[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF PARKE, DAVIS AND CO. AND THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

The Synthesis of Pantetheine–Pantethine[1a]

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Pantetheine (N-(+)-pantothenyl-2-aminoethanethiol) (I) and pantethine (the disulfide) (II) have been prepared by the condensation of methyl pantothenate or pantothenyl azide with β-mercaptoethylamine, or alternatively from (−)-pantolactone and N-(β-alanyl)-2-aminoethanethiol or the disulfide. The synthetic growth factor has been purified by chromatography and solvent distribution. Pantetheine is a colorless viscous oil having a microbiological potency of 20,800 LBF units/mg., and a specific rotation $[\alpha]^{27}D$ +13.5° (water). Pantetheine furnishes a crystalline adduct with juglone, an amorphous silver mercaptide and a crystalline mercuric mercaptide. The latter two derivatives can be reconverted to pantethine.

In a previous communication[2] the characterization of the *Lactobacillus bulgaricus* factor (LBF) as N-(+)-pantothenyl-2-aminoethanethiol (pantetheine) (I) or the disulfide (pantethine) (II) and the synthesis of this latter compound was reported. Concurrent work on natural LBF has shown that several forms of this growth factor exist; the details of the isolation[3] and characterization[4] of one of these forms and the relation[5] of the various forms to pantetheine–pantethine are being described in separate papers. In this paper the details of the synthesis and purification of pantetheine (I) and pantethine (II) are presented.

The first synthetic approach investigated for the formation of pantetheine (ine) was the condensation of methyl or ethyl pantothenate (III) with β-mercaptoethylamine or bis-(β-aminoethyl) disulfide. Of the various conditions which were tried, refluxing a methanol solution of the methyl ester and mercaptamine for 10 to 20 hours appeared the most satisfactory. After an alkaline treatment to saponify unreacted ester, the yield of pantetheine, based on microbiological LBF assay, was 20–30%. The pantetheine was then extracted with butanol and fractionated on Superfiltrol columns. About 10% of the material was obtained in the highest potency fraction, 17,500 LBF units/mg., which represents a purity of 84% based on the potency of 20,800 units/mg. which has been obtained for material giving the correct elementary analysis (3, Table I).

The use of the azide of pantothenic acid (IV) offered the possibility of a more efficient condensation and was accordingly studied. No attempt was made to isolate the azide, which was separated in the butanol layer and coupled directly with β-mercaptoethylamine. Since this process involved four consecutive reactions in which no intermediates could be isolated and no yields determined, the results were sometimes erratic. In several preparations, however, over-all yields of 30–40%, based on microbiological assay of the final product, were realized.[6] The pantetheine obtained by this procedure was purified by partition between butanol and water. In this way, 60% of the active material was recovered in solution in a purity of 90–95%. Further purification could be effected by removing the butanol and precipitating the pantetheine as an oil by the addition of ether.

An alternative approach to the synthesis of the growth factor is the condensation of (−)-pantolactone with N-(β-alanyl)-2-aminoethanethiol (V) or the disulfide (VI).[7]

The reaction of N-carbobenzoxy-β-alanyl chloride with bis-(β-aminoethyl) disulfide, followed by reduction with sodium in ammonia, gave good yields of N-(β-alanyl)-2-aminoethanethiol (V). Acylation of the bis-(β-aminoethyl) disulfide with N-phthalyl-β-alanyl chloride, followed by removal of the protective group with hydrazine, furnished bis-[N-(β-alanyl)-2-aminoethyl] disulfide (VI), isolated as the hydrochloride.[8] Ammonolysis of bis-(β-bromopropionyl-2-aminoethyl) disulfide was also investigated, but this reaction was not as satisfactory as either of the other methods.

The condensation of V or VI with (−)-pantolactone, carried out by allowing an alcohol solution of the reactants to stand at room temperature, resulted in yields of 80–90% of pantetheine or pantethine. Precipitation of the product by the addition of ether furnished material with a microbiological potency of 19,000–23,000 units/mg.

Pantetheine reacts readily with silver oxide to form a silver derivative which could be precipitated from acetone as a yellow amorphous solid which had no melting point. The crude silver mercaptide could not be purified to any great extent so that preparations from relatively crude pantetheine could not be obtained analytically pure. However, from preparations of pantetheine which were 85–95% pure, a number of samples of the analytically pure derivative were obtained.

Pantetheine also reacts readily with mercuric oxide to furnish a crystalline mercury derivative

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(2) E. E. Snell, G. M. Brown, V. J. Peters, J. A. Craig, E. L. Wittle, J. A. Moore, V. M. McGlohon and O. D. Bird, THIS JOURNAL, **72**, 5349 (1950).

(3) V. J. Peters, G. M. Brown, W. L. Williams and E. E. Snell, *ibid.*, **75**, 1688 (1953).

