## 57. *The Synthesis of Pantothenic Acid-2′ and -4′ Phosphates as Possible Degradation Products of Coenzyme A.*

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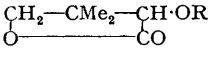
The two monophosphates of pantothenic acid have been synthesised by unambiguous routes for comparison with certain degradation products of coenzyme A, the factor involved in biological acetylation processes. Rates of acid hydrolysis of the phosphate bonds have been measured for both compounds.

Neither of these phosphates stimulated the growth of *Acetobacter suboxydans*. The structure of coenzyme A is discussed in the light of these results.

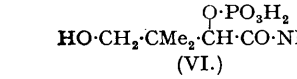
The importance of acetylation in living systems has become increasingly evident during the last few years. Acetylation of choline has long been recognised as a fundamental reaction in the chemistry of nerve action, and acetoacetic acid is a well-known metabolic product of acetic acid, but only lately has it been realised that acetylation is of great significance in other cellular transformations. The discovery by Lipmann (*Enzymologia*, 1937, **4**, 65; *Cold Spring Harbor Symposia*, 1939, **7**, 248; *Advances in Enzymology*, 1946, **6**, 231) of acetyl phosphate in *Lactobacillus delbrückii* and the demonstration that this was an active acetylating agent in enzymic reactions stimulated much subsequent work. "Energy-rich" acetylating agents, possibly chemically related to acetyl phosphate, are believed to participate in the complex cyclic processes involved in the metabolism of carbohydrates (Krebs, *Advances in Enzymology*, 1943, **3**, 191; Martius and Lynen, *ibid.*, 1950, **10**, 167). The presence of a co-factor of general occurrence has been shown to be necessary for the acetylation of aromatic amines by liver preparations (Lipmann, *Fed. Proc.*, 1945, **4**, 97; *J. Biol. Chem.*, 1945, **160**, 173) and of choline in brain (Lipmann and Kaplan, *J. Biol. Chem.*, 1946, **162**, 743). This factor, which is also active in other biological acetylation systems (Stern and Ochoa, *ibid.*, 1949, **179**, 491), has been called "coenzyme A," and hydrolysis of its highly active concentrates yields pantothenic acid (Lipmann, Kaplan, Novelli, Tuttle, and Guirard, *ibid.*, 1947, **167**, 869). The importance of this finding was increased when it was shown that most cellular pantothenic acid is present as either coenzyme A or closely related factors in both animals and plants (Kaplan and Lipmann, *ibid.*, 1948, **174**, 37; Novelli, Kaplan, and Lipmann, *ibid.*, 1949, **177**, 97; King, Fels, and Cheldelin, *J. Amer. Chem. Soc.*, 1949, **71**, 131). Intestinal phosphatase and pigeon-liver extracts inactivate coenzyme A, liberating pantothenic and phosphoric acids (Novelli, Kaplan, and Lipmann, *loc. cit.*), whence it seems probable that the coenzyme is a phosphorylated derivative of pantothenic acid. Coenzyme A itself stimulates the growth of *Lactobacillus arabinosus*, but the liver extract degradation product does not. However, this fragment is active in stimulating the growth of *Acetobacter suboxydans*, a property which is destroyed by treatment with intestinal phosphatase (Novelli, Flynn, and Lipmann, *J. Biol. Chem.*, 1949, **177**, 493). It would seem, therefore, that *A. suboxydans* can utilise a fragment of coenzyme A which exhibits the properties of a phosphoric ester of pantothenic acid. Recent enzymic experiments suggest that the coenzyme itself is a dinucleotide (Novelli, Kaplan, and Lipmann, *Fed. Proc.*, 1950, **9**, 209). The present work describes the synthesis of two isomeric phosphoric esters of pantothenic acid (I; R = R″ = H, R′ = $PO_3H_2$) and (I; R = R′ = H, R″ = $PO_3H_2$), with the expectation that one of them might be identical with the liver-extract degradation product of coenzyme A. Results of biological tests on the two esters are discussed below.

