SYNTHESIS AND BIOLOGICAL ACTIVITY OF PANTETHEINE ANALOGS AND DERIVATIVES

by

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INTRODUCTION

The enzymatic studies (9,25) of an unidentified growth factor for Lactobacillus bulgaricus (L. bulgaricus factor; LBF (43)), revealed the presence of pantothenic acid as an integral part of the molecule. The chemical structure was fully elucidated by Snell et al, (33) as N-pantothenylmercaptoethylamine,

$$CH_2OH\ C(CH_3)_2CHOHCONHCH_2CH_2CONHCH_2CH_2SH$$

They proposed the name pantetheine for the free thiol and pantethine for the dimeric disulfide.

The demonstration by King and Strong (20) of the enzymatic conversion of pantetheine into coenzyme A and the microbial growth studies of Craig and Snell (13) aroused further interest in pantetheine. The inadequacies of the original synthetic method (33) soon became apparent and several laboratories developed more reliable preparative methods (2,18,31,38,39,40,45).

As a result of the foregoing syntheses, the ready availability of pantetheine facilitated the investigation of its relationship to coenzyme A. The synthetically produced 4'-pantetheine phosphate was shown to be

identical to a degradation product of CoA[1] (3) and could be enzymatically converted into the coenzyme (5). Pantetheine was then demonstrated by Novelli (28) to be an essential intermediate in the biosynthesis of CoA.

The recent observations of King and Cheldelin (17) and of Brown and Snell (10,11) that N-pantothenylcysteine had high growth stimulatory effects for Acetobacter suboxydans led the latter workers to conclude that the immediate precursor of pantetheine in vivo is pantothenylcysteine. Hoagland and Novelli (15) have confirmed this in their enzymatic biosynthesis of CoA and have carefully investigated the requirements of their system. The enzymatic formation of pantetheine from pantothenic acid fails to occur unless cysteine is added. In the complete system the formation of CoA involves five individual reactions starting with pantothenic acid:

1) pantothenic acid + cysteine + ATP $\longrightarrow$ pantothenylcysteine + Pi + ADP

2) pantothenylcysteine $\longrightarrow$ pantetheine + CO_2

3) pantetheine + ATP $\longrightarrow$ pantetheinephosphate + ADP

4) pantetheinephosphate + ATP $\longrightarrow$ desphospho CoA + PP

5) desphospho CoA + ATP $\longrightarrow$ CoA + ADP

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[1]The following abbreviations are used throughout this Thesis. CoA - coenzyme A, desphospho CoA - desphospho coenzyme A, ATP - adenosine triphosphate, ADP - adenosine diphosphate, AMP - adenosine monophosphate, Pi - inorganic phosphate, PP - pyrophosphate.

The ubiquitous requirement for CoA in biological systems could be more easily studied if some substance could be prepared which would inhibit CoA dependent reactions. The inhibited reactions might accumulate the unreacted intermediates and thus indicate still unknown mechanisms of metabolism.

CoA is a relatively large dinucleotide (28) which will be extremely difficult to synthesize by chemical means. The most facile method to prepare a CoA inhibitor would be to produce a compound that could block the synthesis of this coenzyme, or that might even be transformed *in vivo* into a CoA analog of inhibitory action. By the proper alteration in the structure of pantetheine it should be possible to prepare a compound which would have this action.

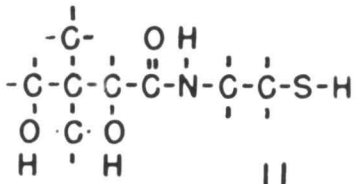
Moreover, the biological activity of these "altered pantetheines" would yield information regarding the steric specificity and points of enzymatic attachment of the intermediate compounds involved in the biosynthesis of CoA and CoA catalyzed reactions.

For the purpose of this investigation the compounds shown in Table I, were prepared and their biological activities determined.

The importance of CoA as a transacylating agent (23,24,35) together with the acetylating ability of diacetyl mercaptoethylamine (1) and S-acetyl glutathione

TABLE I

Pantetheine and Analogs



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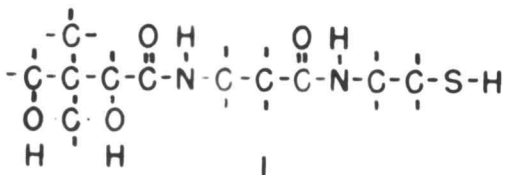

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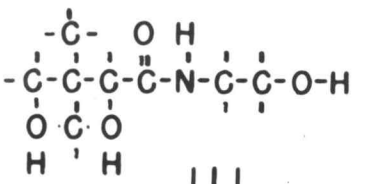

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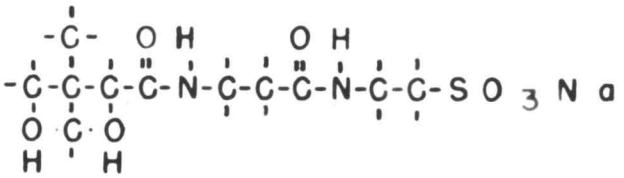

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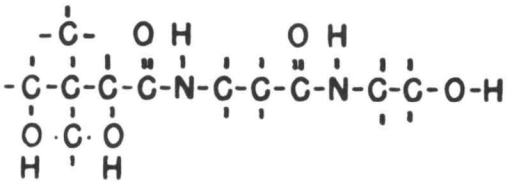
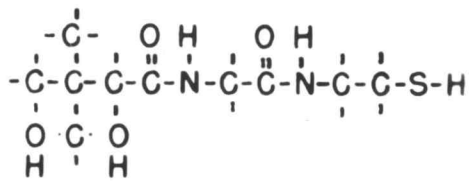
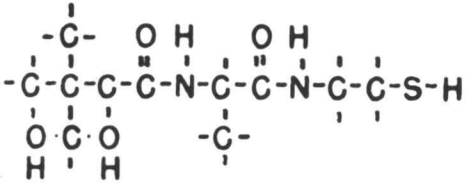
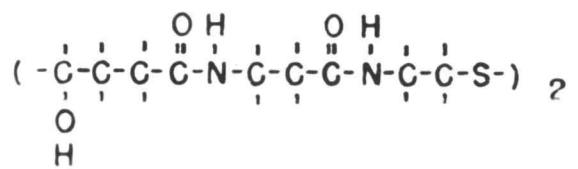