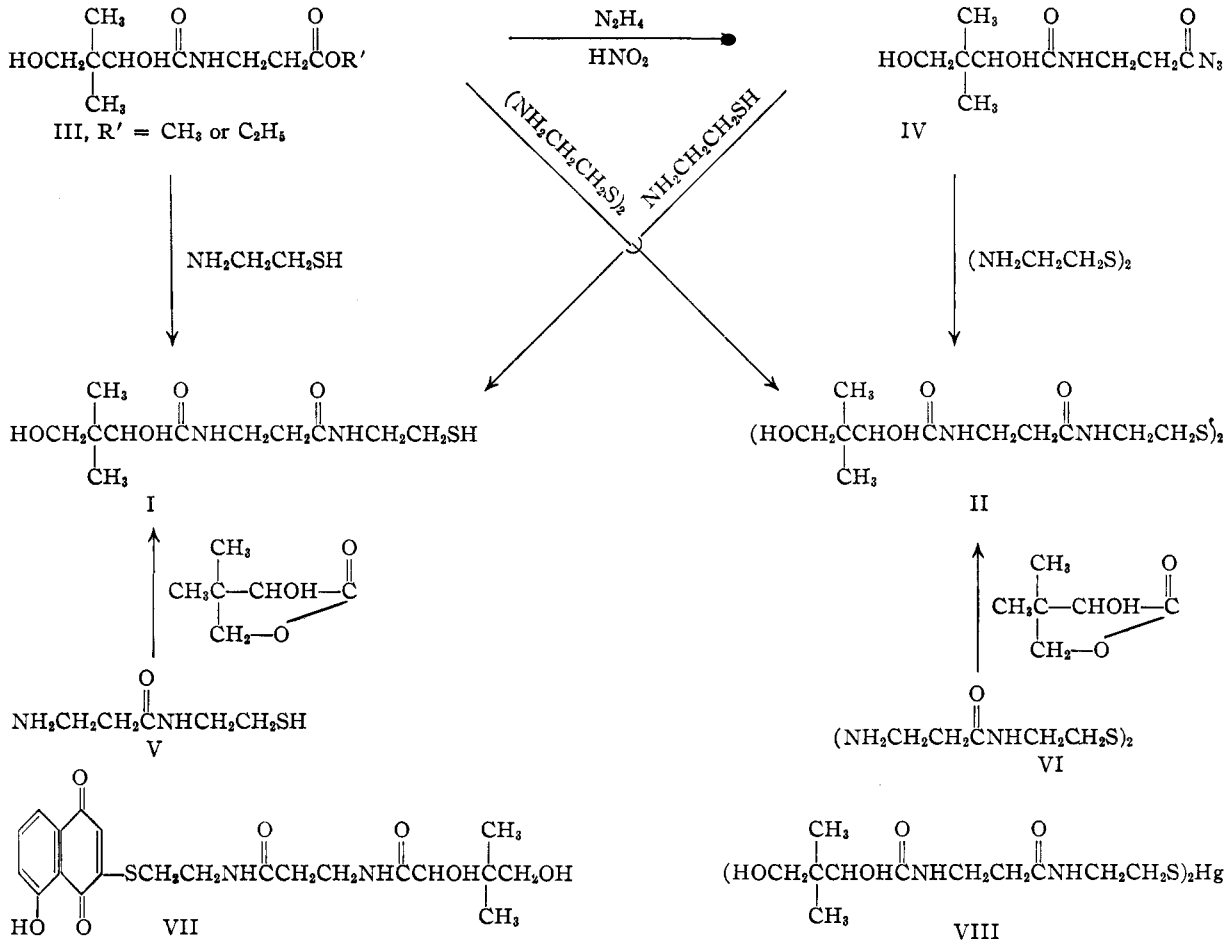
(4) G. M. Brown and E. E. Snell, *ibid.*, **75**, 1691 (1953).

(5) G. M. Brown and E. E. Snell, *J. Biol. Chem.*, **198**, 375 (1952).

(6) T. Wieland and E. Bokelmann, *Naturwissenschaften*, **38**, 384 (1951), have reported the synthesis of pantethine *via* the mixed anhydride of pantothenic acid and ethyl hydrogen carbonate. This reaction in our hands did not appear superior to the azide method.

(7) Since the appearance of our first communication, a synthesis of pantetheine employing this approach has been reported by J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 800 (1952).

(8) This reaction was first performed by Dr. J. F. Cavalla in the research laboratories of Parke, Davis and Co., Ltd., London, England; details of the preparation will be reported elsewhere.



(VIII), containing acetone of crystallization, m.p. 95–98°, $[\alpha]^{27}D$ + 9.6°.

Another characteristic derivative was obtained by the addition of pantetheine to 5-hydroxy-1,4-naphthoquinone (juglone).[9] The resulting 3-[N-(+)-pantothenyl-2-aminoethylthio]-5-hydroxy-1,4-naphthoquinone (VII) was isolated by crystallization from methanolic solution.

The best preparations of pantethine (or pantetheine) exist as a clear colorless sirup or glass which have shown no tendency to crystallize. Since neither the mercaptan nor the disulfide has any characteristic physical properties, the only criterion available for following the course or yield of reactions, or the progress of fractions is the microbiological assay. Despite the usual inherent variations in the assay, the potency of pure pantetheine or pantethine, expressed as LBF units/mg., is a critical value since it is the only means other than elementary analysis by which the purity of any given sample can be determined.

By drying samples of natural LBF (LBF – 1A) at 100° for total solids determination, a value of 29,000 units/mg. was obtained,[2,3,10] although this treatment inactivated the material. It became apparent in the present work that pyrolysis occurred at this temperature, so that this value does not represent the potency of pure pantethine. It has now been established that samples of pantethine can be dried to reproducible constant weight at 65° *in vacuo* without loss in activity, and this procedure has been used throughout most of the present work in assigning potencies. Values for total solids obtained with these drying conditions are approximately double those obtained at 100° and the potencies are correspondingly lowered. With the 65° drying, the best sample of pantethine prepared in the present work has a potency of 20,800 units/mg.

It was expected that regeneration of pantetheine or pantethine from the purified mercaptides would furnish material with satisfactory and reproducible analyses, but this procedure has not been entirely successful. Treatment of the silver mercaptide with hydrogen sulfide liberated pantethine with the correct total activity calculated for the pantetheine content of the mercaptide, but the product had consistently low carbon analyses. The pantetheine obtained from the mercury mercaptide and hydrogen sulfide (8, Table I), had a low sulfur value and also a low potency. No reason is known for these discrepancies. The pantethine obtained by treating the silver mercaptide with iodine (4, 5, 6, Table I) represents some of the better samples which have been obtained, although the nitrogen and sulfur values are somewhat low.

In Table I are listed the properties of the best samples of pantetheine (ine) obtained by the vari-

(9) R. H. Thompsen, *J. Org. Chem.*, **16**, 1082 (1951).

(10) Since the LBF-1A used in this work was probably a mixed disulfide of a low-molecular-weight mercaptan and pantetheine, the value of 29,000 units/mg. would indicate a potency of about 35,000 units/mg for pantetheine, if determined by this procedure.

TABLE I

ANALYSES ON PANTETHEINE-PANTETHINE[a]

	C	H	N	S	Potency, u./mg.	Rotation $[\alpha]^t_D$	t
$C_{11}H_{22}O_4N_2S$	47.46	7.97	10.07	11.52			
$C_{22}H_{42}O_8N_4S_2$	47.64	7.63	10.10	11.56			
1, From methyl pantothenate	47.36	7.58	9.17	11.85	17,500		
	47.86	7.78	9.23	12.78			
2, From azide	48.80	7.98	9.35		20,400[b]		
3, From azide	47.41	7.68	9.89	11.42	20,800	+13.5°	27
			9.82				
4, Ag mercaptide + I_2	47.92	7.98	9.63	10.75	21,700	+16.2	26
5, Ag mercaptide + I_2	47.17	7.19	9.51	11.53	23,150	+17	27
6, Ag mercaptide + I_2	47.98	7.80	9.46	10.92	20,000	+13.6	26
7, From (−)-pantolactone + VI	47.22	7.58	9.33		20,840[c]	+13.5	27
8, Hg mercaptide + H_2S	48.10	7.66	9.74	10.62	18,000		
9, Calcd. from the aver. of 14 assays on Hg mercaptide					21,000		

[a] All samples were dried at 65° *in vacuo* for 48–65 hr. [b] Average of fifteen assays. [c] Average of five different preparations.

ous methods. Only 3 shows completely acceptable analytical results. It has a potency of 20,800 units/mg. based on twelve assays and a specific rotation $[\alpha]^{27}D$ +13.5°. While a series of fifteen assays on a single solution of one preparation (2, Table I) gave a standard deviation of ±825, the problem of drying the samples to constant weight increases the variation in potency between different preparations.[11]

It seems possible from some of the higher potencies which have been obtained (5, Table I and others) that this material still contains a small amount of impurity or is partially racemized, so that this potency and rotation may not represent the highest values that can be obtained.

