323. *Coenzyme A. Part VIII.* The Synthesis of Pantetheine 4′-Phosphate* (Acetobacter *Stimulatory Factor*), *a Degradation Product of the Coenzyme.*

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Pantetheine $O^{2'}S$-dibenzyl ether (III; R = H) was prepared from pantothenic acid 2′-benzyl ether (II; R = H) by reaction with ethyl chloroformate followed by 2-benzylthioethylamine. Phosphorylation with dibenzyl phosphorochloridate,† then removal of benzyl groups with sodium in liquid ammonia, gave DL-pantetheine 4′-(dihydrogen phosphate) (I).

D(+)-Pantetheine 4′-(dihydrogen phosphate) was synthesised by direct phosphorylation of pantethine with dibenzyl phosphorochloridate, followed by removal of benzyl groups with sodium in liquid ammonia, and was identical with the product obtained by the action of nucleotide pyrophosphatase or dilute acids on coenzyme A. It was converted into coenzyme A in the presence of adenosine triphosphate and an enzyme system from pigeon liver.

From a study of the hydrolysis products of coenzyme A it was concluded that a pantothenic acid 4′-phosphate structure was present in the molecule (Baddiley and Thain, *J.*, 1951, 2253). This was confirmed later by the isolation of the 4′-phosphate and other products from an alkaline hydrolysate (*idem*, *J.*, 1952, 3783) and on the basis of these and other findings a structural formula was given to the coenzyme. One observation, however, was not readily explained on this or any other formula, namely, the nature of a degradation product of coenzyme A which promoted growth of *Acetobacter suboxydans* (cf. King, Locher, and Cheldelin, *Arch. Biochem.*, 1948, **17**, 483). This fragment, originally produced from coenzyme A by the action of unpurified liver enzymes (Novelli, Kaplan, and Lipmann, *J. Biol. Chem.*, 1949, **177**, 97; Novelli, Flynn, and Lipmann, *ibid.*, p. 493) is a product of pyrophosphatase action (Novelli, " Phosphorus Metabolism," The Johns Hopkins Press,

* Part VII, *J.*, 1953, 903. † For nomenclature see *J.*, 1952, 5122.

Baltimore, 1951, Vol. I, p. 414). It may be formed also by short acid hydrolysis of the coenzyme and is known as the "*Acetobacter* stimulatory factor" (ASF). As it contained no adenine and gave pantothenic acid and phosphate in a ratio of about 1 : 1 on enzymic hydrolysis it was thought to be a simple phosphate of pantothenic acid. None of the synthetic monophosphates of pantothenic acid stimulated growth of *A. suboxydans*; consequently we suggested (*J.*, 1951, 2253) that ASF was pantetheine 4′-(dihydrogen phosphate) (I). Although this was questioned later (*ibid.*, p. 3421) because of indications that the active substance contained no sulphur, Novelli (personal communication) has found recently that the 2-mercaptoethylamine residue is probably still intact in ASF. In view of the difficulties of isolating such a substance in pure form from a coenzyme A hydrolysate, unequivocal synthesis of (I) was desirable. This has been achieved.

$$H_2O_3PO{\cdot}CH_2{\cdot}CMe_2{\cdot}CH(OH){\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}SH \quad (I)$$

$$RO{\cdot}CH_2{\cdot}CMe_2{\cdot}CH(O{\cdot}CH_2Ph){\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2H \quad (II)$$

Phosphorylation of pantothenic acid 2′-benzyl ether (II; R = H) with diphenyl phosphorochloridate in pyridine yielded a syrupy diphenyl phosphate from which the phenyl groups were removed by alkaline hydrolysis; in accordance with earlier observations on pantothenic acid phosphates (Baddiley and Thain, *J.*, 1951, 246), the amide linkage remained substantially intact during the alkali treatment. The product, $O^{2'}$-benzyl pantothenic acid 4′-phosphate (II; R = PO_3H_2), in the form of its 4-methylmorpholinium salt, was treated with ethyl chloroformate and then 2-mercaptoethylamine under conditions similar to those described in a recent synthesis of pantetheine (Wieland and Bokelmann, *Naturwiss.*, 1951, **38**, 384). The reaction appeared to be complex, however, and no recognisable products were isolated.