VI VII





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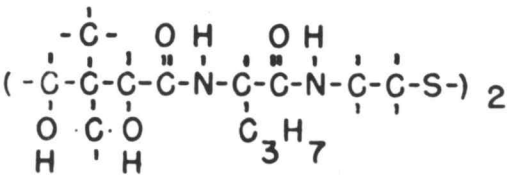

Table I - Legend

I = pantetheine[1]

II = N-pantoyl-2-aminoethanthiol

III = N-pantoylethanolamine

IV = sodium N-pantothenyl taurine

V = N-pantothenylethanolamine

VI = N-(pantoylglycyl)-2-aminoethanthiol

VII = N-(pantoylalanyl)-2-aminoethanthiol

VIII = bis(N-(γ-hydroxybutyryl-β-alanyl)-2-aminoethyl) disulfide

IX = bis(N-(pantoylnorvalyl)aminoethyl) disulfide

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[1]Each of these compounds has been prepared for use in the present study. All except Nos. I, II and III are new compounds. No. VIII has recently been prepared independently in another laboratory (8).

(41) prompted the preparation of S-acetylpantetheine as a model compound of acetyl coenzyme A. The transacetylation method of Wieland and Bakelmann (41) employing phenylthioacetate was found to be satisfactory for the preparation of S-acetylpantetheine as well as acetyl CoA.

EXPERIMENTAL

Synthetic Methods

For use as intermediates or reagents in the following syntheses, several known compounds had to be prepared. This was done, as follows:

1) performic acid was produced by adding 2 ml. of 30% hydrogen peroxide to 18 ml. of 90% formic acid (37); 2) carbobenzoxy chloride according to the directions of Bergmann and Zervas (7); 3) 2-aminoethanthiol as described by Mills and Bogert (26); 4) β-aletheine and 5) panthetheine, by the method of King, Stewart, and Cheldelin (18); 6) N-pantoylethanolamine (III) by fusion of pantoyl lactone with ethanolamine (34); 7) N-pantoyl-2-aminoethanthiol (II) by fusion of pantoyl lactone and 2-aminoethanthiol according to Barnett (6).

Pantothenyl taurine-sodium salt (IV)

The sodium salt of pantetheine was prepared by dissolving 1.95 g. of crude pantetheine (obtained as a fusion mixture) in 4 ml. of 1.118N sodium methoxide. This mixture was added to 20 ml. of performic acid. After standing at room temperature for 15 minutes the reaction solution was poured into 200 ml. of acetone. To encourage precipitation of sodium formate approximately 50 ml. of ether were added to the acetone solution. The turbid

mixture was left at room temperature for three days. The solid was centrifuged off and the supernatant liquid was concentrated to dryness at 1 mm. pressure. Traces of formic acid in this residue were removed by resolution with absolute ethanol and again concentrating to dryness at 1 mm. pressure. The residue was dissolved in about 5 ml. methyl alcohol, poured into ether. The resultant solid gave a qualitative test for sulfur and nitrogen. The carbon, hydrogen and ash determinations indicated purity of about 80 per cent.

A precipitate formed during the final removal of ethanol. Advantage was taken of this phenomenon in purification by dissolving the crude salt of pantothenyltaurine in a small volume of ethanol, centrifuging out the insoluble sodium formate and pouring the clear supernatant into ether. The solid formed was redissolved in methanol and precipitated by ether. This process was repeated twice. One hundred mg. of a white powder were formed which softened at 102°C. formed a semi-molten mass by 104°C. and effervesced on further heating.

$C_{11}H_{21}NaN_2O_7S$.[1] Calculated. C 37.92, H 6.08

Found. C 38.31, H 6.28

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[1]Chemical analyses were either performed by Drs. Weiler and Strauss, Microanalytical Laboratory, Oxford, England, or by Micro-Tech. Laboratories, Skokie, Ill.

bis(γ-Hydroxybutyryl-β-alanyl-2-aminoethyl) disulfide (VIII)[1]

Five hundred mg. of β-aletheine (7) were heated together with 350 mg. of γ-butyrolactone at 100°C. for 10 minutes in order to melt the β-aletheine. Then the material was fused at 70-80°C. for one hour. The fusion mixture was dissolved in 1 ml. methanol and permitted to stand overnight for air oxidation to occur. The material that crystallized out was redissolved by adding 2 ml. of methanol. About 3 ml. of ether were then added to incipient turbidity. After two days the crystals were centrifuged off and recrystallized successively from absolute ethanol, isopropyl alcohol and finally from absolute ethanol. Yield 100 mg. of yellowish-white crystals; m.p. 196.5 - 198°C.[2]

$C_{18}H_{34}N_4O_6S_2$. Calculated. C 46.32, H 7.34, N 12.00

Found. C 46.33, H 7.31, N 12.12

"Oxy-pantetheine" (N-pantothenylethanolamine) (V)

Carbobenzoxy-β-alanine. The synthesis by Sifferd and du Vigneaud (32) was employed with the exception that pure

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[1]While this work was in progress preparation of this compound was reported by Bowman and Cavalla (8).

[2]All melting points were obtained on a Fisher melting point block and are reported as uncorrected.

β-alanine rather than succinimide was the starting material.

Carbobenzoxy-β-alanyl chloride. The method of Dyer and Ballard (14) was used to prepare an ether solution of this compound, which was immediately used in the next step of the synthesis.