The method we envisaged for the synthesis of (±)-*N*-(4′-hydroxy-3′ : 3′-dimethyl-2′-phosphonoxybutyro)-β-alanine,* for convenience called (±)-pantothenic acid-2′ phosphate * (I; R = R″ = H, R′ = $PO_3H_2$), involved the direct phosphorylation of pantolactone (II) and reaction of the resulting phosphate with a suitable derivative of β-alanine, as in the synthesis of pantothenic acid from pantolactone (Williams, Mitchell, Weinstock, and Snell, *J. Amer. Chem. Soc.*, 1940, **62**, 1784). Phosphorylation of pantolactone with phosphoryl chloride did not give any recognisable product, and experiments with dibenzyl chlorophosphonate were equally unsuccessful since the unreactive secondary hydroxyl group in pantolactone is not phosphorylated at the low temperatures required. However, with diphenyl chlorophosphonate in anhydrous pyridine at 0° pantolactone was converted smoothly and in good yield into the crystalline diphenyl phosphate ester (III), which could be purified by distillation at low pressure. When this ester was heated with the sodium salt of β-alanine under conditions similar to those employed in the preparation of pantothenic acid from pantolactone (Williams, *et al.*, *loc. cit.*) a complex reaction occurred. On addition of water, phenol was liberated and from the aqueous solution only nitrogen-free phosphorylated products could be isolated by precipitation with lead acetate. The reaction was repeated under a wide variety of conditions, employing both sodium and lithium salts of β-alanine, but without success. In an effort to clarify the nature of this unexpected reaction, (III) was heated with an excess of *cyclo*hexylamine. A water-soluble, crystalline product, $C_{18}H_{35}O_5N_2P$, was isolated, which can be regarded as derived from one molecule of (III) and two molecules of base with elimination of both phenyl groups and an oxygen atom. One molecule of base is probably involved in amide linkage with the carbonyl group of (III) while the other may have displaced a phenoxy-group to give an aminophosphonate. With ammonia in alcohol at 30° the lactone (III) yielded small amounts of a substance, $C_{12}H_{19}O_5N_2P$, which is probably an aminophosphonate containing a residual phenoxy-group. Further work on the structure of these substances is in progress.

(I.) $R''O{\cdot}CH_2{\cdot}CMe_2{\cdot}CH(OR'){\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2R$



(II; R = H.) (III; R = $PO_3Ph_2$.)



(VI.)

(IV; R = $PO_3H_2$.) (V; R = $CH_2Ph$.)

It seemed likely that the difficulties encountered in the reaction of β-alanine derivatives with (III) arose through the presence of the two phenyl groups. Consequently, these were removed by hydrogenolysis and the resulting pantolactone-2′ phosphate (IV) was characterised by its behaviour with alkali. It consumed two equivalents of sodium hydroxide in the cold and a further equivalent on warming; thereafter, pantolactone-2′ phosphate could be recovered as its crystalline *cyclo*hexylamine salt. It followed that (IV) was both a monophosphate and a lactone. In order to demonstrate that it would behave normally with amines a sample was heated with *cyclo*hexylamine, and the product, isolated *via* the lead salt, was converted into its monostrychnine salt. Analysis of this agreed with that expected for the strychnine salt of (VI).

A more satisfactory method of forming the amide link between (IV) and β-alanine was then sought utilising the benzyl ester of β-alanine (Kuhn and Wieland, *Ber.*, 1940, **73**, 971). A syrup was obtained which presumably consisted of the benzyl ester (I; R = $CH_2Ph$, R′ = $PO_3H_2$, R″ = H) of pantothenic acid 2′-phosphate in the form of its salt with β-alanine benzyl ester, together with the decomposition products of β-alanine benzyl ester. A barium salt obtained from the crude benzyl ester appeared from its barium content to have lost a part of its benzyl group by hydrolysis during isolation. Removal of the benzyl group was then completed by hydrogenolysis, and (±)-pantothenic acid 2′-phosphate (I; R = R″ = H, R′ = $PO_3H_2$) was isolated in good yield as its barium salt.