Acknowledgment.—The authors wish to thank Mr. C. E. Childs, Mrs. G. D. Koch and Miss V. M. Pawlik for the microanalytical determinations and Mr. Y. J. L'Italien and Mr. W. A. Pearlman for generously supplying some of the intermediates and preparations which were used.

Experimental

Assay Procedure.—The organism used was *L. helveticus* and the procedure that of Craig and Snell.[12] In this assay procedure the standard was Basamin Busch yeast extract, one LBF unit being defined as one milligram of yeast extract. The incubation temperature was 37.5° and the incubation period was 17 hours. The incubation period for assays run in the later phases of this work was 40 hours, since this longer period was shown to give more consistent results.

Preparation of Pantethine from the Methyl Ester Process. Condensation of Methyl (+)-Pantothenate and β-Mercaptoethylamine.—A solution of pantothenic acid was prepared by treating 361 g. (1.5 moles) of sodium (+)-pantothenate[13] in 500 cc. of methanol with 1.5 moles of alcoholic hydrogen chloride. The precipitated sodium chloride was filtered off and the acid was converted to the methyl ester by treating with a slight excess of diazomethane. After standing for 30 minutes, the ester solution was concentrated *in vacuo* to a viscous sirup which was filtered through Supercel to remove traces of sodium chloride and then diluted with 2 l. of methanol. To this solution was added 116 g. (1.51 moles) of β-mercaptoethylamine.[14] The solution was refluxed for 16 hours, and was then evaporated as completely as possible at 70° to give 405 g. of a viscous sirup.

A 50-g. sample of this material was dissolved in methanol and made up to 100 cc. of solution. To this was added an equal volume of 2 *N* methanolic potassium hydroxide. The reaction mixture was allowed to stand at room temperature for one hour and was then acidified to *p*H 5.0 (determined by diluting an aliquot with water) with methanolic hydrogen chloride. The solvent was removed by distillation *in vacuo* at a temperature below 50° and the residue was taken up in 375 cc. of butanol. This solution contained 2.22×10^8 LBF units, representing 0.0383 equivalent of LBF (based on a potency of 20,800 units/mg. or 5.80×10^9 units/eq. for pure pantetheine). The total yield of pantetheine–pantethine from the condensation was thus 0.31 eq. or 20%.

The butanol solution was then washed six times with one-third volumes of water (until free of chloride ion). This washed butanol solution was designated as fraction A and contained 38.6% of the LBF activity at a potency of 16,900 units/mg. The water washes were combined and extracted twice with one-half volumes of butanol. These butanol extracts (fraction B) contained material at a potency of 14,600 units/mg., amounting to 50.3% of the activity.

Fraction A from the above distribution was concentrated to one-eighth volume *in vacuo* below 50° and the concentrate was chromatographed over a mixture of 125 g. of Superfiltrol (Thiamine grade) and 125 g. of High Flo Supercel in a column 6.5 cm. in diameter. The column was washed with three liters of anhydrous butanol; this treatment removed some of the impurities. Water-saturated butanol (2.5 l.) was then used to develop the column. Nine effluent fractions were obtained, representing a recovery of 80%. Fraction B was chromatographed in a similar way; the recovery was nearly quantitative. All but the last fractions from both of these columns showed some enrichment in potency over that of the starting material.

The most potent fraction from each of these chromatograms, amounting to 6 and 8% of the total recoveries, were combined and concentrated *in vacuo* at 40° to 15 cc. volume. This solution was then chromatographed on a column of 12.3 g. of Superfiltrol and 12.3 g. of Supercel, which was washed with 800 cc. of butanol and developed with 350 cc. of water-saturated butanol. The main fraction from this chromatogram contained 47% of the starting material at a potency of 34,900 units/mg.

The potencies indicated in this purification, as well as those given in our previous communication,[2] were determined by assaying one aliquot of the solution for LBF units/cc. and drying another aliquot of the same fraction to constant weight at 100°. When an aliquot of the main fraction from the last column was dried at 100° to constant weight and then taken up in solution for assay, however, the sample had no growth promoting properties for *L. helveticus*.

Pantethine is stable and microbiologically active when dried *in vacuo* at temperatures below 80°. Additional losses in weight occur when dried at higher temperatures. Therefore, drying in all subsequent determinations has been done at 65° *in vacuo* to constant weight.

Redetermination of the dry weight of the most potent fraction from the last column at 65° gave 4.46 mg./cc.

(11) The potency of pantetheine reported by Baddiley and Thain[7] was 26,000 units/mg. Since neither the drying conditions nor the assay standard are stated, no comparison can be made.

(12) J. Craig and E. E. Snell, *J. Bact.*, **61**, 283 (1951).

(13) For a review of the chemistry of pantothenic acid, see Rosenberg, "The Chemistry and Physiology of the Vitamins," Interscience Publ., Inc., New York, N. Y., 1945, p. 253.

(14) E. J. Mills and M. T. Bogert, THIS JOURNAL, **62**, 1173 (1940).

(instead of 2.26 mg./cc.), and hence 17,500 units/mg. (instead of 34,900 units/mg.) as the potency. The analysis of this material is given in Table I, 1.

Due to this change in dry weight determination, all of the potencies recorded above, and those reported in our earlier communication[2] are high by a similar factor. This weight loss at higher drying temperatures is not as great with more highly purified samples.

Preparation of Pantethine by the Azide Process. Ethyl (+)-Pantothenate.—To a solution of 612 g. (4.0 moles) of ethyl β-alanate hydrochloride in 1 l. of methanol was added a solution of 92 g. of sodium in 1.5 l. of methanol. To the cold solution of ethyl β-alanate was added a solution of 520 g. (4.0 moles) of (−)-pantolactone in 1.5 l. of methanol. After standing at 25° for 60 hours, the solution was filtered from the sodium chloride and evaporated *in vacuo* to a volume of 1.8 l.