N-(carbobenzoxy-β-alanyl) ethanolamine. Five g. sodium bicarbonate (0.06 moles) and 2.8 g. ethanolamine (0.046 moles) were dissolved in 40 ml. water and cooled by a salt-ice bath to 0 - 5°C. To the cold solution 5 ml. portions of a 50 ml. ether solution of carbobenzoxy-β-alanyl chloride, prepared from 10 g. carbobenzoxy-β-alanine (0.045 moles), were added slowly with stirring. The temperature was maintained at 0 - 5°C. at all times. The product precipitated from the cold reaction mixture. Stirring was continued for 15 minutes after all the acid chloride had been added. The mixture was filtered and the product air dried overnight. The dry crude product was crystallized from chloroform. Yield, 6 g; m.p. 119 - 121°C.

Further purification was obtained by recrystallization from ethyl acetate. Yield, 4 gms; m.p. 126 - 126.5°C.

$C_{13}H_{18}N_2O_4$. Calculated. C 58.63, H 6.81, N 10.5

Found. C 58.48, H 6.43, N 11.2

N-β-alanylethanolamine oxalate. Seven g. of carbobenzoxy-β-alanylethanolamine (.026 moles) were added to about 100 ml. of anhydrous liquid ammonia. Small pieces of metallic sodium (1.3 g., .056 moles) were added until a blue color persisted for ten minutes. To neutralize the sodium and form the free base, 3.5 g. ammonium chloride (.06 moles) were added and the liquid ammonia was permitted to boil to dryness at room temperature. The free base was separated from sodium chloride by extraction with 300 ml. of 95% ethanol. The traces of ammonia in the alcohol solution were removed by concentrating to dryness under reduced pressure (1 mm.). The residual oil was redissolved in 30 ml. absolute ethanol and added to 3 g. of oxalic acid dihydrate dissolved in about 30 ml. isopropyl alcohol. The initial precipitate of the oxalate was redissolved by heating to obtain uniform crystallization. Ether was added to incipient turbidity and the solution placed at -4°C. for crystallization. The product appeared to contain alcohol of crystallization. It melted at 70 - 80°C. without drying, but when dried overnight at 1 mm. pressure over P_2O_5 the melting point rose to 118 - 121°C. Yield, 3.1 gms. After recrystallization from absolute ethanol it melted at 122 - 123°C.

$C_7H_{14}N_2O_6$. Calculated. C 37.83, H 6.35

Found. C 37.77, H 6.36

"Oxy-pantetheine" (N-pantothenylethanolamine). Eight hundred eighty eight mg. of the above oxalate salt (4 m moles) were dissolved in 15 ml. of absolute methanol and to this were added 7.38 ml. of 1.0813 N sodium methoxide solution. The sodium oxalate was removed by centrifugation and washed once with a small volume of methanol. The combined washing and supernatant was concentrated to dryness under vacuum (1 mm.) to yield a white powder. The powder was transferred to a tube, 600 mg. (4.6 m mole) pantoyl lactone[1] were added, and the tube sealed. The sealed tube was heated at 65 - 70°C. for four hours.

Purification was attempted by two-plate counter current extraction with a solvent system of butanol:acetic acid:water (4:1:5). The fusion product was dissolved in 10 ml. of the upper phase, extracted with 10 ml. of the lower phase and transferred for re-extraction with a fresh 10 ml. portion of the lower phase. The lower phase used in the first extraction was extracted with a fresh 10 ml. portion of the upper phase which was in turn re-extracted by the second lower phase. The combined upper phases were freeze-dried in an attempt to remove the excess pantoyl lactone by sublimation under a high vacuum. A highly viscous hygroscopic syrup was obtained in this manner.

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[1]The natural (-) isomer of pantoyl lactone was used throughout the course of this work.

Analysis for the total nitrogen and pantoyl lactone content (36) indicated a purity of 85 percent.

$C_{11}H_{22}N_2O_5$.

Calculated. Pantoyl lactone 49.6, C 50.36, H 8.45, N 10.68

Found. Pantoyl lactone 57.3, C 50.98, H 8.55, N 8.17

N(Pantoylglycyl)-2-aminoethanthiol (VI)

Carbobenzoxyglycine and carbobenzoxyglycyl chloride were prepared by the method of Bergmann and Zervas (7).

bis(N-(Carbobenzoxyglycyl)-2-aminoethyl) disulfide.[1] Three and four tenths grams of 2-aminoethanthiol were dissolved in 40 ml. water and cooled to 0°C. Hydrogen peroxide was added dropwise until a positive nitroprusside reaction was no longer given by a drop of the solution. Sodium hydroxide (6 N) was added until the mixture was alkaline to phenolphthalein. Fifty ml. of an ether solution of carbobenzoxyglycyl chloride, prepared from 10 g. carbobenzoxyglycine, were added in portions in a salt-ice bath, with constant stirring and cooling. Throughout the course of the reaction the solution was maintained alkaline to phenolphthalein by addition of sodium hydroxide and the temperature was kept below 5°C. After all the acid chloride had been added, stirring was

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[1]One sample of this intermediate was kindly furnished by H. J. Sharkey.

continued for ten minutes, then the product which had risen to the surface was removed by filtration, washed with water and air dried overnight. Three grams were obtained after recrystallization from 95% ethanol; m.p. 175 - 178°C.

$C_{24}H_{30}N_4O_6S_2$. Calculated. N 10.5

Found. N 10.8

N-glycyl-2-aminoethanthiol. Three and eight tenths grams of bis(N-carbobenzoxyglycylaminoethyl) disulfide were suspended in approximately 50 ml. of liquid ammonia in a three neck flask. The flask was fitted with a soda lime (drying) tube for exclusion of carbon dioxide and moisture and fitted with a stirring blade. The ammonia suspension of the carbobenzoxy compound was vigorously stirred while a total of 1.0 g. of sodium was added in small portions until the blue color of excess sodium persisted for ten minutes. Two and five-tenths g. of ammonium chloride were added, after which the liquid solution was left at room temperature until the ammonia had evaporated. The residue was extracted with alcohol and the sodium chloride removed by filtration and washed with alcohol. Two and one half grams of oxalic acid dihydrate were dissolved in about 25 ml. absolute alcohol and added to the combined filtrate and washings.