The synthesis of the isomeric 4′-phosphate (I; R = R′ = H, R″ = $PO_3H_2$) necessitated protection of the secondary hydroxyl group before phosphorylation. Pantolactone readily formed a sodio-derivative when treated with powdered sodium or sodium ethoxide (cf. Fleck and Schinz, *Helv. Chim. Acta*, 1950, **33**, 140); with benzyl chloride in hot xylene this gave the crystalline benzyl ether (V) in good yield. The presence of a lactone ring in this substance was demonstrated by its behaviour with alkali. Reaction of (V) with the sodium salt of β-alanine gave the benzyl ether (I; R = R″ = H, R′ = $CH_2Ph$) as a syrup, the structure of which followed from hydrogenolysis to pantothenic acid. Diphenyl chlorophosphonate converted

* Geneva numbering ($CO_2H$ = 1) is used throughout this paper for the butyric acid derivatives.

(I; R = R″ = H, R′ = $CH_2Ph$) in anhydrous pyridine into a syrup (I; R = H, R′ = $CH_2Ph$, R″ = $PO_3Ph_2$). Hydrogenolysis of benzyl and phenyl groups from this proceeded smoothly and the resulting (±)-pantothenic acid 4′-phosphate was purified through either its silver or its lead salt and isolated as its barium salt. Alternatively the benzyl ether (V) was treated with β-alanine benzyl ester giving (I; R = R′ = $CH_2Ph$, R″ = H) which had the expected structure since it could be hydrogenated to pantothenic acid; this ester was phosphorylated with diphenyl chlorophosphonate to (I; R = R′ = $CH_2Ph$, R″ = $PO_3Ph_2$), and both benzyl and phenyl groups were removed by hydrogenolysis; the resulting (±)-pantothenic acid 4′-phosphate was isolated and purified as before

During this work it was observed that both diphenyl and dibenzyl phosphates form readily crystalline *S*-benzylthiuronium salts which have sharp melting points and are quite useful alternatives to the *cyclo*hexylamine salts for identification of these esters.

The two phosphates of pantothenic acid resembled each other closely in general physical properties. However, the phosphate group of the 4′-phosphate is more labile to acid than is that of the 2′-phosphate. In 2N-hydrochloric acid at 100° the 4′-phosphate was 50% and the 2′-phosphate was only 11% hydrolysed in 150 minutes. The 2′-phosphate showed first-order kinetics, $k$ being $1{\cdot}1 \times 10^{-5}$ sec.$^{-1}$; pantolactone 2′-phosphate was hydrolysed at the same rate and is thus probably an intermediate in the hydrolysis of pantothenic acid 2′-phosphate. The 4′-phosphate gave a first-order velocity constant of $6{\cdot}9 \times 10^{-5}$ sec.$^{-1}$ up to approximately 60% hydrolysis, but subsequently the rate fell to approximately that of the 2′-phosphate. The 2′- and the 4′-phosphate showed considerable stability toward alkali, no measurable hydrolysis occurring in 2 hours at 100° in 0·5N-potassium hydroxide.

The two pantothenic acid phosphates have been examined as stimulants for the growth of *A. suboxydans* by Dr. F. Lipmann, to whom we express our thanks. He reports that neither isomer showed growth stimulation or replaced free pantothenic acid. No inhibition was observed in either case and after treatment with alkaline phosphatase the expected growth response was observed from both substances corresponding to the liberation of free pantothenic acid. It would seem then that neither phosphate represents an active degradation product of coenzyme A and we are inclined to conclude that either the active substance is more complex and contains further substituents on the pantothenic acid residue besides phosphate or that phosphate is linked to pantothenic acid through some other component. However, through the kindness of Dr. F. Bergel, the two substances have been examined by Roche Products Ltd., for pharmacological effects on gut: in preliminary experiments both phosphates caused a slow but quite marked contraction of isolated rabbit and guinea-pig ileum. Under the conditions of the experiment neither pantothenic acid nor phosphate showed the effect. It is not possible at this stage to discuss the mechanism of this action. Further work on the relation between these phosphates and coenzyme A is in progress.