Attention was directed next to the phosphorylation of derivatives of pantetheine rather than of pantothenic acid. Pantothenic acid 2′-benzyl ether (II; R = H), which has been prepared by an improved method (see Experimental section), reacted smoothly with ethyl chloroformate in the presence of 4-methylmorpholine. The resulting mixed carbonic anhydride evolved carbon dioxide on treatment with 2-benzylthioethylamine, giving pantetheine $O^{2'}S$-dibenzyl ether (III; R = H) in good yield. The structure of the product was indicated by its ready reduction with sodium in liquid ammonia to pantetheine. Phosphorylation with dibenzyl phosphorochloridate in pyridine then gave a neutral syrup, presumably the tetrabenzyl compound [III; R = $PO(O{\cdot}CH_2Ph)_2$]. Removal of benzyl groups from this substance could not be effected by catalytic methods owing to the presence of sulphur in the molecule. Although it was known from the experiments recorded above that benzyloxy- and benzylthio-groups could be reduced smoothly with sodium in liquid ammonia in similar compounds, it was not known whether both benzyl groups could be removed from a dibenzyl phosphate by this method. Nor was it known whether the resulting phosphates would be stable towards the reagents. Qualitative experiments showed that both dibenzyl and diphenyl phosphate were converted into inorganic phosphate by sodium in liquid ammonia. The scope and limitations of this procedure for removing protecting phenyl and benzyl groups from organic phosphates are under investigation. Certain advantages have already been found over hydrolytic or catalytic methods with compounds containing sulphur or possessing acid-lability.

$$RO{\cdot}CH_2{\cdot}CMe_2{\cdot}CH(O{\cdot}CH_2Ph){\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}S{\cdot}CH_2Ph \quad (III)$$

$$[RO{\cdot}CH_2{\cdot}CMe_2{\cdot}CH(OH){\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}S{\cdot}]_2 \quad (IV)$$

When [III; R = $PO(O{\cdot}CH_2Ph)_2$] in the presence of a little alcohol was treated with sodium in liquid ammonia four benzyl groups were removed and the product (I) was purified by successive conversion into lithium, silver, and finally barium salts. It was homogeneous when examined by paper chromatography in the two solvent systems described in the Experimental section and on hydrolysis yielded pantothenic acid 4′-phosphate. Examination of the crude reaction product showed the presence of pantothenic acid 4′-phosphate as the main impurity. This must have arisen through partial hydrolysis of the rather labile amide linkage between the β-alanine and 2-mercaptoethylamine residues during hydrogenolysis. Purification through the silver salt removed this contaminant. Attempts

to prepare pantetheine 4′-phosphate by phosphorylating (III; R = H) with diphenyl phosphorochloridate, with subsequent removal of phenyl and benzyl groups with sodium in liquid ammonia, gave only an impure product. Although paper chromatography of the product showed the presence of some 4′-phosphate other unidentified substances were also formed.

Pantetheine 4′-phosphate has been examined by Drs. Novelli and Lipmann with a partly purified enzyme system from pigeon liver. This is a mixture of at least two enzymes which, together and in the presence of adenosine triphosphate, are capable of synthesising coenzyme A from ASF (Levintow and Novelli, Abs. Papers, Atlantic City Meeting, Amer. Chem. Soc., 1952, p. 33c). Synthetic pantetheine 4′-phosphate showed an activity in this system equivalent to 265 units CoA/mg. which represents a conversion of 48%, in excellent agreement with the calculated 50% conversion for a DL mixture. Pantetheine 2′-phosphate and the cyclic 2′ : 4′-phosphate described in the previous paper (*J.*, 1953, 903) were inactive in this test.*

The above synthesis is unequivocal and provides a rigid proof of the structure of ASF and strong support for the proposed formula for coenzyme A. However, the product was a DL-mixture and in order to prepare moderate quantities of an optically pure material the somewhat simpler but structurally ambiguous procedure of direct phosphorylation of D(+)-pantethine (IV; R = H) was investigated. It was expected that the primary hydroxyl group at position 4′ in pantethine would be phosphorylated more readily than would the sterically hindered secondary 2′-hydroxyl group. This was shown to be so by treating D(+)-pantethine with dibenzyl phosphorochloridate in pyridine and reducing the neutral dibenzyl ester [IV; R = $PO(O{\cdot}CH_2Ph)_2$] with sodium in liquid ammonia. The product, purified through its barium and silver salts, was identical with pantetheine 4′-phosphate prepared by the first method. Its activity in the enzyme test was equivalent to 450 units CoA/mg., representing an 82% conversion. This work is being extended to include the synthesis of mixed pyrophosphates of pantetheine and adenosine more closely related to coenzyme A.