A small amount of ammonium oxalate, which formed, was removed by filtration after heating the solution on

a boiling water bath to dissolve any precipitated glycylaminoethanthiol oxalate. To the clear warm filtrate, ether was added to incipient turbidity. Crystallization occurred at -4°C. Yield: 1 g. The product melted with decomposition at 165 - 167°C.

$C_6H_{12}N_2O_5S$. Calculated. N 12.49

Found. N 12.43, 12.40

N-(pantoylglycyl)-2-aminoethanthiol. Four hundred and forty-eight mg. of glycylaminoethanthiol oxalate were dissolved in dry methanol. An equivalent amount of sodium methoxide was added (4 mg.). Sodium oxalate was removed by centrifugation and washed once with 1 ml. of dry methanol. The supernatant and washings were concentrated at 1 mm. pressure to a small volume and transferred to a test tube narrowed at the top for sealing. The methanol solution was then evaporated to dryness in vacuo over P_2O_5. Three hundred milligrams of pantoyl lactone were added to the dry material. The tube was sealed and the contents were fused in a glycerol bath at 65 - 70°C. for four hours.

Purification was attempted through use of counter-current extraction in a butanol-acetic acid-water system. The fusion product was dissolved in ten ml. of the upper layer and carried through 12 plates in an all glass Craig counter-current distribution apparatus. Plates 4 to 8 inclusive, which exhibited a positive nitroprusside and

negative ninhydrin test, were concentrated to dryness. Nitrogen analysis data indicated a purity of about 80 percent. Analysis (36) revealed pantoyl lactone to be the major contaminant.

$C_{10}H_{20}N_2O_4S$.

Calculated. C 45.43, H 7.62, N 10.59, S 12.13

Found. C 46.51, H 7.71, N 8.24, S 10.28

Calculated. Pantoyl lactone 49.3

Found. Pantoyl lactone 53.4

N-(pantoylalanyl)-2-aminoethanthiol. (VII)

Phthalyl alanine. Ten grams of DL-alanine were fused with 16.7 g. of phthalic anhydride for 30 minutes at 170-180°C. according to Beilstein (29). Crystallization was effected by dissolving the fusion mixture in approximately 100 ml. boiling ethanol and adding water to the first appearance of turbidity. Eighteen and four tenths grams comprised the first crop of crystals, with a melting point of 162.5-164°C. Concentration of the mother liquor yielded 4.3 g. additional for a total yield of 22.7 g. Beilstein (29) reports a melting point of 160 - 162°C.

Phthalylalanyl chloride was prepared by suspending 15 g. of phthalylalanine in 25 ml. of thionyl chloride and heating at 40°C. until a homogeneous solution was formed. The bulk of unreacted thionyl chloride was removed *in vacuo*, and traces remaining in the solid residue were

removed by adding chloroform and concentrating to dryness. The white solid residue was not purified further. It was dissolved in ether and used immediately in the next step of the synthesis.

bis(N-Phthalylalanyl)-2-aminoethyl) disulfide. Three and one half grams of 2-aminoethanthiol were added to 40 ml. of water at 0 - 5°C. The solution was oxidized by the dropwise addition of hydrogen peroxide with stirring until a negative nitroprusside was obtained without added cyanide. Five and one half grams of sodium bicarbonate were dissolved in the mixture. Stirring and chilling were continued while small portions of the ether solution of phthalylalanyl chloride, prepared from 10 g. phthalyl-alanine, were poured into the reaction vessel. Fifteen minutes after all the acid chloride had been added, stirring was discontinued and the product was removed by filtration and air dried. This material contained traces of salts and had a melting point range of 219 - 225°C. Recrystallization was achieved through solution in boiling pyridine and addition of ethanol to incipient turbidity; m.p. 217 - 221°C.

$C_{26}H_{26}N_4O_6S_2$. Calculated. C 56.30, H 4.72, N 10.10

Found. C 56.25, H 4.92, N 9.76

N-alanyl-2-aminoethanthiol oxalate. Eleven and one tenth grams (0.02 moles) of the foregoing phthalyl compound was suspended in 120 ml. of absolute ethanol, and 40 ml. of

1 M hydrazine hydrate in ethanol was added. The reaction mixture was refluxed for one and one half hours. The undissolved solid was separated by filtration and the clear supernatant liquid concentrated (in vacuo) at 1 mm. pressure. After removal of ethanol, the residue was combined with the solid obtained by filtration and suspended in 30 ml. of 2 N HCl. The acid suspension was heated for five minutes at 53°C. to extract all acid soluble material, and then placed at room temperature for 100 minutes. Solid phthalyl hydrazide (5.8 g.) was removed by filtration and the clear filtrate freeze-dried.

The solid left after removal of all water was dissolved in about 100 ml. of liquid ammonia. Two and eight tenths grams of sodium were added, with constant stirring, to the ammonia solution. Complete reduction was indicated by the blue color of excess sodium persisting for several minutes. Seven grams of ammonium chloride were placed in the reaction vessles to neutralize the sodium ion and the ammonia was permitted to boil to dryness. The dry residue was extracted with 50 ml. of absolute ethanol and the sodium chloride (9 g.) was filtered off. Traces of ammonia were removed from the clear extract by evaporation to dryness at 1 mm. pressure. The residue was dissolved in 30 ml. of ethanol and poured into 20 ml. of ethanol containing 2.6 g. oxalic acid dihydrate. No precipitate formed on

cooling. Addition of 75 to 100 ml. ether precipitated a brown hygroscopic oil. The ether-ethanol was decanted and more ether was added to this clear liquid until the solution was slightly turbid. Fine needle crystals formed after standing at room temperature for several days. These were removed by filtration. Yield 2 g; m.p. 141 - 143°C. This material exhibited positive ninhydrin and nitroprusside tests (without cyanide) but failed to yield the correct analytical results.

