzyme experiments appears to be bound as a monoester, has been assigned provisionally to either position 2′ or 3′ in the adenosine residue (cf. Part II, *loc. cit.*). Further evidence in support of this view has been forthcoming from a study of the behaviour of coenzyme A towards periodate. The method of Buchanan, Dekker, and Long (*J.*, 1950, 3162) was employed. In this method the nucleotide is oxidised on filter paper with a solution of sodium metaperiodate and resulting dialdehydes detected by spraying with Schiff's reagent. Nucleotides substituted at position 2′ or 3′ give no dialdehydes while those unsubstituted in these positions are oxidised readily under these conditions. Coenzyme A gave no dialdehyde when oxidised in this way whereas after hydrolysis in acid and oxidation under identical conditions dialdehydes were detected quite readily. Control experiments on proportionate amounts of adenosine-5′ phosphate and adenylic acids a and b confirmed that the observations had been made well within the limits of sensitivity of the method. It follows then that the coenzyme bears a substituent, presumably a monophosphate, at position 2′ or 3′ in the adenosine residue. At the present time no method exists for distinguishing between positions 2′ and 3′ in the adenine nucleotides.

EXPERIMENTAL.

D-*Pantothenic Acid*-2′ : 4′ *Phosphate* (II; R = R′ = H).—Benzyl pantothenate (14·8 g.) (Kuhn and Wieland, *Ber.*, 1940, **73**, 971) prepared from D(−)-pantolactone was dissolved in anhydrous pyridine (600 c.c.), and the solution cooled in a bath of acetone–solid carbon dioxide until the pyridine started to crystallise. A cold solution of phenyl dichlorophosphinate (10·6 g.) (Brigl and Müller, *ibid.*, 1939, **72**, 2121) in a little anhydrous pyridine was added in one lot. The solution was kept in the cooling-bath during 10 minutes, then allowed to reach room temperature. Reaction was completed by heating at 60—70° for 4 hours, then storage overnight. Most of the pyridine was removed by evaporation under reduced pressure and the remaining reddish-brown syrup dissolved in chloroform, washed with water, cold N-sulphuric acid, sodium hydrogen carbonate solution, and finally water. Chloroform was removed by evaporation under reduced pressure, and the residue dissolved in alcohol, decolorised with charcoal, and evaporated, to a pale amber resin (15·5 g.). A solution of this in 80% alcohol was hydrogenated at room temperature and atmospheric pressure in the presence of hydrogen and a palladium oxide catalyst. Hydrogen was absorbed quite rapidly until the benzyl group had been removed, then absorption ceased abruptly. Catalyst was removed by filtration, and solvent evaporated under reduced pressure. The resulting colourless resin was dissolved in chloroform, washed twice with water, and freed from solvent. The resin was dissolved in acetic acid, and the phenyl group removed by hydrogenolysis at room temperature and atmospheric pressure with hydrogen and a platinum oxide catalyst. Reduction was slow unless a relatively large amount of catalyst was used. Catalyst was removed by filtration and a solution of barium acetate (11 g.) in water (25 c.c.) added. Solvent was removed by evaporation under reduced pressure and most of the acetic acid by evaporation twice with water. The residue was dissolved in water, adjusted to pH 8 with barium hydroxide solution, and diluted with 2 volumes of alcohol. The small precipitate was removed by centrifugation and the clear supernatant liquid evaporated to small volume. Impure barium salt (10 g.) was obtained by addition of a large volume of acetone and collection of the insoluble salt by centrifugation. This salt was dissolved in a little water and passed through a column of Amberlite I.R. 120 (H), to remove barium ions. The eluate was evaporated to dryness from the frozen state, to remove water and acetic acid, and the residue dissolved in water and adjusted to pH 8 with barium hydroxide solution. After evaporation to small volume under reduced pressure the barium salt was precipitated by addition of a large volume of acetone. The product was washed well with acetone and dried over phosphoric oxide.

Preparation of strychnine salt. The barium salt of pantothenic acid-2′ : 4′ phosphate (2·5 g.) was freed from barium by quantitative precipitation with sulphuric acid, barium sulphate removed by centrifugation, strychnine (4 g., 2 mols.) added to the clear solution, and the mixture warmed to effect dissolution. The crystalline solid remaining on evaporation of the water was recrystallised from a small volume of hot water to which acetone was added to incipient opalescence. The strychnine salt crystallised as tiny plates, m. p. 175—177° (decomp.). Analyses indicated that this material was a mixture of the mono- and di-strychnine salts (Found : C, 60·8; H, 6·6; N, 7·0; P, 3·2. Calc. for $C_{51}H_{60}O_{11}N_5P$: C, 64·6; H, 6·3; N, 7·4; P, 3·3. Calc. for $C_{30}H_{38}O_9N_3P$: C, 58·5; H, 6·2; N, 6·8; P, 5·0%). Attempts to prepare the monostrychnine salt only yielded uncrystallisable resins.