## Experimental.

(±)-3 : 3-*Dimethylbutyro-γ-lactone* 2-(*Diphenyl Phosphate*) (III).—Diphenyl chlorophosphonate (8·5 g.) was added slowly to a stirred solution of pantolactone (4 g.) in anhydrous pyridine (40 c.c.) at 0°. After the mixture had been kept for 1 hour at 0° and 12 hours at room temperature water (10 c.c.) was added and the resulting solution evaporated under reduced pressure below 45°. The residue was dissolved in chloroform (60 c.c.), washed with dilute sulphuric acid, sodium hydrogen carbonate solution, and water, and finally evaporated under reduced pressure. The syrupy residue crystallised slowly. The *phosphate* was purified either by recrystallisation from ether–light petroleum (b. p. 40—60°), as prisms, m. p. 70°, or by sublimation in a short-path still at 150°/$10^{-5}$ mm. (yield 10 g., 90%) (Found: C, 60·0; H, 5·1; P, 8·3. $C_{18}H_{19}O_6P$ requires C, 59·7; H, 5·2; P, 8·6%).

*Reaction with* cyclo*Hexylamine.*—A mixture of *cyclo*hexylamine (1·1 g.) and (III) (1 g.) was heated on a steam-bath for 5½ hours. The resulting syrup was dissolved in ether and shaken with water (3 × 10 c.c.). Evaporation of the combined aqueous extracts under reduced pressure left a resin which was dissolved in a little alcohol, and ether was added to incipient opalescence. Fine needles separated. Recrystallised from alcohol–ether the *substance* had m. p. 253° (0·5 g.) (Found: C, 55·2; 55·3; H, 9·1, 9·0; N, 6·7. $C_{18}H_{35}O_5N_2P$ requires C, 55·3; H, 9·0; N, 7·1%).

*Reaction with Ammonia.*—A solution of (III) (1 g.) in 10% alcoholic ammonia (100 c.c.) was kept at 30° for 5 minutes, then at room temperature for 1 hour. A crystalline *substance* was filtered off and recrystallised from acetic acid; it had m. p. 220° (Found, in substance dried at 110°/$10^{-1}$ mm.: C, 47·1; H, 6·4; N, 9·1; P, 9·8. $C_{12}H_{19}O_5N_2P$ requires C, 47·5; H, 6·3; N, 9·2; P, 10·2%).

(±)-3 : 3-*Dimethylbutyro-γ-lactone* 2-*Phosphate* (IV).—A solution of the diphenyl ester (III) (11 g.) in acetic acid (200 c.c.) was hydrogenated at atmospheric pressure and room temperature with a platinum oxide catalyst. After absorption of hydrogen had ceased (8 mols. absorbed at about 900 c.c. per hour) the catalyst was filtered off and solvent removed by distillation at reduced pressure. The residue crystal-

lised slowly as needles and traces of acetic acid were removed by rubbing them with ether. The *phosphate* had m. p. 130—140° (5·6 g., 90%) (Found : C, 35·0 ; H, 5·0 ; P, 14·9. $C_6H_{11}O_6P$ requires C, 34·5; H, 5·2; P, 14·8%). It readily formed a cyclo*hexylamine* salt which, recrystallised from alcohol, had m. p. 202—203° (Found : C, 46·5; H, 7·6; N, 4·5. $C_6H_{11}O_6P,C_6H_{13}N$ requires C, 46·5; H, 7·7; N, 4·5%). On titration (phenolphthalein) at room temperature the phosphate (100 mg.) consumed 9·31 ml. of 0·1N-sodium hydroxide (theor., 9·52 ml.). A further 5 ml. were consumed slowly on warming. The resulting solution did not give a precipitate with barium acetate solution, indicating the absence of hydrolysis of the phosphate residue during titration. Hydrochloric acid (1·23 ml.; 1·124N.), equivalent to the alkali consumed, was added and the solution evaporated to dryness under reduced pressure. The residue was treated with *cyclo*hexylamine and the *cyclo*hexylamine salt of (IV) extracted with hot alcohol. Recrystallised from alcohol it had m. p. 190—193° and mixed with authentic material, m. p. 195—196°.