Pantothenyl Hydrazide, Azide and Pantethine.—One-half of the above solution of ethyl (+)-pantothenate was treated with 120 cc. of 85% hydrazine hydrate (2.1 moles of hydrazine). The base was added in several portions with stirring and cooling, so that the temperature did not rise above 25°. After standing for one week at room temperature the viscous solution was heated to 60° for one hour and then diluted with an equal volume of butanol. The hydrazide was then acidified with 90 g. (2.5 moles) of hydrogen chloride dissolved in 500 cc. of ethanol. (In a number of the preparations, unreacted hydrazine was separated at this point as the hydrochloride, which precipitated from the alcohol solution. In this experiment, the conversion to the hydrazide was apparently complete, since no hydrazine hydrochloride was obtained.)

The solution was then diluted with 2 l. of water and 3 l. of butanol to obtain two layers and the mixture was cooled to 5°. A solution of 138 g. (2 moles) of sodium nitrite in 200 cc. of water was then added dropwise, keeping the temperature at 5–10°. A pale pink color developed. The butanol layer was then separated and washed with one liter of water. The combined aqueous layers were now washed with one liter of butanol. To the combined butanol layers, containing the pantothenyl azide, next was added 155 g. (2 moles) of β-mercaptoethylamine dissolved in 150 cc. of methanol. The *p*H of the solution was then raised to 9.0 by the addition of 50 g. of sodium hydroxide. After standing for 65 hr., at 25°, the solution was evaporated *in vacuo* to a volume of 4.30 l. The yield by microbiological assay was 39% based on the ethyl β-alanate. When the azide was coupled with bis-(β-aminoethyl) disulfide, somewhat lower yields were obtained.

Fractionation.—By preliminary experiments it was found that solvent distribution between butanol and water was very adaptable to purification of this type of synthetic material. The preparation described above, which had a potency of 12,700 units/mg., was fractionated in this way. No attempt was made to protect the pantetheine obtained in these syntheses from oxidation, so that material from the purification steps involving prolonged exposure to air was nearly all in the form of pantethine.

The butanol solution of pantethine was washed eight times with one-third volumes of water. The washed solution (fraction I) had a potency of 14,100 units/mg. and contained 14% of the initial pantethine, thus indicating that most of the material had been transferred to the water washes. The water washes were combined and extracted three times with one-half volumes of butanol. These extracts (fraction II) contained 66.6% of the pantethine and had a potency of 15,500 units/mg. The water residue (fraction III) was discarded. The butanol extracts were then washed sixteen times with 2% of their volume portions of water. This step removed considerable impurities and gave a fraction containing 60% of the activity with a potency of 20,400 units/mg. (fraction IV). A sample of this solution was evaporated to an oil; for analysis see 2, Table I.

The R_f values of this material in several solvent systems on Whatman No. 1 paper are: *n*-butanol–water, 0.87; *n*-butanol–acetic acid–water (54:17:29), 0.92; *n*-butanol–*n*-hexane (60:40)–water, 0.64; ethyl acetate–water, 0.26; ethyl acetate–acetic acid–water (70:15:15), 0.75.

An aliquot of fraction IV was concentrated *in vacuo* to an oil and taken up in a small volume of methanol. Eight volumes of absolute ethyl ether were added. The oil which separated was dissolved in methanol and reprecipitated with ten volumes of absolute ethyl ether. This fraction had a potency of 20,800 units/mg., $[\alpha]^{27}D$ +13.5° (*c* 3.75 in water). The analysis, which is in very good agreement with the calculated value, is given in 3, Table I.

Pantethine is freely miscible with all lower alcohols and water, but very sparingly soluble in ethyl acetate and ether. It has been found to be stable on standing for twelve months in a butanol solution at room temperature. It has also shown complete stability for eleven months in buffers at *p*H 4.5, 6.0 and 7.1 after being autoclaved for six minutes and then being stored at room temperature in ampoules.

Preparation of Pantethine from (−)-Pantolactone and Bis-(β-alanyl-2-aminoethyl) Disulfide. Bis-[N-(N-carbobenzoxy-β-alanyl)-2-aminoethyl] Disulfide.—N-Carbobenzoxy-β-alanyl chloride was prepared by treating a solution of 3.36 g. (0.015 mole) of N-carbobenzoxy-β-alanine[15] in 15 cc. of dry benzene with 3.5 cc. of thionyl chloride. The solution was warmed until gas evolution ceased and then evaporated to small volume and the oily acid chloride was taken up in 25 cc. of anhydrous dioxane.

This solution was then added, concurrently with 15 cc. of 1 *N* sodium hydroxide, to a stirred and cooled solution of 1.69 g. (0.0075 mole) of bis-(β-aminoethyl) disulfide dihydrochloride[14] in 15 cc. of 1 *N* sodium hydroxide. The amide separated as a white solid, yield 4.5 g. The material was recrystallized from methanol; m.p. 179–180°.

Anal. Calcd. for $C_{26}H_{34}O_6N_4S_2$: C, 55.50; H, 6.09; N, 9.96. Found: C, 55.70; H, 6.18; N, 10.14.

N-(β-Alanyl)-2-aminoethanethiol.—A solution of 890 mg. (0.0016 mole) of the bis-carbobenzoxy compound in 30 cc. of liquid ammonia was treated with 253 mg. (0.011 mole) of sodium in small portions. The last portion of sodium produced a blue color which faded very slowly. Then 687 mg. (0.011 mole) of ammonium chloride was added and the ammonia was allowed to evaporate. The residue was dissolved in absolute ethanol and the sodium chloride was filtered off. The ethanol was evaporated, leaving a clear colorless oil which had an alkaline reaction and gave a positive test for the thiol group with sodium nitroprusside.

A sample of this material was distilled at 80° at 10^{-3} mm. in a short-path still, and was obtained as a white crystalline solid, m.p. 95–98°.[16]

Anal. Calcd. for $C_5H_{12}ON_2S$: C, 40.63; H, 8.19. Found: C, 40.91; H, 7.99.

Reduction of the bis-[N-(N-carbobenzoxy-β-alanyl)-2-aminoethyl] disulfide with hydrogen and palladium black catalyst furnished **N-(N-carbobenzoxy-β-alanyl)-2-aminoethanethiol** in 60% yield. This compound was crystallized from methanol–ether, m.p. 109–112°. It reacted with 5-hydroxy-1,4-naphthoquinone (juglone) to give the sulfide, **3-[N-(N-carbobenzoxy-β-alanyl)-2-aminoethylthio]-5-hydroxy-1,4-naphthoquinone**, yellow plates from ethanol, m.p. 198–200° (dec.).