Reconversion of the strychnine salt into the barium salt. The foregoing strychnine salt (4 g.) was dissolved in water (*ca.* 5 c.c.), and barium hydroxide solution (0·3N) added to pH 9. Carbon dioxide was passed through the solution to remove excess barium hydroxide, and the strychnine and barium carbonate were removed by filtration. The filtrate was concentrated under reduced pressure and the *barium* salt (1·5 g.; theory, 1·75 g.) precipitated by the addition of excess acetone [Found, in substance dried at 140°: Ba, 31·7; P, 7·4; N(Kjeldahl), 3·1. $C_9H_{16}O_7NPBa,H_2O$ requires Ba, 31·7; P, 7·2; N, 3·2%], $[\alpha]^{D}_{24}$ +29° (*c*, 2·12 in. water).

Preparation of the brucine salt. The barium salt (0·8 g.) was freed from barium by sulphuric acid as before, and brucine (1·6 g., 2 mols.) added to the aqueous solution of the free acid. The solid remaining on evaporation of the water was crystallised by dissolving it in the minimum of hot water and adding acetone to incipient opalescence. The *brucine* salt crystallised as well-formed, rectangular prisms (1·65 g., 80%), m. p. 135° with effervescence; after drying in a vacuum at 100° it had m. p. *ca.* 165° (to a resinous material) (Found: C, 54·5; H, 7·0; N, 6·0; P, 2·7; loss on drying at 140°, 12·0. $C_{55}H_{68}O_{15}N_5P,8H_2O$ requires C, 54·5; H, 6·9; N, 5·8; P, 2·6; H_2O, 11·9%).

Hydrolysis of Pantothenic Acid-2′ : 4′ Phosphate.—(a) *Alkali.* The barium salt (1 mg.) of the cyclic phosphate was heated with barium hydroxide solution (0·25 c.c.; 0·3N) at 100° for 1·5 hours in a sealed tube. Barium hydroxide was precipitated by carbon dioxide as barium carbonate which was removed by centrifugation. The barium from soluble barium salts was removed by addition of ammonium sulphate. After removal of barium sulphate the solution was concentrated and run on paper using *n*-propyl alcohol–ammonia–water as solvent. The chromatogram was developed by Hanes and Isherwood's molybdate spray (*Nature*, 1949, **164**, 1107). It was found that pantothenic acid-2′ : 4′ phosphate, R_F 0·62, was converted entirely into pantothenic acid-4′ phosphate, R_F 0·32, by this hydrolysis.

(b) *Acid.* The barium salt (1 mg.) of the cyclic phosphate, dissolved in 1 drop of water, was heated with sulphuric acid (0·25 c.c.; N) at 100° for 20 minutes in a sealed tube. The cooled solution was neutralised with barium hydroxide, and the precipitate of barium sulphate removed by centrifugation. Barium was removed from soluble barium salts as before, and after removal of barium sulphate the solution was run on paper with *n*-propyl alcohol–ammonia–water. The acid-treated material gave a spot identical with that of the cyclic phosphate; no trace of either pantothenic acid-2′ or -4′ phosphate was visible.

Prolonged Alkaline Hydrolysis of Coenzyme A, Pantothenic Acid-4′ Phosphate, and Adenosine Phosphates.—Two samples each of coenzyme A (1·5 mg.; 170 units/mg.), the barium salt of pantothenic acid-4′ phosphate (1 mg.), adenosine-5′ phosphate (1 mg.), and commercial yeast adenylic acid (1 mg.) were separately dissolved in barium hydroxide solution (0·5 c.c.; 0·3N) and sealed in glass tubes. One series of tubes was heated at 100° for 45 minutes, the other at 100° for 24 hours. The contents were treated in the usual way for the removal of barium, and the residues run on paper in *n*-propyl alcohol–ammonia–water and *iso*butyric acid–ammonium *iso*butyrate–water. In both solvent systems the two adenosine phosphates were mainly hydrolysed after 45 minutes and completely after 24 hours. Coenzyme A after 45 minutes' hydrolysis and running in *n*-propyl alcohol–ammmonia–water gave 4 spots, the slowest-moving of which corresponded to pantothenic acid-4′ phosphate (R_F 0·36), the faster-moving spots having R_F 0·60, 0·68, and 0·76; in *iso*butyric acid–ammonium *iso*butyrate only one spot was observed, having an R_F, 0·47, which is slightly faster than pantothenic acid-4′ phosphate, R_F 0·43. In both solvent systems the hydrolysate after 24 hours gave one spot only, corresponding to pantothenic acid-4′ phosphate, which is stable to these conditions of hydrolysis.

Action of Sodium Metaperiodate on Coenzyme A.—Coenzyme A (1 mg.; 330 units/mg.) was dissolved in water (0·02 c.c.), and the solution divided into two parts. One part was hydrolysed with hydrochloric acid (0·05 c.c.; N) for 1 hour at 100° and then evaporated. Adenylic acid b (50 mg.) was similarly treated. Parts of the resulting solutions were put on paper, and the chromatogram developed with *n*-propyl alcohol–ammonia–water; the remaining parts were spotted out on paper and treated with sodium metaperiodate solution directly, according to the method of Buchanan, Dekker, and Long (*loc. cit.*). The paper which was treated directly showed that unhydrolysed coenzyme A and adenylic acid b gave no reaction, whereas both after hydrolysis gave positive reactions for aldehyde. A similar result was obtained with the developed chromatogram. Coenzyme A, which gave 5 spots when examined in ultra-violet light, gave no Schiff's reaction whilst both coenzyme A and adenylic acid b after hydrolysis gave strong Schiff-reacting spots at identical positions on the paper.

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