186. *Coenzyme A. Part VII.* Pantetheine-2′ and -2′ : 4′ Phosphates and a New Method for the Synthesis of Cyclic Phosphates.*

By J. Baddiley and E. M. Thain.

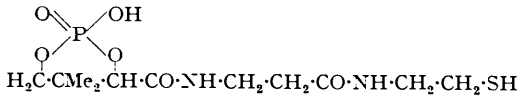
Pantetheine-2′ : 4′ hydrogen phosphate (I) and its disulphide have been synthesised by two different routes. The first involves direct phosphorylation of pantethine with phosphoryl chloride in moist pyridine, giving the cyclic phosphate directly. In the second route pantolactone-2 diphenyl phosphate is heated with an excess of the 2-mercaptoethylamide of β-alanine.

Synthetic pantetheine-2′ : 4′ hydrogen phosphate possessed the same properties as a degradation product obtained from coenzyme A and the two are considered identical.

Pantetheine-2′ phosphate (V) was prepared by heating together pantolactone-2 phosphate and the 2-mercaptoethylamide of β-alanine.

The mechanism of the reaction between a diphenyl phosphoric ester bearing a suitably placed hydroxyl group and amines is discussed.

In Part VI * we described the isolation and identification of several degradation products of coenzyme A. To one of these was assigned the tentative structure (I), namely pantetheine-2′ : 4′ hydrogen phosphate. The formation of this substance during alkaline hydrolysis of the coenzyme was significant in the development of our thesis. As insufficient material was available for analysis the evidence for its structure was confined to the recognition of products of alkaline hydrolysis. A synthesis of (I) and comparison with the natural material was clearly desirable.



(I)

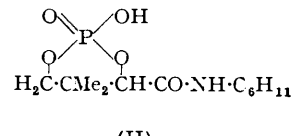
Forrest and T odd (., 1950, 3295) noted the formation of riboflavin-4′ : 5′ hydrogen phosphate from flavin-adenine dinucleotide under conditions similar to those employed by us for the formation of pantetheine-2′ : 4′ hydrogen phosphate from coenzyme A. These authors synthesised their cyclic phosphate by the action of phosphoryl chloride on riboflavin in moist pyridine. In the application of a similar technique to the synthesis of (I) from pantetheine we considered it essential to protect the mercapto-group. This was effected conveniently by oxidation to the disulphide. A solution of pantetheine in pyridine was oxidised by passing in oxygen until it no longer gave a nitroprusside reaction. During this oxidation ½ mol. of water was produced and this was found to be sufficient for the subsequent reaction with phosphoryl chloride. The phosphorylated product was isolated through its lithium and barium salts. It was indistinguishable on paper chromatography from the substance isolated from coenzyme A. Furthermore, on cautious alkaline hydrolysis it yielded a mixture of pantothenic acid-4′ phosphate and -2′ : 4′ hydrogen phosphate in the same manner as did the natural degradation product. It is concluded that the two substances are identical.

A second method for the synthesis of (I) depends on observations made at an early stage in this work. In Part I (Baddiley and Thain, *ibid.*, 1951, 246) a water-soluble substance, $C_{18}H_{35}O_5N_2P$, was obtained from pantolactone-2 diphenyl phosphate and *cyclo*hexylamine at 100°. The empirical formula indicated that the phosphate had reacted with 2 mols. of *cyclo*hexylamine and that both phenyl groups had been eliminated. While it was recognised that one mol. of *cyclo*hexylamine was probably involved in opening the lactone ring to give an amide the nature of the final product was not determined. Re-examination of this substance has established that it is a *cyclo*hexylamine salt and elementary analysis suggests that it is a salt of the cyclic phosphate (II). Further support for this formulation

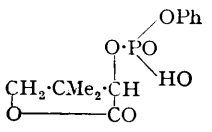
* Part VI, *J.*, 1952, 3783.

was obtained by passing its aqueous solution through a column of Amberlite IR-120 resin to remove the *cyclo*hexylamine. The eluate was strongly acidic and after neutralisation with barium hydroxide a salt was obtained containing the expected amount of barium. The relatively high R_F value of the substance in *n*-propyl alcohol–ammonia supports the cyclic rather than acyclic phosphate formulation. A similar product, also described in Part I (*loc. cit.*), was obtained by the action of ammonia in methanol at 30° on pantolactone-2 diphenyl phosphate. To this was assigned the formula, $C_{12}H_{19}O_5N_2P$, but further examination has shown this to be untenable. The product was formed in low and variable yield by this method, a better yield (1·8 g. from 5 g.) being obtained by passing ammonia through a boiling dioxan solution of pantolactone-2 diphenyl phosphate.