Anal. Calcd. for $C_{23}H_{22}O_6N_2S$: C, 60.78; H, 4.88. Found: C, 60.85; H, 4.92.

Bis-[N-(β-alanyl)-2-aminoethyl] Disulfide Dihydrochloride.—A solution of 1.48 g. (0.01 mole) of N-(β-alanyl)-2-aminoethanethiol in 20 cc. of methanol was acidified with hydrochloric acid and treated at 0° with 1.0 cc. of 15% hydrogen peroxide. The solution gave a negative test for mercaptan. The solution was concentrated to a small volume and diluted with ether until faintly turbid. White crystals separated on cooling; 1.3 g., m.p. 205–208°. The material was recrystallized from methanol–acetone; m.p. 218–221°.

Anal. Calcd. for $C_{10}H_{24}N_4O_2S_2Cl_2$: C, 32.69; H, 6.59; N, 15.25. Found: C, 32.83; H, 6.40; N, 15.20.

A sample of this amine hydrochloride, 200 mg., was dissolved in 5 cc. of water and 2.5 cc. of 1 *N* sodium hydroxide and 0.15 cc. of benzoyl chloride was added. The mixture was shaken for several minutes and the solid was filtered, washed with water and dilute acid and dried, weight 280 mg. The material was recrystallized from ethanol to furnish the dibenzoate of bis-[N-(β-alanyl)-2-aminoethyl] disulfide, m.p. 198–199°.

Anal. Calcd. for $C_{24}H_{30}N_4S_2O_4$: C, 57.35; H, 6.02; N, 11.15. Found: C, 57.09; H, 6.21; N, 11.08.

(15) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).

(16) Baddiley[7] reports m.p. 93–95° for this compound.

Bis-[N-(β-bromopropionyl)-2-aminoethyl] Disulfide.—To a solution of 70 g. (0.31 mole) of bis-(β-aminoethyl) disulfide dihydrochloride in 200 cc. of water was added 71 g. of potassium hydroxide. This solution was treated dropwise with 117 g. (0.66 mole) of β-bromopropionyl chloride. The acid chloride was added quite slowly, with good stirring at a temperature of 0–5°. The solid which separated was filtered, washed with water and recrystallized from methanol to give 72 g. of white crystals, m.p. 121–124°. The melting point was 130–131° after a second recrystallization.

Anal. Calcd. for $C_{10}H_{18}N_2O_2S_2Br_2$: C, 28.44; H, 4.30; N, 6.64; Br, 37.86. Found: C, 28.95; H, 4.23; N, 6.88; Br, 37.28.

Bis-[N-(β-alanyl)-2-aminoethyl] Disulfide Dihydrobromide.—Two grams of bis-(β-bromopropionyl-2-aminoethyl) disulfide and 40 cc. of liquid ammonia were placed in a bomb tube. The tube was sealed and the mixture was allowed to warm to room temperature; the solid starting material dissolved. After standing for 12 days, the tube was opened and the solution was evaporated to dryness. The semi-solid residue was dissolved in methanol and again evaporated to remove traces of ammonia. The material was again dissolved in methanol and allowed to crystallize slowly. Two crops of crystals were obtained. After combining these and recrystallizing from methanol, 500 mg. of material was obtained, m.p. 170–180°.

Anal. Calcd. for $C_{10}H_{24}O_2N_4S_2Br_2 \cdot CH_3OH$: C, 27.08; H, 5.78; N, 11.48; Br, 32.73. Found: C, 27.04; H, 5.60; N, 11.56.

A sample of this compound prepared by the hydrazine cleavage of bis-[N-(β-phthalimidopropionyl)-2-aminoethyl] disulfide followed by treatment with hydrogen bromide had m.p. 205–207°.

Anal. Found: C, 27.35; H, 5.25; N, 11.84; Br, 32.85.

Most of the bis-[N-(β-alanyl)-2-aminoethyl] disulfide dihydrochloride which was used in this work was prepared *via* the bis-[N-(β-phthalimidopropionyl)-2-aminoethyl] disulfide. The details of this process will be reported elsewhere.

An attempt was made to isolate the free base, bis-[N-(β-alanyl)-2-aminoethyl] disulfide by neutralizing an ethanol solution of the bis-amine hydrochloride with sodium ethoxide. After removing the sodium chloride, the ethanol was evaporated. The oily residue on exposure to air formed a white solid, m.p. 140–165° (capillary). This material liberated carbon dioxide on treatment with concd. hydrochloric acid and gave a precipitate of barium carbonate after standing for some time with barium hydroxide. This compound is the stable carbonate of the amine.

Anal. Calcd. for $C_{10}H_{22}O_2N_4S_2 \cdot H_2CO_3$: C, 37.10; H, 6.78. Found: C, 36.68, 36.65; H, 6.48, 6.46.

Pantetheine.—A solution of 164 mg. of N-(β-alanyl)-2-aminoethanethiol (1.1 meq.) and 155 mg. of (−)-pantolactone (1.1 meq.) were mixed in 1.8 cc. of methanol and allowed to stand at 27° for 24 hours in a closed flask. The solution was then heated in a 50° bath for 30 minutes in a closed flask. The solution was cooled, made up to 2 cc. in volume and an aliquot assayed microbiologically; yield 78% based on 20,00 units/mg. for pure pantetheine. 1.3 cc. of this solution was evaporated to dryness *in vacuo* at 60° for 30 minutes to yield a clear colorless oil, 200 mg., which is the theoretical amount. By microbiological assay the potency was 17,600 units/mg. which represents a purity of 84%. Similar runs gave yields of 65–92% based on microbiological assay.

Pantethine.—A solution of 2.76 g. of bis-[N-(β-alanyl)-2-aminoethyl] disulfide dihydrochloride (15 meq.) in 15 cc. of absolute ethanol reacted with a solution of 345 mg. of sodium (15 meq.) in 30 cc. of absolute ethanol until the dihydrochloride was converted to the free amine and insoluble sodium chloride. 1.95 g. (15 meq.) of (−)-pantolactone was then added and the solution was well mixed and allowed to stand at 25° for 24 hours. The solution was heated to 60° for 30 minutes and again allowed to stand for 24 hours at 25°. The sodium chloride was filtered off and rinsed with a small amount of ethanol; weight 900 mg. Microbiological assay of an aliquot of the filtrate (62.7 cc.) showed a yield of 79%. 59.7 cc. of this solution was evaporated to dryness to leave 4.1 g. of a clear oil, potency 20,600 units/mg. The oil was dissolved in 20 cc. of methanol and precipitated by the gradual addition of 200 cc. of ether with shaking. The ether was decanted and the process was repeated. The oil so obtained was dried in a vacuum desiccator by the gradual reduction of pressure so that it slowly foamed to fill a 200-cc. flask. This white foam on continued drying became solid; it was very hygroscopic. It had a potency of 21,700 units/mg. and gave analysis (7, Table I) which indicates it is not completely pure; yield 3 g. (73%).