N-(pantoylalanyl)-2-aminoethanthiol. One and eight hundredths grams of the foregoing oxalate salt were dissolved in 15 ml. of dry methanol. To this solution was added 8.4 ml. of 1.0813 N sodium methoxide in dry methanol. The sodium oxalate that precipitated was separated by centrifugation. The clear supernatant solution was decanted and concentrated at 1 mm. pressure. As the concentration proceeded more solid precipitated from the methanol solution, which would not redissolve in methanol. When a volume of about 2 ml. was reached, the solution and combined rinses were transferred to a test tube with a constricted neck for ease of sealing. Evaporation at 1 mm. pressure over P_2O_5 was resumed until a dry residue remained in the tube. Six hundred and fifty milligrams of pantoyl lactone were placed in the tube. The tube was sealed and the mixture was

placed in a glycerol bath at 85 - 90°C. for four hours for the ensuing fusion reaction.

Purification was attempted by dissolving the fusion mixture in 10 ml. of the upper phase of a butanol-acetic acid-water system (4:1:5) and subjecting it to a 16-plate counter-current extraction. Plates 9 to 15 inclusive exhibited a positive nitroprusside reaction with added cyanide, but failed to show a ninhydrin test. The respective phases from these plates were combined. The combined (water) phases were washed once with 20 ml. of fresh upper (butanol) phase. The combined upper phases and washings were freeze-dried. The viscous sirupy residue, thus obtained was dissolved in water and again freeze-dried to remove traces of solvent. A purity of about 80 percent was obtained by this procedure, as shown by the following analysis:

$C_{11}H_{22}N_2O_4S$. Calculated. C 47.45, H 7.96, N 10.06, S 11.51

Found. C 49.52, H 7.93, N 8.03, S 9.87 (ash free)

Mercuric mercaptide of the alanine analog. The combined water phases from the counter-current extraction plates 9 to 15, were also lyophilized. The sirupy residue was dissolved in 5 ml. of water and stirred for 20 minutes with 200 mg. mercuric oxide. The unreacted mercuric oxide was removed by centrifugation, and the clear water solution was lyophilized. The solid left after removal

of water was dissolved in four ml. butanol and 12 ml. acetone were added. After standing overnight at -4°C. the solution was centrifuged and the clear mother liquor removed by decantation. The solid was dried over P_2O_5 at room temperature and 1 mm. pressure, overnight.

A purity of 88.5 percent was indicated by elemental analysis, as carbon, hydrogen and nitrogen were in the proper ratio although low by a factor of .885.

$C_{22}H_{42}N_4O_0S_2Hg$. Calculated. C 34.98, H 5.61, N 7.42, Hg 26.6
Found. C 30.89, H 4.93, N 6.52, Hg 31.1
Calculated. pantoyl lactone 34.4
Found. pantoyl lactone 32.5

Mercury is the average of eight determinations (16); 34.0, 34.0, 32.0, 33.4, 26.7, 25.6, 31.4, 31.6 .

bis(N-(pantoylnorvalyl)-2-aminoethyl) disulfide (IX)

Phthalylnorvaline. Ten grams of DL norvaline were fused with phthalic anhydride at 170 - 180°C. for 30 minutes. At the end of this period the molten mass was poured into about 80 ml. of benzene. Undissolved material was removed by filtration. Petroleum ether (b.p. 36°C.) was added to the clear benzene until incipient turbidity was obtained. Fine needle crystals formed overnight, which were separated from the mother liquor by filtration.

Yield, 18 g; m.p. 103 - 104°C.

$C_{13}H_{13}NO_4$. Calculated. C 63.14, H 5.30, N 5.66

Found. C 63.10, H 4.88, N 5.60, 5.44

Phthalylnorvalyl chloride. Fifteen grams of phthalylnorvaline were suspended in approximately 30 ml. of thionyl chloride. This suspension mixture was heated at 40°C. to final solution of solids and termination of gas evolution. The excess thionyl chloride was removed by evaporation at 1 mm. pressure. The oily residue was dissolved in a small amount of chloroform and again concentrated to dryness to remove traces of thionyl chloride. The oily residue was not further purified but was dissolved in about 60 ml. of ether for immediate use in the next step of the synthesis.

bis(N-(phthalylnorvalyl)-2-aminoethyl)disulfide. Four and seven tenths grams of 2-aminoethanthiol were dissolved in 75 ml. of H_2O. The solution was chilled to 0 - 5°C. in a salt-ice bath. Hydrogen peroxide (30%) was added dropwise, with continual stirring, until oxidation to the disulfide was indicated by failure to give a positive sulfhydryl test with sodium nitroprusside solution. Ten grams of sodium bicarbonate were then added and 5 - 10 ml. portions of the foregoing ethereal solution of phthalylnorvalyl chloride were added to the reaction mixture. Vigorous stirring and chilling were maintained so that the temperature remained at 0 - 5°C. throughout the

course of the reaction. Ten minutes after all the acid chloride solution had been added, the solid product was separated by filtration and air-dried. Purification was acquired by recrystallization from ethanol. Yield, 7 g; m.p. 173 - 175°C.

