Coenzyme A. Part IX. The Synthesis of Pantothenoylcysteine, its 4′-Phosphate, and Related Compounds as Possible Precursors of the Coenzyme.*

By J. BADDILEY and A. P. MATHIAS.

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Several derivatives of pantothenic acid have been synthesised and examined as possible precursors of coenzyme A in micro-organisms. These include pantothenoylcysteine and its 4′-phosphate, dipantothenoylcystine and its 4′-phosphate, pantothenamide, pantothenamidoacetaldehyde, *N*-2-hydroxyethylpantothenamide, and peptides of pantothenic acid with glycine, α-alanine, β-alanine, serine, and glutamic acid.

Brown and Snell's observation (*J. Amer. Chem. Soc.*, 1953, **75**, 2782) that pantothenoylcysteine is a precursor of coenzyme A in *Acetobacter suboxydans* is confirmed. Some bacteria can utilise other peptides of pantothenic acid.

IT is now known (Levintow and Novelli, Abs. Amer. Chem. Soc. Meeting, Atlantic City, 1952, p. 33c; Baddiley, Thain, Novelli, and Lipmann, *Nature*, 1953, **171**, 76) that intermediate stages in the biological synthesis of coenzyme A (CoA) (I) involve pantetheine (II), pantetheine-4′-phosphate, and 3′-dephospho CoA. However, when this work was started little was known about the stages in the conversion of pantothenic acid into pantetheine (II)

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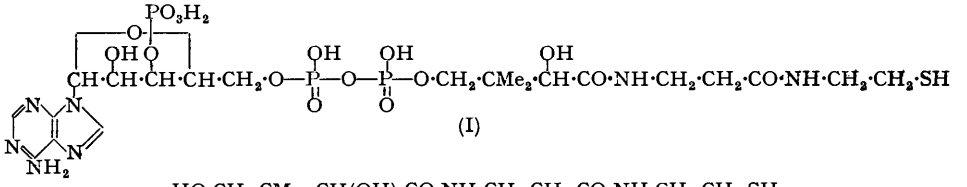
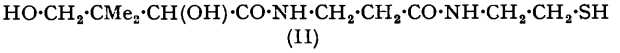
or its 4′-phosphate. This paper describes the synthesis of several derivatives of pantothenic acid which, for various reasons, were considered as possible CoA precursors.

Lactobacillus arabinosus can convert pantetheine into CoA. However, it does not produce CoA from pantothenic acid unless cystine is present in the medium (Pierpoint and Hughes, *Biochem. J.*, 1954, **56**, 130). The fact that cystine cannot be replaced by 2-mercaptoethylamine implies that pantetheine is not formed by the condensation of pantothenic acid with 2-mercaptoethylamine, the latter arising from the decarboxylation of cysteine. Possible mechanisms for pantetheine synthesis include the formation of pantothenoylcysteine (III*a*) and subsequent decarboxylation, or the formation of a pantothenoyl-peptide followed by transfer of a thiol group from cysteine. In an effort to decide between these alternatives we have synthesised pantothenoylcysteine and several sulphur-free peptides of pantothenic acid, and their behaviour towards growth and CoA-production in micro-organisms has been examined.

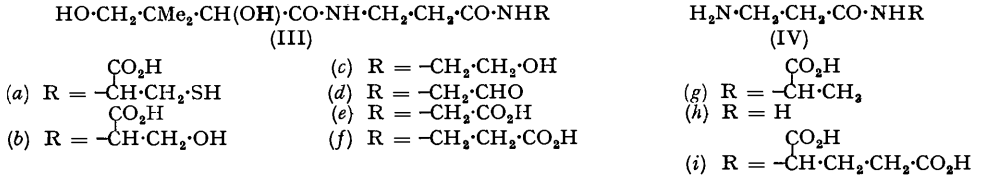
Pantothenoylcysteine (III*a*) or the corrresponding disulphide was synthesised by four different routes. In the first route the mixed carbonic anhydride obtained from pantothenic acid and ethyl chloroformate (Wieland and Bokelmann, *Naturwiss.*, 1951, **38**, 384) was allowed to react with *S*-benzylcysteine to give impure *S*-benzylpantothenoylcysteine (V). The benzyl group was removed by reduction with sodium in liquid ammonia, yielding impure pantothenoylcysteine. A pure sample of pantothenoylcysteine, necessary for biological evaluation, was obtained by chromatography on a cellulose column in butanol–water. The pure substance contained no free amino-group, gave a strong thiol reaction with nitroprusside, and a slowly developing ninhydrin reaction typical of a peptide. All the pantothenic acid derivatives and peptides described in this paper gave very weak ninhydrin reactions but their presence could be demonstrated by the chlorine–



(I)



(II)



starch–iodide method (Buchanan, personal communication; Rydon and Smith, *Nature*, 1952, **169**, 922).

Two other routes to pantothenoylcysteine involve the preparation of β-alanylcysteine (IV*a*) and its reaction with pantolactone (VI). The general peptide synthesis in which amino-groups are protected as their phthaloyl derivatives (Sheehan and Frank, *J. Amer.*

Chem. Soc., 1949, **71**, 1856; Kidd and King, *Nature*, 1948, **162**, 776) was successful but inconvenient. Although phthaloyl-β-alanine chloride and cysteine readily gave phthaloyl-β-alanylcysteine (VII), removal of the phthaloyl residue was difficult. β-Alanylcysteine and its disulphide were obtained more easily by the benzyloxycarbonyl peptide synthesis. Benzyloxycarbonyl-β-alanine chloride and *S*-benzylcysteine gave *S*-benzyl-*N*-(benzyloxycarbonyl-β-alanyl)cysteine (VIII). Both the benzyloxycarbonyl and the benzyl group were removed by reduction with sodium in liquid ammonia, and the purified product consisted of a mixture of β-alanylcysteine (IV*a*) and its disulphide. These were readily interconvertible by oxidation with iodine or reduction with sodium in liquid ammonia, and their constitutions were established by acid hydrolysis to β-alanine and cystine.