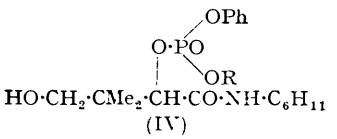
Examination by paper chromatography showed the presence of two substances in this crude ammonium salt. The faster-moving main component (R_F, 0·8) was isolated as its crystalline *cyclo*hexylamine salt and was assigned the formula (III) on the basis of its analysis, the liberation of phenol on acid hydrolysis, and its ready conversion into the salt of the *cyclo*hexylamide (IV; R = H) when heated with *cyclo*hexylamine. The nature of the slower-moving substance formed in the ammonia reaction has not been established.



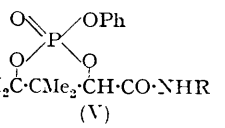
(II)



(III)



(IV)



(V)

It appears that the reaction between pantolactone-2 diphenyl phosphate and a base may take either of two courses according to the nature of the base. With strong bases, *e.g.*, *cyclo*hexylamine, opening of the lactone ring is rapid and a neutral amide (IV; R = Ph) must be the first reaction product. Cyclisation to form a neutral phenyl ester (V) may then occur and the second phenyl group would be removed by a further mol. of base. When a weak base, *e.g.*, ammonia, is employed, opening of the lactone ring is slower and until this has occurred cyclisation cannot take place. However, removal of phenyl groups may be less dependent on the basic strength of the amine and (III) would be formed. It is unlikely that (III) could be an intermediate in the formation of cyclic phosphates in the presence of strong bases since when heated with *cyclo*hexylamine (III) was converted into (IV; R = H) without cyclisation. The main requirement for cyclic phosphate formation by this method seems to be production at some stage of a neutral ester bearing a hydroxyl group suitably placed for esterification, *e.g.*, (IV; R = Ph). In this connection the reaction mechanism probably bears a similarity to the proposed mechanism of alkaline hydrolysis of ribonucleic acid (Brown and Todd, *J.*, 1952, 52) and glycerophosphate isomerisation (Baer and Kates, *J. Biol. Chem.*, 1948, **175**, 79).

While the general scope of the above reaction is still under investigation its use has been demonstrated in an alternative synthesis of pantetheine-2′ : 4′ hydrogen phosphate. Pantolactone-2 diphenyl phosphate was heated with an excess of the 2-mercaptoethylamide of β-alanine and the product converted into a barium salt. Although this was not obtained analytically pure, paper chromatography showing the presence of traces of several unidentified impurities, the main component was pantetheine-2′ : 4′ hydrogen phosphate (I). This was identified by comparison on paper with the substance obtained by the first method and by its conversion on alkaline hydrolysis into a mixture of pantothenic acid-4′ phosphate and the -2′ : 4′ hydrogen phosphate. It should be emphasised here that the preparation of analytically pure specimens of the cyclic phosphate is experimentally difficult.

A possible by-product in the reaction between pantolactone-2 diphenyl phosphate and

the mercaptoethylamide is pantetheine-2′ phosphate (VI), which might have arisen by removal of both phenyl groups from the intermediate pantetheine-2′ diphenyl phosphate without cyclisation. A synthesis of (VI) was accomplished by heating pantolactone-2

$$\begin{array}{c} \mathrm{O{\cdot}PO_3H_2} \\ | \\ \mathrm{HO{\cdot}CH_2{\cdot}CMe_2{\cdot}CH{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}SH} \\ \text{(VI)} \end{array}$$

phosphate with an excess of the mercaptoethylamide. The product was isolated in good yield as its barium salt. Examination of this substance on paper showed that it differed from any of the contaminant phosphates present in the crude cyclic phosphate obtained by the second synthetic method described above. Confirmation of the structure (VI) followed from ready hydrolysis to pantothenic acid-2′ phosphate, identified by comparison on paper with the synthetic substance (Part I, *loc. cit.*).