Mercuric Mercaptide (VIII). A.—A water-saturated butanol solution (250 cc.) of pantethine from azide preparation above (20,400 units/mg., 1.73 g. of pantethine) and 500 mg. of palladium black (American Platinum Works) was shaken under hydrogen at 40 lb. for 18 hours. The catalyst was filtered off with mild suction. Titration of 2 cc. of this solution required the theoretical amount (1 cc.) of 0.05 *N* iodine. To the solution was added 680 mg. of mercuric oxide and 10 cc. of water and it was shaken until practically all of the oxide had disappeared. The solution was filtered through filter aid and evaporated to an oil *in vacuo* in a 65° bath. The residual oil was dissolved in 5 cc. of butanol and then added dropwise with stirring to 350 cc. of acetone. An oil precipitated which on further stirring turned completely solid. The white solid was filtered off, washed with 75 cc. of acetone, and air-dried; yield 900 mg. (38%). The acetone filtrate on concentration and cooling gave two successive crops (1.2 g.) of sticky solid; the total yield of solid was 2.15 g., 91%. The first crop amounted to 900 mg., m.p. 95–98° on the Fisher–Johns block; microbiological potency of 16,050 units/mg., $[\alpha]^{27}D$ +10.8° (*c* 2.23 in H_2O). For analysis the sample was dried *in vacuo* at 30° for 2 hours.

Anal. Calcd. for $C_{22}H_{42}O_8N_4S_2Hg$: C, 34.98; H, 5.61; N, 7.42; S, 8.49. Found: C, 34.81; H, 5.89; N, 7.43; S, 8.49, 8.67.

B.—A water-saturated butanol solution (515 cc.) of pantethine (from the azide route, 24.8 mg./cc., 19,000 units/mg.) was reduced as above and the reduced solution reacted with 6 g. of mercuric oxide and 100 cc. of water by vigorous shaking. The insoluble solid was filtered off with a filter aid and the clear filtrate was evaporated to dryness *in vacuo* at 60°. The residue was dissolved in 200 cc. of methanol and acetone was added slowly with vigorous shaking to precipitate an oil. After 1.5 l. of acetone had been added no further oil precipitated and the clear solution was decanted from the oil. On standing overnight at 25° characteristic white crystals of mercury compound formed over the surface of the flask. They were filtered off and air-dried; yield 4.5 g. (29%), m.p. 95–100° (Fisher–Johns block), $[\alpha]^{27}D$ +11.4° (*c* 4.0 in H_2O) microbiological potency 15,250 units/mg. For analysis the sample was dried *in vacuo* at 30°. All samples which were crystallized from acetone solution contained a molecule of acetone of crystallization, which appeared to be partially lost on drying above 30°.

Anal. Calcd. for $C_{22}H_{42}O_8N_4S_2Hg \cdot C_3H_6O$: C, 36.91; H, 5.95; N, 6.89; S, 7.88. Found: C, 36.89; H, 5.94; N, 7.14.

C.—A sample of pantethine prepared from methyl (+)-pantothenate, potency 17,400 units/mg. (see above), was converted in the same manner to the mercury derivative; m.p. 95–100°, $[\alpha]^{27}D$ +8.8° (*c* 4 in H_2O), microbiological potency 14,450 units/mg.

Anal. Found: C, 36.62; H, 6.03; N, 6.91; S, 7.87.

D.—Pantethine (4 g., potency 17,200 units/mg.) prepared from (−)-pantolactone and bis-(N-[β-alanyl]-2-aminoethyl) disulfide was dissolved in 50 cc. of liquid ammonia and 640 mg. of sodium was added in small portions. 1.5 g. of ammonium chloride was added slowly and the solution was allowed to evaporate to dryness, warming finally to 60°. The residue was boiled for a few minutes with 50 cc. of absolute ethanol, cooled and filtered. Ten cc. of water and 2 g. of mercuric oxide were added and the mixture was well shaken. The solution was filtered and the filtrate was evaporated to dryness *in vacuo* at 60°. The residue was dissolved in 100 cc. of methanol and fractionally precipitated by continued addition of acetone. When further addition of acetone produced only a turbidity, the solution was treated with norite and filtered with Supercel. The clear white acetone filtrate on standing gave 1.4 g. (24%), of white crystalline mercury mercaptide; m.p. 96–98° (Fisher–Johns block), $[\alpha]^{27}D$ +8.8° (*c* 4 in H_2O), microbiological potency 13,900 units/mg. Recrystallization of the material from methanol–acetone produced no change. The

average of three rotations on the crystalline air-dried mercaptide containing acetone of crystallization (pantethine prepared by three different methods) is $[\alpha]^{27}D$ +9.6° (*c* 4 in H_2O). The average of all the microbiological assays (14 assays on different batches by various methods of preparation) on the air-dried crystalline mercury derivative containing acetone of crystallization was 14,370 units/mg. This gives a calculated value of 15,500 units/mg. for the material without acetone of crystallization, and a value of 21,100 units/mg. for pantethine. The mercaptide is very soluble in water or methanol. It shows only general absorption in the ultraviolet, with a very slight inflection at λ 260–265 mμ, ε 1000 at *p*H 11 or 3. The infrared absorption spectrum of the mercaptide containing acetone of crystallization contains a band at 5.87 μ, indicative of the carbonyl group, which is absent in the derivative containing no acetone.

Conversion of the Mercury Mercaptide to Pantetheine.—Addition of hydrogen sulfide to an aqueous solution of the mercury mercaptide of pantetheine gave a colloidal solution of black mercuric sulfide which would not separate. Dissolved in 0.02–0.05 *N* hydrochloric acid or in the presence of an equal weight of sodium chloride, the mercuric sulfide separated readily in 10 minutes.

A.—One hundred mg. of crystalline mercaptide (14,370 units/mg.) and 100 mg. of sodium chloride were dissolved in 10 cc. of distilled water and treated with a stream of hydrogen sulfide for 2 minutes. After 10 minutes the mercuric sulfide was filtered off rinsing carefully to avoid loss and the hydrogen sulfide removed at 50° *in vacuo*, total volume remaining 12.7 cc., calcd. 113,000 units/cc., found by assay 113,500 units/cc. Three other runs were identical.