The work described in this paper completes the identification of all the monophosphates of pantothenic acid and pantetheine obtained by enzymic or chemical hydrolysis of coenzyme A. The course of acid and alkali hydrolysis is summarised briefly as follows.

The first points of attack by alkali are the pyrophosphate linkage and the amide linkage between the β-alanyl and the 2-mercaptoethylamine residue. The products at this stage are pantetheine 4′-(dihydrogen phosphate), pantothenic acid 4′-(dihydrogen phosphate), and phosphorylated derivatives of adenosine. Alternatively, intramolecular phosphorylation may result in the formation of a cyclic phosphate [pantetheine 2′ : 4′-(hydrogen phosphate)]. Further action of alkali on this gives a mixture of pantothenic acid 2′ : 4′-(hydrogen phosphate) and 4′-(dihydrogen phosphate). In acids, coenzyme A is first hydrolysed at the pyrophosphate linkage to pantetheine 4′-(dihydrogen phosphate), no internal phosphorylation being possible under these conditions. Further action of acids gives pantothenic acid 4′-(dihydrogen phosphate).

Experimental

Pantothenic Acid 2′-Benzyl Ether (II; R = H).—The following improved method is based on a recent synthesis of pantothenic acid (B.P. 660,722). During 4 hr.' refluxing, finely powdered β-alanine (2·8 g.) dissolved in a solution of pantolactone benzyl ether (6·95 g.) (Baddiley and Thain, *J.*, 1951, 246) and dimethylamine (2·5 g.) in anhydrous methanol (50 c.c.). After a further 4 hr.' refluxing, evaporation under reduced pressure gave a syrup, which was dissolved in water. Acidification with dilute sulphuric acid, extraction (3 times) with ethyl acetate, washing with water, and removal of solvent under reduced pressure gave a syrup. This was dissolved in sodium hydrogen carbonate solution, washed (3 times) with ether, then recovered by acidification with sulphuric acid and extraction (3 times) with ethyl acetate. The organic layer was washed with water, dried (Na_2SO_4), and evaporated under reduced pressure to a clear syrup (7·4 g.) (Found: C, 62·2; H, 7·6; N, 4·5. Calc. for $C_{16}H_{23}O_5N$: C, 62·2; H, 7·5; N, 4·5%).

* A preliminary account of these results has been reported already (Baddiley, Thain, Novelli, and Lipmann, *Nature*, 1953, **171**, 76).

$O^{2'}$-*Benzyl Pantothenic Acid 4'-(Dihydrogen Phosphate)* (II; R = PO_3H_2).—The above benzyl ether (3·8 g.), dissolved in anhydrous pyridine (50 c.c.), was cooled to −20°. Diphenyl phosphorochloridate (3·63 g., 1·1 mol.) was added and the resulting solution set aside at room temperature for 20 hr. Water (3 c.c.) was added and after a further 30 min. solvent was removed by distillation under reduced pressure. The residue was dissolved in chloroform, washed twice with N-sulphuric acid, then twice with water, dried ($MgSO_4$), and freed from solvent under reduced pressure. The residual syrup was evaporated twice with benzene to remove traces of chloroform, then dissolved in 2N-sodium hydroxide (100 c.c.). Traces of organic solvents were removed by gentle warming at the water pump. After 1·5 hr.' refluxing the cooled solution was passed through Amberlite IR-120 resin (H form) to remove sodium ions. The acidic eluate was neutralised (pH 8) with barium hydroxide solution, kept at 0° for 12 hr., filtered, and concentrated under reduced pressure to about 35 c.c. This solution was passed through IR-120 and the eluate neutralised (pH 8) with barium hydroxide solution. The clear solution was evaporated to a few c.c. and acetone added to about 70% concentration. A small sticky precipitate (0·5 g.) was removed by centrifugation and a further 200 c.c. of acetone were added. The precipitate (1·95 g.) was removed by centrifugation, washed with acetone, and dried over phosphoric oxide. Evaporation of the mother-liquors yielded a further quantity of solid (0·5 g.). The pure *barium* salt (Found: N, 2·2. $C_{16}H_{21}O_8NPBa_{1·5}$ requires N, 2·4%) was homogeneous when examined by paper chromatography in *n*-propanol–ammonia (R_F 0·82). Its constitution was established by suspending a small sample in liquid ammonia, then reducing it with sodium. The insoluble barium salt was rubbed continually during the reduction. After evaporation of the ammonia the solid residue was dissolved in water and passed through a small column of IR-120 resin. The eluate was neutralised with ammonia and, after concentration, was run on paper in *n*-propanol–ammonia. A single spot was observed, having R_F 0·35, identical with pantothenic acid 4'-(dihydrogen phosphate).