The most satisfactory synthesis of pantothenoylcysteine involved conversion of the *S*-benzyl derivative (VIII) into *S*-benzyl-β-alanylcysteine (IX) by reduction with sodium in liquid ammonia and re-benzylation of the thiol group *in situ*. The benzyl peptide was then condensed with pantolactone to give *S*-benzyl-*N*-(D-pantothenoyl)cysteine (V) from which the benzyl group was removed with sodium in liquid ammonia.

Pantothenoylserine (III*b*) was best prepared by treating benzyloxycarbonyl-β-alanine with ethyl chloroformate followed by serine, to give benzyloxycarbonyl-β-alanylserine. Removal of the benzyloxycarbonyl group by catalytic hydrogenolysis yielded β-alanylserine (IV*b*) which, when heated with pantolactone and dimethylamine in alcohol, gave pantothenoylserine in good yield (cf. B.P. 660,722). Pantothenoylserine containing considerable amounts of pantothenic acid was also prepared by the direct action of serine on the anhydride from pantothenic acid and ethyl chloroformate.

Benzyloxycarbonyl-β-alanine azide reacted readily with 2-hydroxyethylamine. The single neutral product did not evolve nitrogen when treated with nitrous acid and so contained no free amino-group. It was converted into *N*-β-alanyl-2-hydroxyethylamine (IV*c*) by hydrogenolysis with sodium in liquid ammonia. When this amide was heated with pantolactone, *N*-2-hydroxyethylpantothenamide (III*c*) was obtained in high yield. Pantothenoylglycine (III*e*), pantothenoyl-β-alanine (III*f*), pantothenoyl-α-alanine (III*g*), and pantothenoylglutamic acid (III*i*), were prepared by heating the respective β-alanylpeptides (IV) with pantolactone and dimethylamine in alcohol and were purified through their readily crystallisable dimethylamine salts. Pantothenamide (III*h*) was obtained in good yield by heating β-alaninamide (IV*h*) with pantolactone, this being a more convenient method than that of Wieland, Molier, and Dieckelmann (*Chem. Ber.*, 1952, **85**, 1035).

The reaction of aminoacetaldehyde diethyl acetal with the mixed anhydride from pantothenic acid and ethyl chloroformate gave the pantothenoylamino-acetal. Cautious acid hydrolysis then liberated pantothenoyl aminoacetaldehyde (III*d*) which was characterised as its 2 : 4-dinitrophenylhydrazone and with silver oxide gave pantothenoylglycine.

While this work was in progress Brown and Snell (*J. Amer. Chem. Soc.*, 1953, **75**, 2782) and later King and Cheldelin (*Proc. Soc. Exp. Biol. N.Y.*, 1953, **84**, 591) announced that pantothenoylcysteine (synthesised independently by Bowman and Cavalla, unpublished work) is utilised by *A. suboxydans*, but not be *L. arabinosus*, as a CoA precursor. Pantothenoylcysteine, prepared by us, has been examined by Drs. D. E. Hughes and W. S. Pierpoint who confirm these findings.

Brown and Snell suggested that pantothenoylcysteine-4′ phosphate (X) might be a CoA precursor. We have synthesised it by a route essentially similar to the synthesis of

$$H_2O_3PO{\cdot}CH_2{\cdot}CMe_2{\cdot}CH(OH){\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}\underset{CO_2H}{CH}{\cdot}CH_2{\cdot}SH$$
(X)

pantetheine-4′ phosphate (Part VIII). *S*-Benzylpantothenoylcysteine (V) was phosphorylated with dibenzyl phosphorochloridate in pyridine, and benzyl groups were removed with sodium in liquid ammonia. Under these conditions the 2′-hydroxyl group is unaffected. The structure of the product was proved by hydrolysis to cysteine and pantothenic acid-4′ phosphate (Baddiley and Thain, *J.*, 1951, 246).

Pantothenoylcysteine-4′ phosphate had about 40% of the activity of pantothenyl cysteine and twice the activity of pantothenic acid in the growth of *A. suboxydans*. This may mean that dephosphorylation to pantothenoylcysteine occurs before conversion into

CoA. However, it is possible that different micro-organisms adopt different routes or sequences for the synthesis of the coenzyme. In support of this is the observation (Baddiley, Hughes, Mathias, and Pierpoint, *Biochem. J.*, 1954, **56**, xxii) that *L. arabinosus* can phosphorylate pantothenic acid itself to pantothenic acid-4′ phosphate. It is not yet known whether this represents a normal step in the conversion of pantothenic acid into CoA in this organism but, if so, it would seem that pantothenoylcysteine-4′ phosphate must also be an intermediate. The failure of this organism to respond to external supplies of the phosphorylated intermediates would be ascribed to their inability to penetrate the cells.

Of the other peptides described here only two were significantly active towards growth of, or CoA-production in, a variety of organisms tested. Pantothenoyl-β-alanine (III*f*) had 20—30% of the activity of pantothenic acid in supporting the growth of *Proteus morganii*. Pantothenoylglycine (III*e*) was variable in its effect on *L. arabinosus*: it had 30—80% of the activity of pantothenic acid when cystine was present in the medium. It seems likely that both these substances owe their effect to hydrolysis to pantothenic acid by peptidases in these organisms. The complete inactivity of all the other sulphur-free peptides described here suggests that rather specific β-alanine- and glycine-peptidases are present in the respective organisms.