B.—One gram of mercaptide and 1 g. of sodium chloride were dissolved in 15 cc. of distilled water and a stream of hydrogen sulfide was bubbled through the solution for one minute. After 5 minutes the mercuric sulfide was filtered off with Supercel, rinsing carefully, and the colorless filtrate was shell frozen and evaporated to dryness from the frozen state. The residue was dissolved in 3 cc. of methanol and 10 cc. of acetone was added. The solution was separated from the fine precipitate of salt which formed and evaporated to dryness in the cold. The pale yellow oil dissolved slowly in 10 cc. of acetone overnight and was then separated from a trace of insoluble oil. Dilution with 5 cc. more acetone caused a small amount of oil to separate and it was removed. The clear solution was evaporated in a stream of nitrogen and then in high vacuum to give a viscous pale yellow oil, 665 mg. (98% of theory). Microbiological potency on a sample dried at 60° *in vacuo* for 40 hours was 18,000 units/mg. The analysis is given in 8, Table I.

Silver Mercaptide.—A butanol solution of pantethine, 24.2 mg./cc. (440,000 units/cc.) was reduced by shaking with 2 g. of palladium black under hydrogen for 45 hours at 55° and 30 p.s.i. (the reduction was very slow at room temperature). The reduced solution, which was quite dark, contained 0.083 meq. of RSH/cc. (theoretical 0.087 meq./cc.); assay showed no loss in microbiological activity. This solution was shaken with 4.10 g. (0.035 eq.) of silver oxide and 100 cc. of water. After shaking for 30 minutes at 25°, the mixture was filtered to remove unreacted silver oxide and a small amount of green amorphous material which was characteristic of this reaction. Both layers of the filtrate were then poured into 4.5 l. of acetone and the light yellow precipitate settled slowly. After standing overnight, the acetone was decanted and the gummy solid was dissolved in 100 cc. of water and poured into 2 l. of acetone. The yellow precipitate obtained was filtered, washed with acetone and dried in a vacuum desiccator. The material dried to a horn-like consistency, weight 6.95 g. (52%); dried at 65° *in vacuo* for analysis.

Anal. Calcd. for $C_{11}H_{21}O_4N_2SAg$: C, 34.29; H, 5.49; N, 7.27; S, 8.34; ash (as Ag_2O), 32.2. Found: C, 34.45; H, 5.77; N, 7.07; S, 7.98; ash, 32.0.

From another similar preparation, material was obtained which gave the following analysis after drying at 65° *in vacuo:* Found: C, 34.31; H, 5.76; N, 7.38; S, 8.45; ash, 30.8.

This mercaptide as obtained by this procedure is initially very soluble in water, apparently in all proportions. When the solid or an aqueous solution is treated with an organic solvent such as ethanol, butanol or acetone, however, the material gradually becomes less soluble in water. After several reprecipitations from acetone, for example, it becomes practically insoluble in cold water, but is extremely soluble in water containing a trace of electrolyte, such as 0.001 *N* sodium chloride. Either form of the mercaptide forms a stiff gel with pyridine. This change of solubility in an electrolyte suggests association of the mercaptide. A concentration of approximately 0.6 *N* hydrochloric acid was required for the formation of silver chloride from the mercaptide. This reaction is reversible; neutralization caused the silver chloride to disappear.

The optical rotation of the material exhibits some remarkable anomalies which seem to confirm such an association or polymerization. A solution of the mercaptide in pure water or water containing the minimum amount of electrolyte necessary to effect solution shows a profound dependence of rotation with temperature. In one determination, the specific rotation was $[\alpha]^{35}D$ +8°, $[\alpha]^{26}D$ +18°, $[\alpha]^{8}D$ +130° (*c* 5.39 in 0.001 *N* aqueous sodium chloride). When excess sodium chloride was present, the rotation was $[\alpha]^{8}D$ +8°, $[\alpha]^{25}D$ +8° (*c* 4.27 in 0.90 *N* aqueous sodium chloride). This latter value probably represents the true rotation of the mercaptide.

The microbiological activity of the mercaptide had an average value of 11,000 units/mg. in a number of preparations. This corresponds to 72% of the pantetheine content. When the mercaptide was decomposed with iodine or hydrogen sulfide, the calculated amount of LBF activity was obtained, that is, the "recovery" of activity from the derivative was 140%. This demonstrates that the pantetheine is not denatured in any way by mercaptide formation.

The ultraviolet absorption spectrum showed no bands in the region λ 200–400 mμ.

Regeneration of Pantetheine.—A solution of 162 mg. (0.422 meq.) of the silver mercaptide (which assayed 11,000 units/mg., 1.78 × 10^6 units total) in 2.0 cc. of water was treated with a stream of hydrogen sulfide for several minutes. The black precipitate was filtered off through Supercel (in some cases the silver sulfide was colloidal and very difficult to remove). The faintly turbid solution was diluted to 9.0 cc. volume and an aliquot was assayed for LBF. The solution contained 260,000 units/cc., corresponding to a total of 2.34 × 10^6 units of LBF, or 0.39 meq. of pantetheine.

A portion of the solution was then evaporated *in vacuo* to a sirup. This material was redissolved in 5 cc. of methanol and filtered through Supercel to clarify. The water-white solution was then evaporated to an oil and a sample was dried *in vacuo* at 65° for 72 hours for analysis.

Anal. Calcd. for $C_{11}H_{22}O_4N_2S$: C, 47.46; H, 7.97; N, 10.07; S, 11.52. Found: C, 46.10; H, 8.05; N, 9.73.

The low carbon analysis was characteristic of the pantetheine obtained by this reaction; no explanation is evident.

Regeneration of Pantethine.—One gram (2.60 meq.) of analytically pure silver mercaptide was dissolved in 15 cc. of water. To this solution was added, dropwise, a solution of 330 mg. (2.60 meq.) of iodine in 10 cc. of methanol. The iodine was decolorized immediately at first, the last few drops were not completely decolorized. The silver iodide which was formed could be only partially removed by centrifuging. The addition of an equal volume of butanol caused most of the silver iodide to coagulate. The mixture was then centrifuged and the turbid solution was evaporated *in vacuo* to remove the methanol. Two layers separated; the butanol solution was clear and faintly yellow. About 0.5 mg. of sodium sulfite was then added, and the mixture was filtered through Supercel to remove the last traces of silver iodide. The clear colorless butanol layer was then washed with four 5-cc. portions of redistilled water and evaporated at 30° to 2-cc. volume. Redistilled water was added and the solution was evaporated and the oily residue was dissolved in methanol. This solution was faintly turbid, and was filtered through hard paper and then evaporated to a glass. A sample was removed and dried at 65° *in vacuo* for 65 hr. for analysis (see Table I, 4).