$C_{30}H_{34}N_4O_6S_2$. Calculated. C 58.99, H 5.61, N 9.17,
Found. C 58.39, H 5.34, N 9.43, 9.26

bis(N-norvalyl-2-aminoethyl)disulfide dihydrochloride. Six and one tenth grams (0.01 mole) of the intermediate just described were suspended in 40 ml. of absolute ethanol. Twenty milliliters of 1 N ethanolic hydrazine hydrate were added and the mixture was refluxed. Within the first 15 minutes complete solution was effected, followed by slow formation of a precipitate. At the end of two hours the solution was cooled to room temperature. The solid was filtered from the solution and set aside for later extraction with acid. The clear solution was concentrated to dryness under reduced pressure. The filtered solid was added to the residue and from the supernatant solution the combined solids suspended in 40 ml. of 1 N hydrochloric acid. The acidified suspension was heated at 50°C. for ten minutes and then cooled to room temperature. The phthalyl hydrazide that formed (3.1 g.) was removed by filtration. The clear filtrate was concentrated to dryness by freeze-drying. Lyophilization was continued overnight to remove all traces of moisture.

The dihydrochloride was dissolved in 20 ml. absolute ethanol. Ether was added until the solution became cloudy, then placed in an acetone-dry ice bath overnight. After warming to room temperature in a desiccator, the clear supernatant liquid was decanted from the solid. The white crystalline product was washed with ether. Yield, 2 gms. The material started to decompose at 160°C.

$C_{14}H_{28}N_4O_2S_2Cl_2$.

Calculated. C 40.08, H 6.72, N 13.36, S 15.36

Found. C 39.02, H 7.44, N 13.4, S 15.3

bis(N-(pantoylnorvalyl)-2-aminoethyl) disulfide. One and six hundredths grams of the above dihydrochloride salt was dissolved in methanol. An equivalent amount of sodium methoxide in methanol was added. Any precipitated sodium chloride was removed by centrifugation. The supernatant liquid was concentrated to dryness at 1 mm. pressure. Pantoyl lactone (700 mg.) was added to the residual syrup, and fused (sealed tube) at 85 - 90°C. for eight hours. Purification was attempted by subjecting the fusion mixture to a 26 plate counter-current extraction in butanol-acetic acid-water (4:1:5). The solution in plates 21 to 25, which gave a negative ninhydrin reaction, were concentrated to dryness. Paper chromatography indicated the presence of only one sulfur containing component. Formula calculated 42.5% pantoyl lactone

content, found 46.4%. The compound was thus about 93 percent pure.

S-acetyl pantetheine (19). A mixture of 12.9 g. acetylthiophenol in 30 ml. methanol and 5 ml. water was adjusted to pH 3.0 with hydrochloric acid. The solution was added to 2.4 g. of freshly prepared pantetheine (kept in the reduced state under nitrogen) in a methanol solution. The homogeneous mixture was allowed to stand at room temperature for five hours under nitrogen. The solvent was then distilled off in vacuo. Excess thiophenol was removed by three successive ether extractions. The residue after extraction was dried overnight in a vacuum desiccator at 0.01 mm. pressure. Yield, 2.0 g. of pale yellow oil. Sixty and four tenths mg. of the product were dissolved in water and assayed for acetyl (22) and bound pantothenic acid (27) content.

	Acetyl (μmols)	Bound pantothenic acid (μmols)	Molar ratio of pantothenic acid to acetyl
Found	178	180	1.01
Theoretical	189	189	1.00

The active acetyl content corresponded to the bound pantothenic acid, as shown. These data revealed that the product was about 95% pure. It readily

acetylated hydroxylamine. Baddiley and Thain (4) report it to be identical to their product which was prepared independently at the same time.

S-acetyl coenzyme A (19). Thirty milligrams of coenzyme A (Pabst Laboratories - 75% pure) and 0.5 ml. of acetylthiophenol were dissolved in 20 ml. of methanol-water (2:1) acidified to pH 3.2 with 0.1 N H Cl. The reaction solution was permitted to stand at room temperature for four and one half hours under a nitrogen atmosphere. After this time interval the solution was concentrated in vacuo to remove the methanol. When the mixture was about dry, 5 ml. of water were added and the solution was extracted with ether. The solution was then freeze-dried. A pale yellow oil remained. Active acetyl content, calculated 1.21 μM, found 0.66 μM, 0.72 μM.

By this method acetyl CoA in purity of about 60% was obtained from a sample of CoA, 75% pure.

Biological Methods

Lactobacillus helveticus 80(kindly supplied by Dr. E. E. Snell) and Lactobocillus bulgaricus A.T.C.C. 8001 were carried on Difco litmus milk. Lactobacillus fermenti A.T.C.C. 9338 was carried on agar stabs, the composition of which was 0.5% glucose, 1% yeast extract, 0.5% peptonized milk, and 2% agar. Acetobacter suboxydans A.T.C.C. 621 was maintained on slants of medium containing 0.5% yeast extract, 5% glycerol and 2% agar.

The microbiological assays were conducted as Craig and Snell (13) prescribed with the exception of the Acetobacter suboxydans assay which was performed by the method of Sarett and Cheldelin (30). Lactobacilli inocula were grown at 37°C. for 22 - 24 hours on 10 ml. of the basal medium supplemented with either 100 μg. of pantetheine or 100 μg. of calcium pantothenate. Acetobacter inocula were obtained from a 30 to 40 hour culture grown at 30°C. in a 50 ml. Erlenmeyer flask in 10 ml. of basal medium to which 100 μg. of pantetheine had been added.

The amount of bacterial growth was determined turbidimetrically on a Pfaltz and Bauer fluorophotometer and expressed in terms of optical density (O.D. = 2 - log% light transmission).

RESULTS AND DISCUSSION

S-acetylpantetheine and acetyl CoA

The transacetylation of Wieland and Bakelmann (41) is readily applicable to the formation of S-acetylpantetheine and acetyl CoA. The growth supporting ability of S-acetylpantetheine parallels the pantetheine content (cf. Table II). This is presumably due to hydrolysis of the acetyl group. A 14% ammonium hydroxide solution of S-acetyl pantetheine will give a positive sulfhydryl test with sodium nitroprusside within 5 minutes.

The preparation of acetyl CoA by this method is superior to the method of Wilson (44). The acetylating agent, acetylthiophenol, can be removed easily by ether extraction, whereas Wilson's procedure with sodium thioacetate introduces extraneous salt, which is difficult to remove.