(±)-N-cyclo*Hexyl*-4-*hydroxy*-3 : 3-*dimethyl*-2-*phosphonoxybutyramide* (VI).—To a mixture of pantolactone 2-phosphate (0·6 g.) and *cyclo*hexylamine (4 c.c.) on a steam-bath, sufficient alcohol was added to effect dissolution at the b. p. Solvent was then distilled off slowly and the remaining oil heated at 100° for 2 hours. Excess of *cyclo*hexylamine was removed at 100°/$10^{-1}$ mm. The residue was dissolved in water, and addition of lead acetate solution precipitated a lead salt. This was collected by centrifugation, washed well with water, and dried over sulphuric acid in a desiccator (0·9 g.). The lead salt was dissolved in aqueous alcohol, and lead removed by precipitation as the sulphide. The filtered solution was evaporated under reduced pressure, leaving a brittle resin. This did not crystallise but formed a *strychnine* salt, m. p. 160—170°, after recrystallisation from alcohol–ether (Found, in sample dried at 100° : C, 61·8; H, 7·1; N, 6·3. $C_{12}H_{24}O_6NP,C_{21}H_{22}O_2N_2$ requires C, 61·6; H, 7·1; N, 6·5%).

(±)-*Pantothenic Acid* 2′-*Phosphate* (I; R = R″ = H, R′ = $PO_3H_2$).—Pantolactone 2-phosphate (3·8 g.) was mixed with dry, freshly prepared β-alanine benzyl ester (12·8 g.) (prepared by the method of Kuhn and Wieland, *loc. cit.*) and the mixture heated at 100° for 6 hours with the exclusion of moisture and carbon dioxide. The reaction mixture was cooled to room temperature and dissolved in water (50 c.c.), and barium hydroxide solution (107 c.c. of 0·34N.) added. After evaporation of the mixture to small volume under reduced pressure, alcohol (150 c.c.) was added and the precipitated barium salt centrifuged, washed twice with alcohol, then once with ether, and dried in an oven at 100°. Addition of more barium hydroxide solution (60 c.c.) to the mother-liquors precipitated a further quantity of barium salt which was centrifuged and combined with the first lot. The combined barium salts were dissolved in water (60 c.c.), and carbon dioxide was passed through the solution. The small amount of insoluble material produced at this stage was removed by centrifugation, and the crude barium salt of (I; R = $CH_2Ph$, R′ = $PO_3H_2$, R″ = H) precipitated by addition of alcohol, centrifuged, washed with alcohol and then ether, and dried at 100° (6·9 g., 75%) (Found : Ba, 35·6. Calc. for $C_{16}H_{22}O_8NPBa$ : Ba, 26·1%). Barium was removed from the barium salt (5·7 g.) in water (100 c.c.) by dilute sulphuric acid (rhodizonic acid indicator), and the sulphate was separated by centrifugation and washed twice with cold water. Combined washings and supernatant liquor were diluted with alcohol (100 c.c.) and hydrogenated at room temperature and atmospheric pressure with a palladium oxide catalyst. When hydrogen absorption had ceased the catalyst was filtered off and barium hydroxide solution added to pH 8. After evaporation of the solution to about 40 c.c. under reduced pressure, alcohol (150 c.c.) was added and the precipitated barium salt collected by centrifugation. This was redissolved in water (50 c.c.), a small amount of insoluble material removed by centrifugation, and the barium salt reprecipitated by alcohol.

It was collected, washed with alcohol and ether, and dried as before (5·0 g.). Further purification was effected by dissolution in the minimum quantity of cold water, heating to 100°, filtering from some precipitated barium salts (all the barium salts described in this paper are more soluble in cold than in hot water), and addition of alcohol to the filtrate to precipitate the purified material. Repetition of this process yielded a pure *barium* salt (Found : C, 21·7; H, 3·6; N, 2·4; P, 6·5. $C_9H_{15}O_8NPBa_{1·5}$ requires C, 21·5; H, 3·0; N, 2·8; P, 6·2%).