Experimental

Phthaloyl-β-alanylcysteine.—A solution of phthaloyl-β-alanine chloride (2·37 g.) (Gabriel, *Ber.*, 1908, **41**, 243) in dioxan (50 c.c.) was added with stirring to sodium hydrogen carbonate (2·7 g.) in water (100 c.c.) containing L-cysteine hydrochloride (1·57 g.) at 0—5°. The mixture was kept for 1 hr. at room temperature, then acidified (Congo-red) with hydrochloric acid, and evaporated *in vacuo* to a small volume. The crystalline *peptide* was filtered off, washed with water, and dried. Recrystallised from alcohol it had m. p. 164—165° (2·7 g.) (Found: C, 52·0; H, 4·5; N, 8·8; S, 9·5. $C_{14}H_{14}O_5N_2S$ requires C, 52·0; H, 4·4; N, 8·7; S, 10·0%). When examined by paper chromatography in butanol–acetic acid–water it had R_F 0·82.

Di-β-alanylcystine (*Method* I).—Phthaloyl-β-alanylcysteine (1 g.) and 90% hydrazine hydrate (0·18 c.c.) in alcohol (10 c.c.) were refluxed for 3 hr. Solvent was removed under reduced pressure and the residue was triturated with N-hydrochloric acid (5 c.c.) for 20 min. Undissolved material was filtered off and washed with a little dilute hydrochloric acid, then with water. Combined filtrate and washings were passed through a column of Amberlite IRA-400 resin (formate form) to remove chloride ions. The effluent was concentrated under reduced pressure, then acetone and alcohol were added. The white precipitate (0·3 g., 50%) consisted of two components (R_F 0·11 and 0·29), the latter being present only in small amount. The minor component was removed by fractional precipitation from aqueous solution with alcohol and acetone. The more soluble *peptide* (R_F 0·11 in butanol–acetic acid–water) was thus obtained pure (Found: C, 37·0; H, 5·9; N, 14·6; S, 16·8. $C_{12}H_{22}O_6N_4S_2$ requires C, 37·7; H, 5·8; N, 14·7; S, 16·8%).

Hydrolysis. A sample (10 mg.) was heated with 6N-hydrochloric acid in a sealed tube at 100° for 3 hr. The hydrolysate was evaporated to dryness in a desiccator. A second sample was treated with hydrogen peroxide (0·1 c.c.; 30-vol.) at room temperature for 30 min., then gently warmed to decompose excess of peroxide. The solution was diluted with an equal volume of concentrated hydrochloric acid and hydrolysed as above. Examination of these two hydrolysates by paper chromatography in *n*-propanol–ammonia–water (6 : 3 : 1) showed that the first contained β-alanine (R_F 0·29) and cystine (R_F 0·17), and the second β-alanine and cysteic acid (R_F 0·23).

S-*Benzyl*-N-(*benzyloxycarbonyl*-β-*alanyl*)*cysteine.*—Benzyloxycarbonyl-β-alanine chloride (Sifferd and du Vigneaud, *J. Biol. Chem.*, 1935, **108**, 753) (prepared from benzyloxycarbonyl-β-alanine, 2·23 g.) in dry ether (15 c.c.) was added with shaking to a solution of *S*-benzyl-L-cysteine (2·11 g.) (Wood and du Vigneaud, *ibid.*, 1939, **130**, 110) in 2N-sodium hydroxide (10·5 c.c.) at 0°. The sodium salt of the product crystallised during the reaction. After 1 hr. at room temperature water was added to dissolve the precipitate. The ethereal layer was separated and discarded. The aqueous layer was acidified (Congo-red) with 5N-hydrochloric acid, and the precipitate filtered off, washed with water, then dried. The finely powdered material was thoroughly washed with ether to remove benzyloxycarbonyl-β-alanine, then extracted with boiling ethyl acetate (3 × 50 c.c.). The combined extracts were evaporated to dryness under reduced pressure, and the residue was recrystallised from ethyl acetate. The *benzyloxycarbonyl*

compound crystallised as prisms, m. p. 122—124° (3·4 g., 78%) (Found: C, 60·7; H, 5·4; N, 6·6; S, 8·0. $C_{21}H_{24}O_5N_2S$ requires C, 60·6; H, 5·8; N, 6·7; S, 7·7%).

β-Alanylcysteine and its Disulphide. (*Method* II.)—Small pieces of sodium were added to a solution of *S*-benzyl-*N*-(benzyloxycarbonyl-β-alanyl)cysteine (2·0 g.) in liquid ammonia (25 c.c.) until a persistent blue colour was produced. Excess of sodium was destroyed by adding glacial acetic acid in ammonia. Solvent was evaporated under reduced pressure and the residue treated with a suspension of Amberlite IRC-50 resin (ammonium form) in ice-water (50 c.c.). The turbid suspension was poured on to a column of IRC-50 (ammonium form) which was then washed with water. The effluent was washed with ether, concentrated under reduced pressure, and diluted with alcohol and ether, yielding white crystals (0·85 g., 92%). This mixture was resolved by a 55-stage counter-current distribution between the two phases of the butanol–acetic acid–water. Tubes 1 and 2 contained a slow-moving substance (R_F 0·11) and tubes 4—9 a faster-moving substance (R_F 0·29). Hydrolysis and paper chromatography as described above showed that both contained β-alanine and cysteine or cystine. The fractions were evaporated to small volume *in vacuo* and the peptides precipitated by addition of alcohol. *β-Alanylcysteine* (R_F 0·29) (0·5 g.) gave an intense colour with sodium nitroprusside after spraying with ammonia (Found: C, 37·4; H, 6·7; N, 14·2; S, 16·7. $C_6H_{12}O_3N_2S$ requires C, 37·5; H, 6·3; N, 14·6; S, 16·7%). Di-β-alanylcystine (R_F 0·11) (0·2 g.) was indistinguishable from the peptide obtained from phthaloyl-β-alanylcysteine. It gave no colour with nitroprusside but after reduction with potassium hydrogen sulphite–cyanide solution a positive thiol reaction was obtained (Found: C, 37·0; H, 6·0; N, 14·7%). β-Alanylcysteine was readily oxidised to this disulphide by iodine in aqueous sodium iodide.