The glass was then dissolved in water for assay; a solution containing 22.8 mg. solid/cc. assayed 495,000 units/cc., giving a potency of 21,700 units/mg. The solution was slightly turbid and a rotation could not be obtained; on another sample prepared by this iodine regeneration method and having a potency of 21,300 units/mg., the rotation was $[\alpha]^{26}D$ +16.2° (*c* 9.2 in H_2O) (the rotation was not significantly affected by temperature changes).

Preparation of 3-[N-(+)Pantothenyl)-2-aminoethylthio]-5-hydroxy-1,4-naphthoquinone (Juglone–Pantetheine Adduct) (VII).—Seventy cc. of a butanol solution of pantetheine prepared by catalytic reduction of pantethine and containing 0.028 meq. RSH/cc. (2.0 meq. pantetheine) was treated at room temperature with 348 mg. (2 mmoles) of 5-hydroxy-1,4-naphthoquinone. The red quinone dissolved to give a very dark brown solution. The solution was immediately evaporated at 40° (*in vacuo* with slow air stream) to a dark brown solid residue. This residue was dissolved in 5 cc. of methanol and the deep red solution was filtered to remove some black granular insoluble material. The filtrate was cooled and reddish-brown crystals were obtained; 270 mg. (30% yield), m.p. 143–148°. The derivative was recrystallized from methanol, orange-red needles, m.p. 153–155° (dec.). A mixed m.p. with juglone (m.p. 160–161°) was 130–134°; dried at 60° *in vacuo* for analysis.

Anal. Calcd. for $C_{21}H_{26}O_7N_2S$: C, 55.99; H, 5.82; N, 6.22; S, 7.12. Found: C, 55.90; H, 6.01; N, 6.13; S, 7.35.

In some of the preparations which were made, particularly when incompletely reduced pantethine was the starting material, the separation of the black amorphous by-product was quite troublesome. Dilution of the methanol solution with ether caused the material to separate, but since the desired adduct is very sparingly soluble in ether, some loss may be incurred by this treatment. The derivative is fairly soluble in water and absolute ethanol.

The ultraviolet spectrum in ethanol showed three bands: λ 232 mμ, ε 15,900; λ 307 mμ, ε 6,700; λ 413 mμ, ε 7,100. The derivative was so highly colored that an accurate rotation was impossible; a value of $[\alpha]^{25}D$ +13 ± 3° (*c* 0.466 in abs. EtOH) was obtained.

The microbiological activity of this derivative was somewhat erratic, values of 9,100 to 11,300 LBF units/mg. were obtained. The value was not changed significantly when the derivative was added aseptically to the medium and assayed without autoclaving. This potency of 10,000 units/mg. corresponds to 85% of the pantetheine content.

The yield of this derivative was not improved by using excess juglone; the adduct was more difficult to isolate in this case. Attempts to form similar adducts with 2-methylnaphthoquinone or with benzalacetophenone were unsuccessful.

DETROIT 32, MICHIGAN

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY]

Configuration of Steroid Bromoketones. I. Methyl 4β-Bromo-3-ketocholanate

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The configuration of the bromoketone (II) mentioned in the title was established by sodium borohydride reduction to two bromohydrins identified as *cis* (V) and *trans* (VI) by their respective conversion with base to a ketone (I) and an oxide (X), and by hydrogenation of the *cis*-bromohydrin to the 3β-hydroxy compound (IV). Both bromohydrins and their acetates are convertible in excellent yield to methyl Δ^3-cholenate (IX). The free acid corresponds in three constants with an acid tentatively formulated by Wieland as Δ^2-cholenic acid.

Although the last step in the synthesis of several steroid hormones consists in dehydrobromination of a 4-bromo-3-ketone of the normal or bile acid series, chemical evidence of the configuration of the predominant products of bromination has been lacking.[1] In this investigation of the problem we employed as a model compound the 4-bromo derivative (II) of methyl 3-ketocholanate,[2] available in high yield by oxidation of methyl lithocholate[2] with sodium chromate in acetic acid. The bromo ketone, isolated in 58% yield by crystallization, appeared to be homogeneous when chromatographed and its behavior on dehydrohalogenation conformed to the usual pattern. Thus refluxing in pyridine afforded methyl 3-keto-Δ^4-cholenate[3] (III) in low yield, whereas in the non-stereospecific reaction of Mattox and Kendall[4] it gave the 2,4-dinitrophenylhydrazone of III in high yield.

The plan for determination of the orientation of the bromine atom was to reduce the carbonyl group of the bromo ketone, see if the resulting product behaved as a *cis*- or a *trans*-bromohydrin, and determine the orientation of the hydroxyl group by removal of the bromine substituent. Sodium borohydride seemed a promising reagent for effecting the first step because of Chaikin and Brown's[5] brief mention of the successful reduction of ω-bromoacetophenone and because the reagent reduces 3-ketones of the bile acid series in methanol (absolute) without attack of the ester group in the side chain.[6] Reduction of the 4-bromo derivative of methyl 3-ketocholanate with sodium borohydride in methanol at 25° gave a mixture of 3-epimeric bromohydrins from which one isomer was isolated by direct chromatography; by chromatography after acetylation of the total mixture both epimeric acetates were isolated. Each acetate afforded the corresponding bromohydrin on saponification with methanolic alkali at room temperature and also on methanolysis with boron fluoride as catalyst. The observation that deacetylation by the latter method proceeded notably slower with the more dextrorotatory of the two acetates indicated that this isomer probably has the *cis* orientation of the substituents at 3 and 4. Conclusive evidence that the more hindered, more dextrorotatory bromohydrin is indeed *cis* and the epimer *trans* was found in the behavior of the bromohydrins on dehydrohalogenation: refluxing alcoholic alkali converted the former into the 3-ketone I and the latter into an oxide. The validity of this method of diagnosis was established by Bartlett[7] in the cyclohexane series. Finally, the orientation of the hydroxyl group in the *cis*-bromohydrin was established by debromination. Reduction proceeded smoothly in alcoholic potassium hydroxide solution at room temperature and gave 3β-hydroxycholanic acid,[8,9] identified by comparison (as acid and as ester) with a sample

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