Pantetheine Analogs

Snell and Shive (34) report N-pantoylethanolamine (cf. Table I compound II) to competitively inhibit the growth of certain pantothenic acid requiring bacteria, e.g., Leuconostoc mesenteroides P-60. However, Table III demonstrates that for a pantetheine requiring organism such as L. helveticus this compound, as well as

TABLE II

Growth response of Lactobacillus bulgaricus to pantetheine and S-acetylpantetheine

	Growth response (O.D.) to	
µg.	Pantetheine	S-acetylpantetheine
0.1	.14	.08
0.2	.17	.17
0.3	.25	.26
0.4	.30	.32

Growth responses of S-acetylpantetheine are given in terms of their pantetheine content.

TABLE III

Growth response of Lactobacillus helveticus 80 to pantetheine, N-pantoyl-2-aminoethanthiol, and N-pantoylethanol amine

Pantetheine analog (μg. per 10 ml.)	Pantetheine (μg per 10 ml.)		
	0	.15	.20
Pantoyl-aminoethanthiol	Optical	Density	
0	.01	.47	.51
10	.01	.47	.51
100	.02	.47	.51
1000	.02	.51	.56
Pantoyl-ethanolamine			
0	.02	.40	.52
10	.02	.40	.53
100	.02	.40	.52
1000	.02	.43	.57

N-pantoyl-2-aminoethanthiol (III) is biologically inert.

Despite the importance of the thiol group for enzymatic activity in the CoA family of compounds, one is led to speculate that biological activity in pantetheine, and CoA is also dependent upon two amide bonds in the molecular structure, probably as points of attachment to the protein. Such attachment might easily be effected through hydrogen bonding. The inactivity of N-methyl pantothenic acid reported earlier (21) is in line with this idea.

Replacement of the β-alanine fragment of pantetheine with either glycine or DL α-alanine produces analogs with lowered growth sustaining ability. Table IV demonstrates the growth stimulation of L. helveticus by the glycine and α-alanine analogs N-(pantoylglycyl)-2-aminoethanthiol, VI and N-(pantoylalanyl)-2-aminoethanthiol, VII . The relative activity of each compound compared to pantetheine is listed in the extreme right column. Evidence that any residual pantoyl lactone contained in these preparations is biologically inert, is exhibited in Table V.

Apparently the distance between the amide groups in pantetheine is of importance for active utilization. Substitution of glycine for β-alanine decreases the activity to about 0.02 percent of an equal weight of pantetheine. A comparison of the activity of the glycine

TABLE IV

Growth stimulation of L. helveticus by N-(pantoylglycyl)-2-aminoethanthiol, N-(pantoylalanyl)-2-aminoethanthiol, and bis(N-(γ-hydroxybutyryl- β-alanyl)-2-aminoethyl) disulfide

μg.	Pantetheine analog	Pantetheine equivalent (in μg.)	Activity of analog percent
50	glycyl	.012	.024
100	glycyl	.028	.028
250	glycyl	.072	.029
500	glycyl	.092	.018
1000	glycyl	.138	.014
			Average 0.02
50	α-alanyl	.006	.012
100	α-alanyl	.009	.009
250	α-alanyl	.015	.006
500	α-alanyl	.030	.006
1000	α-alanyl	.069	.007
			Average 0.008
100	γ-hydroxy	.075	.075
250	butyryl	.173	.069
500		.32	.064
			Average 0.07

TABLE V

Effect of pantoyl lactone on the growth of *L. helveticus* in the presence of pantetheine

μg. Pantoyl lactone	μg. Pantetheine			
	0	.05	.10	.15
	Optical density			
0	.02	.05	.22	.29
100	.03	.05	.19	.30
250	.03	.05	.21	.30
500	.02	.04	.21	.30
1000	.04	.05	.20	.30

analog, containing two amide bonds separated by a single methylene carbon, to that of pantetheine, possessing two amide bonds separated by two methylene carbons, indicates the exact steric specificity that the CoA synthesizing enzymes have for these sites.

Introduction of a methyl group onto the methylene carbon in the glycine analog, i.e., formation of the α-alanine analog, has no effect upon biological activity. The α-alanine analog possess about 0.01 percent of the activity of pantetheine, which is of the same order of magnitude as the glycine compound. However, when a bulky group is introduced onto this methylene carbon as in the norvaline analog (IX), biological activity is lost altogether. Presumably, IX is incapable of attachment to the receptor enzymes.

The pantoic acid moiety of pantetheine also contributes to the configurational requirements of the molecule, as might be expected from earlier studies with analogs of the free vitamin (12). The γ-hydroxybutyric acid analog (VIII) is only 0.07 percent as active (cf. Table IV) for L. helveticus, and completely inactive for A. suboxydans, which requires the pantoic acid fragment for growth.

The unusual structure of pantoic acid confers not only unique biological properties upon its derivatives, but also characteristic physical behavior. All compounds

containing this residue in their structure are hygroscopic and tend to form viscous syrups, whereas the γ-hydroxybutyryl analog is obtained easily as a stable crystalline compound. It is possible that the α-hydroxy group, which has also been shown previously to be necessary for attachment to enzyme surfaces, (12) may provide a means for hydrogen bonding.

Oxidation of the sulfhydryl group of pantetheine to a sulfonic acid produces a compound (IV), as shown in Table VI, with one percent activity. This slight growth promoting activity is in marked contrast to the behavior of pantoyltaurine, the corresponding analog of the free vitamin; the latter is a competitive inhibitor of pantothenate in many microorganisms, as well as plant and animal systems. The simplest explanation of the failure of IV to inhibit L. helveticus growth may be a hydrolysis by the organism to free pantothenic acid, which is about one percent as active as pantetheine for this organism (13). Panthothenyl taurine is also capable of supporting the growth of L. fermenti, as shown in Table VII. This growth is presumably due to a partial hydrolysis of pantothenyl taurine to free pantothenic acid, since L. fermenti responds to the free vitamin rather than pantetheine.