NN′-*Di*-D-*pantothenoyl*-L-*cystine*.—β-Alanylcysteine (0·384 g.), D-pantolactone (0·26 g.), and dimethylamine (0·2 c.c.) were heated together in methanol (1·5 c.c.) for 8 hr. A small amount of undissolved solid was removed by centrifugation and washed with methanol. Combined supernatant liquid and washings were evaporated to dryness *in vacuo*. The crude product was dissolved in water, and the solution passed through a column of Amberlite IR-120 resin (H^+ form). The effluent was washed with ether, neutralised with dimethylamine, and evaporated to dryness, leaving a syrup (0·6 g., 82%). On paper it moved as a single spot (R_F 0·34), indistinguishable from that given by a sample of pantothenoylcysteine (prepared by the method described below) which had been oxidised with iodine in sodium iodide solution. Similarly, the above disulphide, after reduction with sodium in liquid ammonia, gives a spot (R_F 0·65) indistinguishable from that given by pantothenoylcysteine.

D-*Pantothenoyl*-L-*cysteine*.—Ethyl chloroformate (1·95 c.c.) was added dropwise with shaking to a solution of sodium pantothenate (4·82 g.) in dimethylformamide (30 c.c.) cooled to −5°. After 10 min., a solution of *S*-benzyl-L-cysteine (4·22 g.) in 1·025N-sodium hydroxide (19·5 c.c.) was added with vigorous stirring. The resulting solution was kept at room temperature for 1 hr. Solvents were evaporated under reduced pressure and the residue was dissolved in water. A small amount of *S*-benzylcysteine was filtered off, and the filtrate acidified (Congo-red) with dilute hydrochloric acid and extracted with chloroform (3 × 20 c.c.). The combined extracts were dried ($MgSO_4$) and evaporated to dryness *in vacuo* and the resulting syrup (7·7 g.) was dissolved in liquid ammonia. Metallic sodium was added in small pieces until a blue colour persisted for 30 min. The slight excess of sodium was destroyed with ammonium chloride, and ammonia was allowed to evaporate. The residue was dissolved in ice-water and the pH adjusted to 7 with sulphuric acid. A solution of mercuric sulphate (30%) in sulphuric acid (10%) was added with stirring until no further precipitation occurred. The precipitate was collected by centrifugation, washed with water, suspended in water, and decomposed by hydrogen sulphide. After filtration, the solution was neutralised with barium hydroxide and evaporated to dryness. Paper chromatography of the crude barium salt (8·7 g.) in butanol–acetic acid–water showed that the main component was a sulphur-containing peptide (R_F 0·65). A sulphur-containing peptide of R_F 0·85 and traces of unidentified substances were also present. An aqueous solution of the crude barium salt was passed through a column of Amberlite IR-120 resin (H^+ form), reprecipitated as the mercury salt, and isolated as its barium salt. This consisted of a mixture of the two sulphur-containing peptides only.

Purification by chromatography on cellulose. A column (110 × 2 cm.) of washed, powdered cellulose was equilibrated by thorough washing with butanol saturated with water. The crude barium salt (from above experiment) (250 mg.) was dissolved in water and barium removed by passage through a column of Amberlite IR-120 resin (H^+ form). Most of the solvent was removed *in vacuo* and the residue was dissolved in a little wet butanol. The solution was transferred to the column which was developed with saturated butanol. 100 fractions of

2 c.c. each were collected. Fractions 79—84 contained the substance of R_F 0·85, and fractions 87—100 the substance of R_F 0·65. The appropriate fractions were bulked and evaporated under reduced pressure. The residues were dissolved in water, neutralised with barium hydroxide, and evaporated to dryness. The barium salt of the substance, R_F 0·85 (90 mg.), was precipitated from aqueous alcohol with acetone–ether (Found: C, 27·9; H, 4·0; N, 4·2; S, 7·2; Ba, 23·8%). A sample of the free acid was hydrolysed for 1 hr. at 100° with concentrated hydrochloric acid. Paper chromatography of the hydrolysate in butanol–acetic acid–water and butanol–pyridine–water (65 : 35 : 65) indicated the presence of cysteine but absence of β-alanine. In the latter solvent mixture a second sulphur-containing, ninhydrin positive hydrolysis product (R_F 0·26) besides cysteine was observed. The barium salt of the substance of R_F 0·65 (110 mg.) was precipitated from alcohol by acetone and ether. This consisted of slightly impure *barium pantothenoylcysteine* salt (Found: C, 33·3; H, 5·0; N, 5·2; S, 5·3; Ba, 19·5. $C_{12}H_{21}O_6N_2SBa_{\frac{1}{2}}$ requires C, 36·9; H, 5·4; N, 7·2; S, 8·2; Ba, 17·6%). A sample of the free *acid* was prepared by removing barium ions with an Amberlite IR-120 column (H^+ form), then freeze-drying the eluate (Found: C, 45·3; H, 7·1; N, 7·7. $C_{12}H_{22}O_6N_2S$ requires C, 46·1; H, 6·8; N, 8·7%). Hydrolysis of the free acid and paper chromatography as described above indicated the presence of β-alanine and cysteine.