Pantetheine $O^{2'}$*S-Dibenzyl Ether.*—Ethyl chloroformate (2·4 g., 1 mol.) was added dropwise to a solution of pantothenic acid 2'-benzyl ether (6·8 g., 1 mol.) and 4-methylmorpholine (2·2 g., 1 mol.) in dry dimethylformamide (*ca.* 20 c.c.) which was cooled to −20° in acetone–solid carbon dioxide. 4-Methylmorpholine hydrochloride separated and after the addition the temperature was kept at −5° for 10 min. A chloroform solution of 2-benzylthioethylamine, prepared by liberation of the base from the hydrochloride (4·5 g., 1 mol.) with concentrated sodium hydroxide solution, extraction with chloroform (4 × 15 c.c.), and drying ($MgSO_4$), was cooled to −5° and added to the dimethylformamide solution, which caused a discrete evolution of carbon dioxide. The temperature was kept at −5° for 15 min., then set aside for 12 hr. at room temperature. Solvents were removed under reduced pressure, the residue was dissolved in chloroform (50 c.c.), washed successively with 2N-sulphuric acid (30 c.c.), water (30 c.c.), 2N-sodium hydroxide (30 c.c.), and water (30 c.c.), and dried ($MgSO_4$), and the solvent was removed by distillation under reduced pressure, yielding *pantetheine* $O^{2'}$*S-dibenzyl ether* (8·8 g., 87%) as an almost colourless syrup (Found: C, 65·2; H, 7·6; N, 6·3. $C_{25}H_{34}O_4N_2S$ requires C, 65·4; H, 7·4; N, 6·1%).

Reduction of Pantetheine $O^{2'}$*S-Dibenzyl Ether to Pantetheine.*—The dibenzyl ether (*ca.* 10 mg.) was dissolved in liquid ammonia (15 c.c.), and very small pieces of sodium were added until a blue colour persisted in the solution for 20 min. Excess of sodium was destroyed by gentle swirling in air, and solvent allowed to evaporate. The white, powdery residue was dissolved in water and the solution neutralised (pH 7) with dilute sulphuric acid. Solvent was removed by evaporation *in vacuo* and the residue extracted thrice with butanol. A drop of this solution was examined by paper chromatography in butanol–acetic acid–water. A spot (R_F 0·75), indistinguishable from that of pantetheine, was observed.

Pantetheine 4'-(Dihydrogen Phosphate).—Pantetheine *O*$^{2'}$*S*-dibenzyl ether (6 g.) was dissolved in dry pyridine (20 c.c.), and the solution cooled to the f. p. in acetone–solid carbon dioxide. To the slurry was added dropwise, with shaking, a solution of dibenzyl phosphorochloridate in carbon tetrachloride prepared from dibenzyl phosphite (6·9 g., 2 mol.) (Atherton, Openshaw, and Todd, *J.*, 1945, 382). The temperature was then kept at −5° for 30 min. and the mixture finally left at room temperature overnight. As much pyridine and carbon tetrachloride as possible were removed by distillation under reduced pressure, and the residue was dissolved in chloroform (20 c.c.), washed with 2N-sulphuric acid (2 × 20 c.c.) and water (2 × 20 c.c.), and dried ($MgSO_4$). The syrup obtained on evaporation of the solvent was dissolved in methyl alcohol (10 c.c.). Liquid ammonia (60 c.c.) and then sodium, in small pieces, were added until a transient blue colour was produced. Ammonia was allowed to evaporate off, the residue dissolved in ice-cold water (50 c.c.) and extracted with ether, and the clear aqueous solution

passed through IR-120 resin (H form) to remove bases. The acid eluate was neutralised (pH 7) with lithium hydroxide and evaporated to dryness.