Experimental

Pantetheine-2′ : 4′ Hydrogen Phosphate (First Method).—Oxygen was passed through a solution of pantetheine (3·5 g.) in dry pyridine (20 c.c.) until the nitroprusside test was negative. The oxidised solution was then added dropwise to phosphoryl chloride (1·9 g., 1 mol.) in pyridine (20 c.c.) which had been cooled to the f. p. in acetone–solid carbon dioxide. After the addition the temperature was raised to 0° and maintained thereat for 1 hour. The solution was re-cooled to the f. p. and water (1 c.c.) in pyridine (5 c.c.) added dropwise. As much pyridine as possible was removed under reduced pressure, the residue dissolved in water (50 c.c.), and the solution passed through a column of ion-exchange resin (IR-120, H form) to remove the remaining pyridine. The acid eluate was neutralised with lithium hydroxide solution and evaporated to dryness, yielding lithium chloride and pantetheine-2′ : 4′ lithium phosphate.

Fractionation of the lithium salt and its conversion into the barium salt. The mixed lithium salts were triturated with methyl alcohol (150 c.c.); the insoluble fraction was removed by centrifugation, washed with methyl alcohol (20 c.c.), and discarded. The combined methanolic solutions were diluted with ether (500 c.c.) and the gelatinous precipitate was collected by centrifugation. Since the precipitate still contained traces of halide the process was repeated twice by dissolution in methanol (100 c.c.) and precipitation with ether (500 c.c.). The lithium salt so obtained was free from halide and was converted into the barium salt by dissolving it in water (20 c.c.) and removing lithium on an ion-exchange column (IR-120, H form). The acid eluate was adjusted to pH 9 with barium hydroxide solution, excess of barium removed by carbon dioxide, and the solution concentrated by distillation under reduced pressure to *ca.* 5 c.c. A slight precipitate was removed by centrifugation and the resulting clear solution diluted with acetone (50 c.c.) and ether (50 c.c.), yielding a sticky precipitate of *pantetheine-2′ : 4′ barium phosphate* (2·2 g.) which became powdery on trituration with fresh acetone (Found: C, 32·7; H, 5·4; N, 6·3; P, 7·3; Ba, 16·8. $C_{22}H_{38}O_{12}N_4P_2S_2Ba$ requires C, 32·5; H, 4·7; N, 6·9; P, 7·6; Ba, 16·9%). This substance was homogeneous on running on an ascending paper chromatogram in the *n*-propyl alcohol–ammonia solvent with respect to phosphorus and sulphur, R_F 0·65.

Alkaline Hydrolysis of Pantetheine-2′ : 4′ Barium Phosphate.—A sample of the barium salt (2 mg.) was heated with 0·3N-barium hydroxide (0·2 c.c.) for 2 hours at 100°. Excess of barium was precipitated as barium carbonate, and barium from soluble barium salts removed by cautious addition of ammonium sulphate. An aliquot of the clear solution was run on an ascending paper chromatogram in the *n*-propyl alcohol–ammonia solvent. Two spots were observed corresponding to the standards: pantothenic acid-4′ phosphate, R_F 0·23, and pantothenic acid-2′ : 4′ hydrogen phosphate, R_F 0·55.

Barium Salt of N-cyclo*Hexylpantoamide-2′ : 4′ Hydrogen Phosphate* (II).—An aqueous solution of the *cyclo*hexylamine salt, m. p. 253°, of the cyclic phosphate (Part I, *loc. cit.*) was passed through a column of Amberlite IR-120 resin in the hydrogen form. The strongly acid eluate was neutralised (pH 7·5) with barium hydroxide solution, then evaporated to dryness under reduced pressure. The resulting brittle resin was ground in ether, filtered off, and dried at room temperature (P_2O_5). When the *barium* salt was examined by paper chromatography in *n*-propanol–ammonia in the usual way a single spot (R_F 0·85) was observed (Found, in substance dried at room temperature: C, 37·6; H, 6·0; N, 3·0; P, 7·9. $C_{12}H_{21}O_5NPBa_{\frac{1}{2}},H_2O$ requires C, 38·2; H, 6·1; N, 3·7; P, 8·0. Found, in sample dried at 130° *in vacuo*: Ba, 19·8. $C_{12}H_{21}O_5NPBa_{\frac{1}{2}}$ requires Ba, 19·2%).