S-*Benzyl*-N-β-*alanyl*-L-*cysteine*.—Sodium (slight excess), in small pieces, was added to a solution of benzyloxycarbonyl-β-alanyl-*S*-benzyl-L-cysteine (41·6 g.) in liquid ammonia (500 c.c.), the excess being destroyed by ammonium chloride. Benzyl chloride (23 c.c.) was added dropwise with stirring. Ammonia was removed, finally at a water-pump. The residue was dissolved in ice-water (200 c.c.). The solution was washed with ether (2 × 50 c.c.) and adjusted to pH 6 by 5N-hydrochloric acid. The precipitate was filtered off and washed with water and alcohol. Recrystallisation from boiling water gave S-*benzyl*-N-β-*alanyl*-L-*cysteine* (23 g., 85%) as colourless needles, m. p. 233°, sharply depressed on admixture with *S*-benzylcysteine. In butanol–acetic acid–water it had R_F 0·65 (cf. *S*-benzylcysteine, R_F 0·65). It gave no colour with cyanide–nitroprusside, but was readily detected on paper by reaction with ninhydrin solution (yellow spot) or by the reagent of Winegard, Toennies, and Block (*Science*, 1948, **108**, 506) (Found: C, 55·5; H, 6·0; N, 9·7; S, 11·5. $C_{13}H_{18}O_3N_2S$ requires C, 55·3; H, 6·4; N, 9·9; S, 11·3%).

S-*Benzyl*-N-D-*pantothenoyl*-L-*cysteine*.—D-Pantolactone (8·23 g.), β-alanyl-*S*-benzyl-L-cysteine (17·82 g.), and dimethylamine (6·2 c.c.) were heated together in dry boiling methanol (120 c.c.) for 21 hr. After cooling, the solution was filtered to remove unchanged β-alanyl-*S*-benzyl-L-cysteine (1·5 g.). The filtrate and washings were evaporated to dryness and the residue was dissolved in 20% ethanol. The solution was passed through a column of Amberlite IR-120 resin (H^+ form). The effluent was neutralised with barium hydroxide solution, and evaporated to dryness. Last traces of water were removed by repeated evaporation with dry ethanol. The residue was dissolved in dry ethanol (150 c.c.), and the barium salt precipitated by cautious addition, first of dry acetone, then dry ether. The *barium* salt was filtered off and washed quickly with dry acetone and ether, giving 25 g. (82%) of a white powder which travelled as a single spot (R_F 0·86) in butanol–acetic acid–water (Found: C, 46·0; H, 5·9; N, 5·9; S, 6·5; Ba, 14·5. $C_{19}H_{28}O_6N_2SBa_{\frac{1}{2}}$ requires C, 47·4; H, 5·8; N, 5·8; S, 6·7; Ba, 14·3%).

N-D-*Pantothenoyl*-L-*cysteine*.—Barium *S*-benzyl-*N*-D-pantothenoyl-L-cysteine (4·8 g.) was treated in liquid ammonia (*ca.* 50 c.c.) with sodium, the slight excess being destroyed by swirling the solution in air. Ammonia was removed and the powdery residue treated with a suspension of Amberlite IR-120 resin (H^+ form) in ice-water (this avoided the development of alkaline conditions on dissolution in water). The suspension was poured on to a column of IR-120 resin. The effluent was neutralised with barium hydroxide solution and evaporated to dryness under reduced pressure. The residue was dissolved in dry ethanol (50 c.c.) and *barium pantothenoylcysteine* precipitated by the cautious addition of dry acetone. It was separated by centrifugation, washed with acetone, and dried *in vacuo*, yielding an amorphous white powder (3·2 g., 82%) (Found: C, 36·2; H, 5·5; N, 7·1; S, 7·7. $C_{12}H_{21}O_6N_2SBa_{\frac{1}{2}}$ requires C, 36·9; H, 5·4; N, 7·2; S, 8·2%).

Paper chromatography in butanol–acetic acid–water showed that it was almost entirely in the thiol form (R_F 0·65), only a trace of the disulphide form (R_F 0·34) being present.

N-D-*Pantothenoyl*-L-*cysteine*-4′ *Dihydrogen Phosphate*.—Barium *S*-benzyl-*N*-D-pantothenoyl-L-cysteine (5 g.) was converted into the free acid by passage in aqueous ethanol (4 : 1) through Amberlite IR-120 (H^+ form). The eluate was evaporated to dryness under reduced pressure. The residue was evaporated twice with anhydrous benzene, and then dried (P_2O_5) at 10^{-3} mm. This material was dissolved in anhydrous pyridine (30 c.c.), the solution cooled to just above

the freezing point, and a solution of dibenzyl phosphorochloridate (prepared from dibenzyl phosphite, 4 g.; 1·5 mols.) in carbon tetrachloride was added dropwise with shaking. After 15 min. the flask was removed from the acetone–carbon dioxide bath and set aside at room temperature overnight. Pyridine was removed under reduced pressure, the residue dissolved in chloroform (50 c.c.), and the solution washed with 2N-sulphuric acid (2 × 20 c.c.) and water (2 × 20 c.c.). After being dried ($MgSO_4$), the chloroform was removed by evaporation, and the residue triturated with benzene. It was dissolved in liquid ammonia (*ca.* 50 c.c.) and treated with sodium, the excess being destroyed with ammonium acetate. Ammonia was allowed to evaporate. The powdery residue was added with stirring to a suspension of Amberlite IR-120 (H^+ form) in ice-water, and the suspension poured on to a short column of the same resin. The acid eluate was washed with ether and adjusted to pH 7 by barium hydroxide solution. The small amount of barium phosphate which separated was removed, and the solution evaporated to *ca.* 20 c.c. Ethanol (3 vols.) was added, and the precipitate centrifuged off, washed with ethanol, and dried *in vacuo*, yielding a white powder (2·5 g.). Paper chromatography in butanol–acetic acid–water and propanol–ammonia showed a major spot having the expected properties, a spot which appeared to be the disulphide form, and a number of contaminants, including a sulphur-free phosphate. Purification *via* the silver and copper salts was tried, but without success. The disulphide form was found to be less soluble in aqueous ethanol than the other components of the mixture. Repeated fractionation of the barium salt by precipitation from aqueous solution by ethanol gave a material which was practically homogeneous on paper chromatography (R_F 0·05 in both solvents). Alkaline hydrolysis (Part II, *J.*, 1951, 2253) gave pantothenic acid-4′ phosphate, and hydrolysis with 5N-hydrochloric acid after treatment with hydrogen peroxide gave β-alanine and cysteic acid. The impure fractions from the fractional precipitation were combined and dissolved in water, and the solution was adjusted to pH 8·5 with barium hydroxide solution and aerated with a brisk current of oxygen until the thiol test became negative. The solution was passed through a column of Amberlite IR-120 (H^+ form). The acid eluate was cautiously evaporated to dryness, and the residue submitted to 50-stage counter-current distribution between the two phases of butanol–acetic acid–water. At the end of the run, the tubes which gave a positive reaction with cyanide–nitroprusside (tubes 1—6) were evaporated separately to a small volume, diluted with aqueous ethanol, and again concentrated. This process was repeated until the smell of acetic acid was no longer noticeable. The residues were diluted with water to *ca.* 2 c.c. and neutralised with barium hydroxide solution. Addition of a large excess of ethanol precipitated the barium salts. From tube 1 was obtained an almost pure sample of the *barium* salt of the disulphide (Found : C, 22·4; H, 3·5; N, 4·2; S, 4·9; P, 4·7; Ba, 34·2. $C_{24}H_{38}O_{18}N_4S_2P_2Ba_3$ requires C, 23·8; H, 3·1; N, 4·6; S, 5·3; P, 5·1; Ba, 34·0%). The barium salts obtained from the other tubes were progressively more and more contaminated with impurities.

