CHEMICAL AND BIOCHEMICAL STUDIES OF THE *LACTOBACILLUS BULGARICUS* FACTOR

by

CHARLES JACK STEWART

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APPROVED:

Redacted for Privacy

Professor of Chemistry

Redacted for Privacy

Assistant Professor of Chemistry

In Charge of Major

Redacted for Privacy

Chairman of Department of Chemistry

Redacted for Privacy

Chairman of School Graduate Committee

Redacted for Privacy

Dean of Graduate School

May 17, 1952

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INTRODUCTION

Early in 1949 Williams *et al.* (15) reported the discovery of an unidentified growth factor for *Lactobacillus bulgaricus* and *Lactobacillus helveticus*, to which they ascribed the name *Lactobacillus bulgaricus* Factor or "LBF". Snell *et al.* (14) later traced the structure of LBF as N-pantothenylmercaptoethylamine. They synthesized it by an ammonolytic condensation between methylpantothenate and 2-aminoethylmercaptan. From its ability to exist as a free thiol or a disulfide the names pantetheine and pantethine were proposed (in conformance with the nomenclature of cysteine and cystine).

Prior enzymatic studies (10; 4) of pantethine had revealed that it contained "bound" pantothenic acid and that it was related to a product formed when coenzyme A is treated with intestinal phosphatase. Craig and Snell (5) have summarized the relationship of coenzyme A and pantethine. Coenzyme A has been found to be a large molecule containing 22-28% bound pantothenic acid (2; 7). It is apparently a dinucleotide which will probably be rather difficult to synthesize by chemical means. Its isolation is also tedious from natural sources. Since this coenzyme has been found to participate in a variety of metabolic

reactions of fundamental importance to life processes, its preparation in liberal quantities is highly desirable. One might hope to obtain a biochemical synthesis where a precursor such as pantethine is added to the basal medium, since LBF has been enzymatically converted to coenzyme A in a yield of 25% in 2 hours by King and Strong (8).

Attempts to prepare a large quantity of pantethine by the method of Snell et al. (14) resulted in variable yields and of inconsistent purity. The method involves a protracted chromatographic separation and its application to large scale synthesis was not considered feasible. Other methods were therefore sought which would produce better yields of pantethine.

Recently, Baddiley and Thain (1) have described methods for the preparation of S-acetyl derivatives of 2-aminoethylmercaptan, including the condensation product with N-acetyl-β-alanine. Because of the known ease of coupling of pantoyl lactone with β-alanine (16), it appeared that the condensation of β-alanine with 2-aminoethylmercaptan might be effected, and that this product might then be combined with pantoyl lactone to produce LBF. The peptide of β-alanine and 2-aminoethylmercaptan is hereafter called "β-aletheine", and its dimeric dehydrogenation product, "β-alethine", from their structural relationships to pantetheine and pantethine.

The method of synthesis for LBF would thus be:

$$C_6H_5CH_2OCOCl + H_2NCH_2CH_2COOH \longrightarrow$$

$$C_6H_5CH_2OCONHCH_2CH_2COOH \xrightarrow{SOCl_2}$$

$$C_6H_5CH_2OCONHCH_2CH_2COCl \xrightarrow{(H_2NCH_2CH_2S-)_2}$$

$$(C_6H_5CH_2OCONHCH_2CH_2CONCH_2CH_2S-)_2 \xrightarrow[NH_3\ (liquid)]{6Na}$$

$$H_2NCH_2CH_2CONHCH_2CH_2SH + \underbrace{CH_2C(CH_3)_2CHOHCO}_{O} \longrightarrow$$

$$CH_2OHC(CH_3)_2CHOHCONHCH_2CH_2CONHCH_2CH_2SH$$

The microbial behavior of β-aletheine as well as the crude pantethine made by the present method was investigated.

EXPERIMENTAL

Chemical:

Carbobenzoxy chloride. The procedure of Bergmann (3) was used.

Carbobenzoxy-β-alanine. The synthesis by Siffered and du Vigneaud (14) was employed with the exception that pure β-alanine rather than succinimide was the starting material.

Carbobenzoxy-β-alanyl chloride. The method of Dyer and Ballard (6) was used to prepare an ether solution of this compound, which was immediately used in the next step of the synthesis.

Carbobenzoxy-β-alethine. Two and six tenths g. of 2-aminoethylmercaptan were dissolved in 40 ml. water and cooled at 0° C. Concentrated sodium hydroxide solution was added until the mixture was alkaline to pH 11-12, then the ether solution of carbobenzoxy-β-alanylchloride, prepared from 10 g. carbobenzoxy-β-alanine, was added in small portions with constant stirring. The pH was maintained at 11 to 12 throughout the reaction. After all the acid chloride had been added, stirring was continued for 15 minutes, then the product which had risen to the surface was removed by filtration, washed with water, and

recrystallized from 95% ethanol. Yield 4.3 g., (45% of theory), melting at 153-167° C. After recrystallization from ethylacetate, the melting point raised to 180.5-181.5° C.[1] Further recrystallization did not change the melting point.