Replacement of the SH group by OH forms "oxypantetheine" (V) an active competitive inhibitor for

TABLE VI

Growth stimulation of L. helveticus
by pantothenyl taurine

μg. pantetheine	μg. Panthothenyl taurine			
	0	5	10	15
	Optical density			
0	.05	.18	.31	.41
.1	.30	.37	.41	.49

TABLE VII

Growth stimulation of L. fermenti
by pantothenyl taurine

μg. Pantethenyl taurine	Pantetheine equivalents (in μg.)	Relative activity (percent)
0	0	
10	.045	.45
20	.055	.28
30	.08	.27
40	.16	.40
	Average	.35

L. helveticus (cf. Tables VIII and IX) with an inhibition index, I/S, of 100 - 200 at half maximum growth.

Wieland et al, have shown that S-phenyl pantetheine is also an active competitive inhibitor, while the S-methyl and S-ethyl derivatives are slowly hydrolyzed to pantetheine (42).

The mode of action of "oxy-pantetheine", as well as S-phenyl pantetheine, is probably due to formation of CoA analog which competes with CoA for the enzyme surface. This seems plausible on the basis of the structural features of V, which possesses an unaltered pantothenyl residue (hydroxy and amide groups and carbon skeleton duplicating those in the vitamin). On the other hand, substitution of the thiol group by a hydroxy group evidently prevents the formation of S∿acyl bonds, which are characteristic products of CoA activity. Presumably an altered CoA may be formed, which competes with CoA in normal transacylation reactions.

The synergistic effect that low concentrations (cf. Table VIII, 5 μg. level) of "oxy-pantetheine" exerts with pantetheine is more difficult to explain. Craig and Snell (13) have shown pantothenic acid to have similar synergistic action with pantetheine in L. helveticus. Table X demonstrates that some hydrolysis can occur. "Oxy-pantetheine" has a pantothenic acid

TABLE VIII

Inhibition of L. helveticus
by "oxy-pantetheine" (V)

μg. Pantetheine	μg. "oxy-pantetheine" 0	5	10	25	100
	Optical density				
0	.03	.03	.03	.03	.03
.10	.25	.41	.20	.04	.02
.15	.34	.54	.36	.06	.04
.25	.50	.64	.57	.29	.05

TABLE IX

Competitive inhibition of L. helveticus by "oxy- p antetheine" (V)

µg. Pantetheine	µg. "oxy-pantetheine"	"oxy-pantetheine" / pantetheine	Optical density
0	0	0	.11
0.05	0	0	.34
0.05	5	100	.54
0.05	7.5	150	.47
0.05	10	200	.38
0.10	0	0	.49
0.10	10	100	.52
0.10	15	150	.44
0.10	20	200	.36
0.15	0	0	.53
0.15	15	100	.52
0.15	22.5	150	.47
0.15	30	200	.36
0.25	0	0	.60
0.25	25	100	.53
0.25	37.5	150	.45
0.25	50	200	.38

TABLE X

Stimulation of growth of L. fermenti
by "oxy-pantetheine" (V)

µg. "oxy-pantetheine"	Calcium Pantothenate equivalence	Activity (percent)
10	.014	.14
20	.049	.25
60	.164	.27
100	.270	.27
		Average .23

activity of 0.23 percent for L. fermenti. Whether the growth may result from pantothenic acid, arising from hydrolysis, or a competition of "oxy-pantetheine" for pantetheinehydrolyzing enzymes, thus preventing some normal partial destruction of the vitamin, cannot be determined by these nutritional studies.

SUMMARY

1. The following fifteen new compounds were prepared; sodium N-pantothenyl taurine, bis (N-(γ hydroxybutyryl -β-alanyl)-2-aminoethyl) disulfide, N-pantothenylethanolamine ("oxy-pantetheine"), N-(pantoylglycyl)-2-aminoethanthiol, N-(pantoylalanyl)-2-aminoethanthiol, bis (N-(pantoylnorvalyl)-2-aminoethyl) disulfide, N-carbobenzoxy-β-alanyl)ethanolamine, N-β-alanylethanolamine oxalate, bis (N-(carbobenzoxyglycyl)-2-aminoethyl) disulfide, N-glycyl-2-amino-ethanthiol, bis (N-(phthalylalanyl)-2-aminoethyl) disulfide, phthalyl-norvaline, bis (N-(phthalylnorvalyl)-2-aminoethyl) disulfide, bis (N-norvalyl-2-aminoethyl) disulfide, and S-acetyl pantetheine.

2. A new method for the preparation of S-acetyl coenzyme A was described.

3. S-acetylpantetheine was shown to be as active as pantetheine in promoting the growth of Lactobacillus bulgaricus.

4. N-pantoyl-2-aminoethanthiol, N-pantoylethanolamine and bis (N-(pantoylnorvalyl)-2-aminoethyl) disulfide were unable to stimulate the growth of Lactobacillus helveticus.

5. N-(pantoylglycyl)-2-aminoethanthiol, N-(pantoylalanyl)-2-aminoethanthiol, and

bis (N-(γ-hydroxybutyryl-β-alanylaminoethyl) disulfide were able to support the growth of L. helveticus at a concentration of 100 to 1000 μg. per 10 ml. of culture medium.

6. N-Pantothenyl taurine was able to stimulate the growth of both L. fermenti and L. helveticus at concentrations of 5 to 60 μg. per 10 ml. of culture medium.

7. "Oxy-pantetheine" (N-pantothenylethanolamine) was active as a competetive inhibitor of pantetheine in L. helveticus, with an inhibition index of 100 to 200. A concentration of "oxy-pantetheine of at least 10 μg. per 10 ml. of culture medium was required to support the growth of L. fermenti.

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