*Benzyloxycarbonyl-β-alanyl-*DL*-serine.*—Ethyl chloroformate (2·86 c.c.) was added slowly with shaking to a solution of benzyloxycarbonyl-β-alanine (6·7 g.) and triethylamine (4·22 c.c.) in dimethylformamide (30 c.c.) at −5°. The solution was kept for 15 min. at this temperature, then a solution of serine (3·15 g.) in 1·025N-sodium hydroxide (29·3 c.c.) was added rapidly with shaking. A vigorous evolution of carbon dioxide occurred. The solution, after 2 hr. at room temperature, was evaporated to dryness *in vacuo*. The residue was dissolved in warm water (100 c.c.), acidified (Congo-red) with hydrochloric acid, shaken with ether (3 × 100 c.c.) to remove benzyloxycarbonyl-β-alanine, and extracted with ethyl acetate (3 × 100 c.c.). The extract was dried ($MgSO_4$), then evaporated to dryness. *Benzyloxycarbonyl-β-alanyl-*DL*-serine* (4·5 g., 48%) had m. p. 127° after recrystallisation from ethyl acetate (Found : C, 54·6; H, 6·2; N, 8·9. $C_{14}H_{18}O_6N_2$ requires C, 54·2; H, 5·8; N, 9·0%). It had R_F 0·81 in butanol–acetic acid–water.

*β-Alanyl-*DL*-serine.*—A solution of benzyloxycarbonyl-β-alanylserine (1·2 g.) in methanol–acetic acid–water (44 : 3 : 3) (20 c.c.) was shaken with hydrogen in the presence of palladium oxide. After the uptake of hydrogen had ceased water was added to dissolve the precipitated product, and the catalyst was removed by filtration. The filtrate was evaporated to small bulk *in vacuo* and the peptide precipitated by alcohol. *β-Alanyl-*DL*-serine* crystallised as fine needles, m. p. 208—209° (decomp.) (0·65 g., 95%). It had R_F 0·32 in butanol–acetic acid–water (Found : C, 40·5; H, 6·8; N, 16·0. $C_6H_{12}O_4N_2$ requires C, 40·9; H, 6·8; N, 15·9%).

*Pantothenoyl-*DL*-serine.*—This was prepared from β-alanylserine (0·6 g.), pantolactone (0·443 g.), and dimethylamine (0·3 c.c.) (Found : C, 48·6; H, 8·5; N, 11·0. $C_{14}H_{29}O_7N_3$ requires C, 47·9; H, 8·3; N, 12·0%). The dimethylamine salt did not crystallise.

N-(*Benzyloxycarbonyl-β-alanyl*)-2-*hydroxyethylamine*.—A solution of benzyloxycarbonyl-β-alanine azide, prepared from the hydrazide (3·6 g.) (Sifferd and du Vigneaud, *loc. cit.*), in dry chloroform (20 c.c.), was added to a solution of 2-hydroxyethylamine (0·91 g.) in dry chloroform (5 c.c.). The resulting yellow solution was kept at room temperature overnight, then chloroform and hydrazoic acid were removed under reduced pressure. The *benzyloxycarbonyl* compound was recrystallised from ethyl acetate as fine needles, m. p. 123—124° (2·9 g., 72%) (Found : C, 58·6; H, 6·7; N, 10·2. $C_{13}H_{18}O_4N_2$ requires C, 58·6; H, 6·8; N, 10·5%).

N-β-*Alanyl*-2-*hydroxyethylamine*.—The preceding compound was treated in liquid ammonia with sodium, excess being destroyed with ammonium chloride and the ammonia allowed to evaporate. The residue was dissolved in ice-water and acidified (Congo-red) with hydrochloric acid. The solution was washed with ether, then evaporated to dryness *in vacuo*. The dry, powdered residue was extracted with boiling *iso*propyl alcohol (4 × 6 c.c.), and the combined extracts were evaporated to dryness *in vacuo*. The residual syrup was dissolved in water and acid removed by passing the solution through a column of Amberlite IR-4B resin (OH^- form). Solvent was removed and the resulting syrup sublimed (110°/10^{-4} mm.), forming a very hygroscopic waxy solid, m. p. 49—50°. The β-alanyl compound had R_F 0·30 in butanol–acetic acid–water and formed a crystalline *hydrogen oxolate*, m. p. 115—116°, after recrystallisation from dilute oxalic acid solution (Found : C, 37·6; H, 6·3; N, 12·7. $C_7H_{14}O_6N_2$ requires C, 37·8; H, 6·3; N, 12·6%).