(±)-2-*Benzyloxy*-3 : 3-*dimethylbutyro*-γ-*lactone* (V).—Pantolactone (11·8 g.) was added to a solution of sodium (2·09 g.) in dry alcohol (30 c.c.), and the solvent removed by distillation under reduced pressure. The solid sodio-derivative of pantolactone was dried at 90°/0·2 mm. and powdered under dry xylene (30 c.c.), and the suspension boiled under reflux with benzyl chloride (11·5 g.) for 2 hours. The cooled solution was washed with sulphuric acid (20 c.c.; 2N.) and then water, and dried ($Na_2SO_4$), and solvent removed by distillation at reduced pressure. The residual oil yielded on distillation the *benzyl ether* (14·5 g., 72%), b. p. 100—110°/$10^{-2}$ mm. or 80°/$10^{-4}$ mm. in a short-path still, as a crystalline solid, m. p. 46—47° (Found : C, 70·9; H, 7·4. $C_{13}H_{16}O_3$ requires C, 70·9; H, 7·3%). When heated for 5 minutes at 80° the ether (0·2115 g.) consumed the expected amount of sodium hydroxide (1·05 ml.; 0·911N.). Acidification of the resulting solution with hydrochloric acid precipitated the benzyl ether as an oil which was extracted with ether. The ethereal solution, dried ($Na_2SO_4$) and evaporated, left an oil which crystallised when seeded and rubbed (0·19 g., 90%), m. p. 46—47°, undepressed on admixture with an authentic sample of the benzyl ether, m. p. 46—47°.

(±)-*Pantothenic Acid* 2′-*Benzyl Ether* (I; R = R″ = H, R′ = $CH_2Ph$).—The sodium salt (0·44 g.) of β-alanine and kieselguhr (0·5 g.) were ground together and the intimate mixture was dried over phosphoric oxide. The benzyl ether (V) (0·88 g.) was added and the mixture heated at 120° for 2 hours. The pasty mass which solidified on cooling was triturated with ether and water and filtered from silica. The ethereal layer, on evaporation, yielded unchanged benzyl ether (0·12 g.). The aqueous layer was acidified to Congo-red with hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with water, dried ($Na_2SO_4$), and evaporated. The slightly impure (±)-*pantothenic acid* 2′-*benzyl ether* remained as a pale yellow syrup (1·05 g., 85%) (Found : N, 4·3. $C_{16}H_{23}O_5N$ requires N, 4·5%) (0·513 g. required 1·63 ml. of N-sodium hydroxide for neutralisation to phenolphthalein. Calc.: 1·66 ml.). Hydrogenation of a sample dissolved in alcohol at room temperature and atmospheric pressure with a

palladium catalyst yielded pantothenic acid, identified as its benzylthiuronium salt, m. p. 132—133°, undepressed on admixture with an authentic sample.

*Pantothenic Acid 4′-Phosphate* (I; R = R′ = H, R″ = $PO_3H_2$).—Diphenyl chlorophosphonate (3·1 g., 1·1 mols.) in anhydrous pyridine (8 c.c.) was added dropwise to a solution of pantothenic acid 2′-benzyl ether (3·25 g., 1 mol.) in pyridine (10 c.c.) cooled to −10°. The mixture was kept at −10° for 1 hour and left at room temperature overnight. Water (*ca.* 1 c.c.) was added and as much pyridine as possible removed by distillation under reduced pressure. The residue was dissolved in chloroform, washed with water (15 c.c.), dilute hydrochloric acid (15 c.c.), and finally water (3 × 15 c.c.), and then dried ($Na_2SO_4$). Evaporation of the chloroform left a viscous, pale yellow syrup (5·45 g.). Attempts to prepare a *S*-benzylthiuronium salt from a sample of this material were unsuccessful, the only crystalline Product isolated being a small amount of the *S*-benzylthiuronium salt of diphenyl phosphate, m. p. 195—197°, undepressed when mixed with a sample of the authentic salt prepared by the method described below. The syrup without further purification was hydrogenated in acetic acid solution at room temperature and atmospheric pressure with a platinum oxide catalyst. When hydrogen absorption had ceased, barium acetate (4·0 g.) dissolved in the minimum amount of water was added and solvent removed by distillation under reduced pressure. The residue was dissolved in water and the pH adjusted to 8—9 by saturated barium hydroxide solution. A small precipitate of barium phosphate was centrifuged off and barium removed quantitatively from the clear supernatant liquid by titration with sulphuric acid (rhodizonic acid). Barium sulphate was removed by centrifugation and the volume of the supernatant liquid concentrated under reduced pressure. Barium hydroxide was added again to pH 8 and the crude phosphate precipitated by acetone, washed with acetone and then ether, and dried at 100° (yield, 3·9 g., 82%) (Found : C, 23·2; H, 4·2; N, 2·8. Calc. for $C_9H_{15}O_8NPBa_{1·5}$ : C, 21·5; H, 3·0; N, 2·8%).