Purification via *the lithium and silver salts.* The crude lithium salt was triturated with methyl alcohol (100 c.c.), and the undissolved fraction removed by centrifugation; this fraction was free from halide. The methyl-alcoholic extract was diluted with ether (300 c.c.), and the precipitate collected. Since it still contained a trace of chloride ion it was redissolved in methyl alcohol (50 c.c.) and diluted with ether (200 c.c.). The precipitate was collected by centrifugation, combined with the undissolved fraction, and dried. The lithium salt was dissolved in water (50 c.c.), and acetic acid added (to pH 4), followed by silver nitrate solution until no further precipitate was formed. The yellow silver salt was collected by centrifugation and washed with water (2 × 20 c.c.), after which the washings were almost free from silver ions. The precipitate was suspended in water (50 c.c.), the solution saturated with hydrogen sulphide, excess of hydrogen sulphide removed under reduced pressure, barium hydroxide solution added to pH 7, and the precipitate of silver sulphide removed by centrifugation. The clear aqueous solution was concentrated (*ca.* 10 c.c.) under reduced pressure, and ethyl alcohol (50 c.c.) added. No precipitate was produced. Ether (200 c.c.) was added and the precipitate of the *barium* salt of pantetheine 4′-phosphate collected by centrifugation and dried (1·5 g.) (Found: C, 25·6; H, 4·7; N, 5·6; P, 6·0; Ba, 28·2. $C_{11}H_{21}O_7N_2PSBa,H_2O$ requires C, 25·8; H, 4·1; N, 5·5; P, 6·1; Ba, 26·9%).

Ascending paper chromatography of this material showed it to be homogeneous with respect to phosphorus and sulphur in the propyl alcohol–ammonia–water and *iso*butyric acid–ammonia–water solvents.

	R_F in Pr^nOH–NH_3–H_2O	R_F in $Pr^i{\cdot}CO_2H$–NH_3–H_2O
Pantothenic acid 2′ : 4′-phosphate	0·51	0·40
Pantothenic acid 4′-phosphate	0·22	0·32
Pantetheine 4′-phosphate	0·28	0·55

D(+)-*Pantetheine 4′-(Dihydrogen Phosphate)* (IV; R = PO_3H_2).—A brisk current of oxygen was passed through a sintered bubbler into a solution of pantetheine (3·8 g.) in pyridine (60 c.c.) until a sample no longer gave a positive nitroprusside test for thiol. Anhydrous benzene (*ca.* 30 c.c.) was added and the solution evaporated to a syrup under reduced pressure. The residue was dissolved in anhydrous pyridine (50 c.c.) and cooled to −40°. Dibenzyl phosphorochloridate (from dibenzyl phosphite, 3·6 g., 1 mol.) was added and the solution kept at −40° for 15 min. After 3—4 hours at room temperature pyridine was removed by distillation under reduced pressure and the residue dissolved in chloroform. The chloroform solution was washed with successive portions of N-sulphuric acid, water, and sodium hydrogen carbonate solution, dried ($MgSO_4$), and evaporated. The residual syrup was washed thrice with benzene, during which it changed into a hard resin. The resin was dissolved in alcohol, a small amount of solid removed by centrifugation, and most of the alcohol removed by evaporation. The rather viscous solution was added to liquid ammonia (*ca.* 50 c.c.), and small pieces of sodium were added until a transient blue colour could be observed throughout the solution. Ammonia was evaporated off, and the residue dissolved in cold water (20 c.c.) and passed through IR-120 resin. The eluate was neutralised (pH 8) with barium hydroxide solution, barium phosphate removed by centrifugation, the clear solution evaporated to small volume, and the barium salt (2·0 g.) precipitated with acetone.

Purification through the silver salt. To a solution of the barium salt (1·2 g.) in water (50 c.c.) was added an excess of silver nitrate solution and the pH adjusted to 7 with dilute aqueous sodium hydroxide. The precipitated silver salt was collected by centrifugation, washed with water until washings were free from barium ions (about 4 washings were required), suspended in water, and decomposed with hydrogen sulphide. Excess of hydrogen sulphide was removed by aeration and the solution was neutralised (pH 8) with barium hydroxide solution. Silver sulphide was removed by centrifugation, and the clear supernatant liquid evaporated under reduced pressure to a very small volume. The *barium* salt of D(+)-pantetheine 4′-phosphate (probably containing some of the disulphide) (0·3 g.) was precipitated by addition of acetone and ether. A further quantity (0·6 g.) was extracted by suspending the silver sulphide precipitate in water and saturating it with hydrogen sulphide. During this treatment the precipitate decreased considerably in bulk and darkened. It was removed by centrifugation, and the supernatant liquid evaporated to small volume from which the barium salt was isolated as described above (Found: Ba, 28·3. $C_{11}H_{21}O_7N_2PSBa$ requires Ba, 27·8%). It had $[\alpha]_D^{18}$ +10·8° (*c*, 4·2 in H_2O). When examined on paper in the *iso*butyric acid–ammonia–water system it gave a

single spot (R_F 0·55), containing phosphorus and thiol and indistinguishable from pantetheine 4′-phosphate prepared as described above.

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