2-*Hydroxyethyl*-D-*pantothenamide*.—D(−)-Pantolactone (0·44 g.) and *N*-β-alanyl-2-hydroxyethylamine (0·45 g.) were heated together on a steam-bath for 2·5 hr. and the resinous product was washed by trituration with ether. Its solution in water was passed through a column of Amberlite IR-120 resin (H^+ form) to remove a little amino-compound, and the effluent evaporated to dryness *in vacuo*. 2-*Hydroxyethyl*-D-*p-antothenamide* was a colourless glass (0·8 g.) (Found : C, 49·8; H, 8·5; N, 10·6. $C_{11}H_{22}O_5N_2$ requires C, 50·4; H, 8·4; N, 11·1%). It had R_F 0·65 in butanol–acetic acid–water.

D-*Pantothenoylglycine*.—This synthesis started from D(−)-pantolactone (2·9 g.), β-alanylglycine (3·26 g.) (Hanson and Smith, *J. Biol. Chem.*, 1948, **175**, 833), and dimethylamine (2·3 c.c.), following a similar procedure to that described for the synthesis of pantothenoylcysteine. The free acid (5·5 g., 75%) was a syrup which readily formed a crystalline *dimethylamine* salt, prisms, m. p. 103—105° (from dry alcohol containing a little dimethylamine) (Found : C, 48·3; H, 8·6; N, 12·9. $C_{13}H_{27}O_6N_3$ requires C, 48·6; H, 8·4; N, 13·1%). On paper it had R_F 0·64 (butanol–acetic acid–water).

D-*Pantothenoyl*-β-*alanine*.—This was prepared from D(−)-pantolactone (1·3 g.), β-alanyl-β-alanine (1·6 g.) (Hanson and Smith, *loc. cit.*), and dimethylamine (1·0 c.c.) by a method similar to that described for the synthesis of pantothenoylglycine. The *dimethylamine* salt (2·7 g., 80%) formed needles, m. p. 122—124°, from alcoholic dimethylamine (Found : C, 49·8; H, 8·6; N, 12·2. $C_{14}H_{29}O_6N_3$ requires C, 50·2; H, 8·7; N, 12·5%).

N-(*Benzyloxycarbonyl*-β-*alanyl*)-L-*glutamic Acid*.—*iso*Butyl chloroformate (9·29 g.) was added slowly with shaking to a solution of benzyloxycarbonyl-β-alanine (15·7 g.) and triethylamine (9·55 c.c.) in dimethylformamide (75 c.c.) cooled to −5°. After 15 min. a solution of L-glutamic acid (10 g.) in 1·025N-sodium hydroxide (132·7 c.c.) was added rapidly. The solution was kept at room temperature for 2 hr. and then the solvents were removed *in vacuo*. The residue was dissolved in water (100 c.c.) and the solution acidified (Congo-red) with 5N-hydrochloric acid. After being washed with ether the solution was extracted with ethyl acetate (3 × 100 c.c.), and the combined extracts were dried ($MgSO_4$) and evaporated to dryness *in vacuo*. Unchanged benzyloxycarbonyl-β-alanine was removed by dissolution in ethyl acetate and addition of ether. The product was filtered off and dissolved in 40% ethanol, and the solution was passed through a column of Amberlite IR-120 resin (H^+ form) to remove glutamic acid. The eluate was evaporated to dryness and the syrupy *benzyloxycarbonyl* peptide (13 g., 55%) was dried over phosphoric oxide (Found : C, 53·8; H, 6·3; N, 8·4. $C_{16}H_{20}O_7N_2$ requires C, 54·3; H, 5·7; N, 8·0%). It had R_F 0·07 in butanol–acetic acid–water and contained a trace of benzyloxycarbonyl-β-alanine.

β-*Alanyl*-L-*glutamic Acid*.—The above benzyloxycarbonyl compound (11·5 g.) in methanol–acetic acid–water (44 : 3 : 3; 120 c.c.) was shaken with hydrogen in the presence of palladium. After hydrogenation was complete the catalyst was filtered off and the filtrate was evaporated to small volume *in vacuo*. Addition of alcohol precipitated an oil which on drying became a brittle, hygroscopic solid (6·5 g., 91%) (Found : C, 44·3; H, 6·7; N, 11·9. $C_8H_{14}O_5N_2$ requires C, 44·0; H, 6·4; N, 12·9%). The *peptide* had R_F 0·21 in butanol–acetic acid–water and contained a trace of β-alanine.

N-(D-*Pantothenoyl*)-L-*glutamic Acid.*—This was prepared from pantolactone (1·3 g.), β-alanyl-L-glutamic acid (21·7 g.), and dimethylamine (1·7 c.c.) in a manner similar to that described for pantothenoylglycine. The *dimethylamine* salt (3·5 g., 80%) had R_F 0·56 in butanol–acetic acid–water and contained a trace of pantothenic acid (Found: C, 49·2; H, 8·6; N, 12·9. $C_{14}H_{24}O_8N_2$ requires C, 49·3; H, 8·7; N, 12·8%).

*Benzyloxycarbonyl-β-alanyl-*DL-*α-alanine.*—A solution of benzyloxycarbonyl-β-alanine chloride (from 10 g. of benzyloxycarbonyl-β-alanine) in a little ether was added to a solution of alanine (4 g.) in 2N-sodium hydroxide (25 c.c.) at 0° with continuous addition of sodium hydroxide (to maintain slight alkalinity). After 1 hr. at room temperature the solution was acidified (Congo-red) and the crystalline precipitate collected. The *benzyloxycarbonyl* peptide recrystallised from water as plates, m. p. 142—143° (1·9 g.) (Found: C, 57·3; H, 6·4; N, 10·2. $C_{14}H_{18}O_5N_2$ requires C, 57·2; H, 6·1; N, 9·5%).