*Purification* via *the lead salt.* The barium salt (1·0 g.) was dissolved in water (*ca.* 5 c.c.), and a saturated solution of lead acetate added until precipitation was complete. The heavy white precipitate was centrifuged and washed with a little cold water, in which it was appreciably soluble. The precipitate was re-suspended in water, lead removed by precipitation as the sulphide which was centrifuged off, and the clear solution reduced slightly in volume under reduced pressure. Barium hydroxide solution was added to pH 9 and the slight excess of alkali removed by carbon dioxide and centrifugation. The clear solution, on dilution with alcohol, yielded the pure *barium* salt (0·45 g.) which was washed with alcohol and ether and dried in a desiccator (Found : C, 22·0; H, 3·2; N, 3·0; P, 5·8; Ba, 41·5. $C_9H_{15}O_8NPBa_{1·5}$ requires C, 21·5; H, 3·0; N, 2·8; P, 6·2; Ba, 41·1%).

*Benzyl Pantothenate 2′-Benzyl Ether* (I; R = R′ = $CH_2Ph$, R″ = H).—β-Alanine benzyl ester (5·3 g.) and (V) (6·5 g.) were heated together at 100° for 3 hours, then cooled, and the oily product dissolved in ether and washed with water. After being dried ($MgSO_4$) the solvent was removed under reduced pressure, yielding *benzyl pantothenate 2′-benzyl ether* as a viscous oil (10·7 g., 90%) (Found : C, 68·9; H, 7·4; N, 3·2. $C_{23}H_{29}O_5N$ requires C, 69·2; H, 7·3; N, 3·5%).

*Phosphorylation.*—Diphenyl chlorophosphonate (10 g., 1·5 mols.) was added dropwise to a solution of the benzyl ester (9·85 g., 1 mol.) in anhydrous pyridine (30 c.c.) cooled to −10°. After the mixture had been kept at room temperature overnight, water (*ca.* 5 c.c.) was added and as much pyridine as possible removed by distillation under reduced pressure. The oily residue was dissolved in chloroform (30 c.c.), washed successively with hydrochloric acid (2N.), water, sodium hydrogen carbonate solution (2·5%), and water (30 c.c. of each), and then dried ($MgSO_4$). Removal of the solvent gave the *diphenyl phosphate ester* (I; R = R′ = $CH_2Ph$, R″ = $PO_3Ph_2$) as a golden-yellow mobile oil (10·8 g., 71%) (Found : C, 67·0; H, 6·4; N, 2·1. $C_{35}H_{38}O_7NP$ requires C, 68·3; H, 6·2; N, 2·3%. The low carbon content is possibly caused by contamination with a little chloroform).

The oil (2·1 g.) in pure dioxan (*ca.* 10 c.c.) was passed through a column of Raney nickel and alumina to remove catalyst poisons and coloured impurities. Water (*ca.* 5 c.c.) was added to the eluate which was then hydrogenated at room temperature and atmospheric pressure with a mixed palladium–platinum oxide catalyst. A total of 1080 ml. of hydrogen was absorbed at an average rate of about 1·5 ml./min. (Theor. for 8 mols. = 1330 ml.). The catalyst was filtered off, barium hydroxide solution added to pH 8, and the solution evaporated to small volume under reduced pressure. Alcohol was added and the precipitated barium salt centrifuged off, washed with alcohol and then ether, and dried at room temperature (0·75 g., 44%).