cyclo*Hexylamine Salt of Pantolactone-2 Phenyl Phosphate* (III).—A gentle stream of ammonia was passed through a refluxing solution of pantolactone-2 diphenyl phosphate (5 g.) in dioxan (50 c.c.) during 5 hours. After cooling, the solid product (1·8 g.) was filtered off, washed with ether, and dried in air. Evaporation of the mother-liquors under reduced pressure yielded some unchanged starting material (1·7 g.). The product was dissolved in boiling water, and the clear solution cooled and passed through a column of IR-120 resin in the hydrogen form. The acidic eluate was neutralised (pH 7) with *cyclo*hexylamine and evaporated to small volume under reduced pressure. The crystalline product recrystallised from water as needles, m. p. 235°. When examined by paper chromatography in *n*-propanol–ammonia the cyclo*hexylamine* salt was homogeneous, having R_F 0·85 (Found: C, 55·7; H, 7·4; N, 4·1; P, 8·1. $C_{18}H_{28}O_6NP$ requires C, 56·0; H, 7·3; N, 3·6; P, 8·1%).

cyclo*Hexylamine Salt of* N-cyclo*Hexylpantoamide-2 Phenyl Phosphate* (IV; R = H).—The above *cyclo*hexylamine salt (0·6 g.), m. p. 235°, was heated with *cyclo*hexylamine (3 c.c.) at 100° for 3 hours. The crystalline solid dissolved during the first 15 min. Excess of base was removed under reduced pressure and the resulting syrup warmed with a little acetone to effect crystallisation. The cyclo*hexylamine* salt (0·3 g.) crystallised as fine needles, m. p. 206°, from alcohol–ether (Found: C, 59·0; H, 8·4; N, 6·1; P, 7·0. $C_{24}H_{41}O_6N_2P$ requires C, 59·5; H, 8·5; N, 5·8; P, 6·4%). When treated with cold dilute sodium hydroxide, *cyclo*hexylamine was liberated immediately and phenol was detected readily after the solution had been heated for 10 min. at 100°

Pantetheine-2′ : 4′ Hydrogen Phosphate (*Second Method*).—A mixture of pantolactone-2 diphenyl phosphate (2·3 g.) and the 2-mercaptoethylamide of β-alanine (3·4 g.) (Baddiley and Thain, *J.*, 1952, 800) was heated at 100° for 5½ hours in an inert atmosphere. The resulting viscous syrup was triturated with water (20 c.c.) and sufficient alcohol added to give a clear solution which was then passed through a column of IR-120 resin in the hydrogen form. The cloudy acid eluate was extracted with ether (3 times) and the aqueous layer neutralised (pH 8) with barium hydroxide solution. The impure barium salt (1·8 g.) was isolated by evaporation to small volume under reduced pressure and precipitation with acetone. Chromatographic examination on paper in *n*-propanol–ammonia showed the presence of a main component (R_F 0·63) identical with pantetheine-2′ : 4′ hydrogen phosphate prepared by the first method, together with a smaller amount of an impurity (R_F 0·9) which also gave positive tests for phosphate and mercapto-groups. Alkaline hydrolysis in the manner described previously resulted in the production of two phosphorus-containing spots, R_F 0·23 and 0·55, corresponding to pantothenic acid-4′ and -2′ : 4′ phosphates.

Pantetheine-2′ Phosphate (VI).—A mixture of pantolactone-2 phosphate (Part I, *loc. cit.*) (0·2 g.) and the 2-mercaptoethylamide (0·6 g.) was heated at 100—105° for 4 hours. The resulting brittle resin was dissolved in water and passed through a column of IR-120 resin. The strongly acid eluate was neutralised (pH 8) with barium hydroxide solution and evaporated to small volume. The *barium* salt (0·4 g.) was precipitated with acetone. Paper chromatography in *n*-propanol–ammonia showed that the product was homogeneous (R_F 0·43) (Found: C, 25·4; H, 5·3; N, 5·9; P, 5·0. $C_{11}H_{21}O_7N_2SPBa,2H_2O$ requires C, 25·1; H, 4·8; N, 5·4; P, 5·8. Found, in sample dried at 120°: Ba, 27·4. Anhydrous salt requires Ba, 27·8%).

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