β-*Alanyl-*DL-*α-alanine.*—This was prepared by catalytic hydrogenolysis of the above benzyloxycarbonyl *peptide* (1·7 g.) as described for β-alanylserine. The *peptide*, m. p. 228—230° (0·9 g.), was homogeneous on paper chromatography in butanol–acetic acid–water (R_F 0·4) (Found: C, 41·3; H, 7·8; N, 15·4. $C_6H_{12}O_3N_2,H_2O$ requires C, 40·5; H, 7·9; N, 15·7%).

D-*Pantothenoyl-*DL-*α-alanine.*—This was obtained from pantolactone and β-alanyl-α-alanine by the method used for pantothenoylglycine. The *dimethylamine* salt had m. p. 132° after softening at 100° (Found: C, 50·1; H, 8·8; N, 12·3. $C_{14}H_{29}O_6N_3$ requires C, 50·2; H, 8·7; N, 12·5%).

D-*Pantothenamide.*—β-Alanine amide acetate was prepared by catalytic hydrogenation of benzyloxycarbonyl-β-alanine amide (Hanson and Smith, *loc. cit.*) under conditions similar to those described for the preparation of β-alanyl-DL-serine. The acetate (1·5 g.), D(−)-pantolactone (1·3 g.), and dimethylamine (1 c.c.) were refluxed together in methanol (10 c.c.) for 8 hr. Solvent was distilled off and an aqueous solution of the residue passed through a column of Amberlite IRC-50 resin (H^+ form), then Amberlite IR-4B (OH^- form). The neutral eluate was washed with ether and evaporated to dryness under reduced pressure. The glassy *product* (2 g.) had R_F 0·60 in butanol–acetic acid–water (Found: C, 49·1; H, 8·8; N, 12·7. Calc. for $C_9H_{18}O_4N_2$: C, 49·5; H, 8·3; N, 12·8%). Traces of contaminants, R_F 0·35 and 0·88, were observed.

2-D-*Pantothenamidoacetaldehyde Diethyl Acetal.*—To a solution of the mixed anhydride, prepared from sodium pantothenate (4·82 g.) and ethyl chloroformate (1·95 c.c.) in dioxan (20 c.c.), was added aminoacetaldehyde diethyl acetal (2·66 g.) in dioxan (10 c.c.). The mixture was kept at 0° for 5 min., then allowed to warm to room temperature. The solvent was evaporated under reduced pressure and an aqueous solution of the residue passed through a column of Amberlite IR-4B resin (OH^- form), then through Amberlite IR-120 resin (H^+ form). The neutral, halogen-free effluent was evaporated to dryness, leaving a syrup (3·8 g., 57%) (Found: C, 53·6; H, 8·9; N, 8·6; OEt, 25·8. $C_{15}H_{30}O_6N_2$ requires C, 53·8; H, 9·0; N, 8·4; OEt, 26·9%). The *acetal* was homogeneous on paper chromatography in butanol–pyridine–water (R_F 0·94) and in butanol–acetic acid–water (R_F 0·84). It was detected on the paper by Rydon and Smith's method (*loc. cit.*) or by spraying with 0·1N-hydrochloric acid, heating at 100° for 5 min., then spraying with Schiff's reagent. The acetal gave a 2 : 4-*dinitrophenylhydrazone*, m. p. 114—115° (from alcohol) (Found: C, 46·0; H, 5·7; N, 18·7. $C_{17}H_{24}O_8N_6$ requires C, 46·3; H, 5·5; N, 19·1%).

2-D-*Pantothenamidoacetaldehyde.*—Qualitative experiments indicated that, although the above acetal did not reduce ammoniacal silver nitrate, rapid reduction was observed on samples which had been boiled with N-hydrochloric acid for 1 min. Paper chromatography of hydrolysates established that the acetal (R_F 0·94 in butanol–pyridine–water, 0·84 in butanol–water) was completely converted into the aldehyde (R_F 0·77 in butanol–pyridine–water, 0·60 in butanol–water) when heated at 100° for 2 min. in water containing a trace of sulphuric acid. The aldehyde appeared as a brown spot on paper after spraying with ammoniacal silver nitrate and then heating at 100° for 2 min. The following method was adopted for preparing quantities of the aldehyde.

A solution of the acetal (1 g.) in water (100 c.c.) was acidified by adding 1 drop of 50% sulphuric acid and then boiled for 2 min. The solution was neutralised with barium hydroxide solution, filtered, and evaporated to dryness *in vacuo*. The residual syrup was dissolved in dry alcohol and D-*pantothenamidoacetaldehyde* was precipitated as a white solid by addition of dry ethyl acetate and ether in almost quantitative yield (Found: C, 50·2; H, 8·2; N, 10·3. $C_{11}H_{20}O_5N_2$ requires C, 50·7; H, 7·7; N, 10·8%).

Oxidation to D-*Pantothenoylglycine.*—A solution of the aldehyde, prepared as above from

the acetal (0·167 g.), in water (2 c.c.) was treated with freshly prepared silver oxide (from 0·085 g. of silver nitrate) and sodium hydrogen carbonate (0·042 g.). The mixture was heated on a steam-bath with constant stirring for 4 hr. The solution was filtered and passed through a column of Amberlite IR-120 resin (H^+ form), and the acidic eluate evaporated to dryness *in vacuo*. Paper chromatography of the residue showed that the main product was indistinguishable from pantothenoylglycine (R_F 0·63 in butanol–acetic acid–water) prepared by the first method. Two slow-moving contaminants were observed.

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THE LISTER INSTITUTE OF PREVENTIVE MEDICINE,
LONDON, S.W.1.

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