*Purification* via *the silver and barium salts.* The impure barium salt (0·37 g.) was dissolved in water (*ca.* 5 c.c.), and a concentrated aqueous solution of silver nitrate (0·35 g.) added. The precipitated silver salt was centrifuged off, washed with a little water, suspended in water, and decomposed with hydrogen sulphide. Silver sulphide was centrifuged off and washed with water, and the combined supernatant liquid and washings were freed from hydrogen sulphide by evaporation under reduced pressure. Barium hydroxide solution was added to pH 9, carbon dioxide passed through the solution to remove excess of barium ions, and a small precipitate of barium carbonate centrifuged off. The clear aqueous solution was diluted with alcohol and the precipitated barium salt removed by centrifugation. Final purification was effected by redissolving the salt in water, centrifuging off a small precipitate, and precipitating the product with alcohol. The pure *salt* (0·22 g.) was washed with alcohol and ether and dried at 100°/0·1 mm. (Found : C, 21·3; H, 3·7; N, 2·8; P, 5·9; Ba, 40·8. $C_9H_{15}O_8NPBa_{1·5}$ requires C, 31·5; H, 3·0; N, 2·8; P, 6·2; Ba, 41·1%). A sample of the solution of barium salt obtained directly from the silver salt was evaporated to dryness from the frozen state but analysis indicated that this was not pure.

S-*Benzylthiuronium Salts of Diphenyl and Dibenzyl Phosphate.*—Prepared from the corresponding sodium salts and benzylthiuronium chloride in aqueous alcohol in the usual way and recrystallised from aqueous alcohol, S-*benzylthiuronium diphenyl phosphate* crystallised as needles, m. p. 195—197° (Found : C, 57·8; H, 5·4; N, 6·5; S, 7·4. $C_{20}H_{21}O_4N_2PS$ requires C, 57·7; H, 5·1; N, 6·7; S, 7·7%), and

S-*benzylthiuronium dibenzyl phosphate* as fine silky needles, m. p. 163—164° (Found : C, 59·3; H, 5·5; N, 6·0; S, 6·8. $C_{22}H_{25}O_4N_2PS$ requires C, 59·5; H, 5·6; N, 6·3; S, 7·2%).

*Acid Hydrolysis of Pantothenic Acid* 2′- and 4′-*Phosphate and Pantolactone* 2-*Phosphate.*—The barium salts of pantothenic acid 2′- and 4′-phosphate (*ca.* 6 mg.) and of pantolactone 2-phosphate (*ca.* 3 mg.) were severally dissolved in hydrochloric acid (20 ml.; 2N.), and the solutions heated in hard glass flasks in a boiling water-bath. Free phosphate was measured in aliquots at intervals photometrically by the method of Allen (*Biochem. J.*, 1940, **34**, 858), with the following results :

| Time (mins.) | | 30 | 60 | 105 | 160 | 255 | 335 | 400 |
|---|---|---|---|---|---|---|---|---|
| Free phosphorus, % : | 2′-ester | 2·4 | 6·0 | 6·8 | 9·7 | 18·4 | 20·8 | 21·8 |
| | 4′-ester | 10·1 | 25·1 | 40·2 | 55·9 | 63·0 | 67·0 | — |
| | lactone 2-phosphate | 4·4 | 6·0 | 8·4 | 9·3 | 17·2 | 19·9 | 20·8 |

*Alkaline Hydrolysis.*—The barium salts (*ca.* 6 mg.) were severally dissolved in water (2—3 c.c.), and a slight excess of sodium sulphate was added to precipitate barium as its sulphate. The precipitates were centrifuged off and washed with water. Supernatant liquids and washings were made up to 20 ml. with sodium hydroxide solution so that the final concentration was 0·5N. The alkaline solutions were heated at 100° in silver tubes, and aliquots withdrawn at intervals. Free phosphate was determined as above. After 2 hours there was no measurable phosphate liberated from either pantothenic acid 2′- or 4′-phosphate.

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