Calculated for $C_{26}H_{34}N_4O_6S_2$: C, 55.50%; H, 6.09%; N, 9.95%.
Found:[2] C, 56.13%; H, 6.26%; N, 9.78%.

The broad melting range of the crude product obtained above suggested that it was a mixture of the thiol and the corresponding disulfide forms. The following revised procedure was therefore developed, which apparently gave principally the disulfide form.

Three and one half grams 2-aminoethylmercaptan were dissolved in 100 ml. water and hydrogen peroxide was added dropwise until a positive nitroprusside test was no longer given. Ice was added directly to the reaction vessel and the solution made alkaline by adding 14 ml. of 2 N sodium hydroxide. The carbobenzoxy-β-alanylchloride was added in small portions with constant stirring. When about one third of the acid chloride had been added, 36 ml. of 2 N sodium hydroxide were added. After all the acid chloride had been introduced, stirring was maintained until the ice in the reaction beaker had melted. The product was

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[1] All melting points were determined on a Fisher-Johns Block.
[2] Samples analyzed by Micro-Tech. Laboratories, Stokie, Ill.

separated by filtration, washed and recrystallized from 95% ethanol. Yield 9.2 g. (73% of theory), melting point 180-182° C.

<u>β-aletheine oxalate</u>. To a three-neck flask placed in a chloroform-dry ice bath and fitted with a soda lime tube, 3 g. of carbobenzoxy-β-alethine were suspended in about 75 ml. of liquid amonia with stirring. Eight hundred seventeen mg. of metallic sodium were added in small pieces until an excess was indicated by the formation of a dark blue color. After the addition of sodium was complete the stirring was continued. After 15 minutes, an amount of ammonium sulfate, 2.34 g., was introduced which was exactly equivalent to the sodium added. The stirring was continued until all the ammonia had boiled off. The solid material was extracted with boiling absolute alcohol. With the removal of sodium sulfate by filtration, a 10% excess of an alcoholic solution of oxalic acid dihydrate (1.5 g.) was added. The solution was then placed in the cold over night for crystalization.

The needle-like crystals were filtered, washed with ether, and dried in a desiccator. Yield 1.86 g. (73% of theory), melting point 121-122° C. with decomposition, rate of heating 1°/min. Analytical data suggested a monohydrate.

Calculated for $C_5H_{12}N_2OS \cdot H_2C_2O_4 \cdot H_2O$: N, 10.93%.

Found: 10.66%.

Pantetheine. The crude β-aletheine used in the following experiments was formed by dissolving the oxalate salt in water, adding an equivalent amount of calcium hydroxide, centrifuging off the precipitate, evaporating to dryness, redissolving the β-aletheine in methanol, and removing the insolubles by filtration and re-evaporating to dryness.

Direct Fusion. Seventy nine mg. of β-aletheine and 70 mg. DL pantoyl lactone were placed in a sealed tube and heated for 2 hours at 92° C. The yield, 4.4%, of pantetheine was determined by microbiological assay.

Coupling in Methanol Solution. One hundred mg. of β-aletheine, 88 mg. of DL pantoyl lactone, and 0.05 ml. of diethylamine dissolved in 0.17 ml. of methanol were refluxed together for 2 hours. The yield, 4.5-15%, of pantetheine was determined by microbiological assay.

Biochemical:

All bacterial cultures were carried on an agar stab, the composition of which is 0.5% glucose, 1% yeast extract, 0.5% peptonized milk, and 2% agar. Inocula were grown at 37° C for 22-24 hours on the basal medium supplemented with 10 mg. yeast extract, 10 mg. liver extract and 2 g. calcium pantothenate. The yeast culture was maintained on

molasses agar slants.

The microbiological assays were conducted as Craig and Snell (5) prescribed with the exception of the yeast assay, which was preformed by the method of Sarett and Cheldelin (12). Bound pantothenate was determined through the use of the chicken liver enzyme procedure of Neilands and Strong (11).

All growth was determined turbidimetrically on a Pfaltz and Bauer fluoro-photometer and expressed in terms of optical density (O.D. = 2 - log. % light transmission).

RESULTS AND DISCUSSION

β-aletheine exhibited a very low response to Sacchromyces cervisiae Lash Miller (Table I). Since the response of 1 mg. of β-aletheine was corresponding to 0.02 g. β-alanine, it was presumably not metabolized as such. This might likely be due to cell impermeability. The small response was evidently due to the partial hydrolysis of the β-aletheine with formation of β-alanine in the heat sterilization. Such a view was supported by the facts shown in Table II. The difference in response, between the sample that was filter sterilized and the one that was autoclaved with the medium, was very pronounced. The growth response which was increased about ten-fold by autoclaving indicated the ease of hydrolysis of this compound.

β-aletheine was not inhibitory to the growth of yeast when β-alanine was used as the limiting growth factor as shown in Table I. This is in agreement with the view that β-aletheine is a moiety of a metabolically active form of pantothenic acid.

Evidence supporting the condensation of pantoyl lactone with β-aletheine in the formation of LBF (although in small yield) was provided by the successful demonstration of "bound" pantothenic acid (II) activity in the crude

Table I

Growth Response of
Saccharomyces cervisiae Lash Miller
to β-aletheine and β-alanine

β-aletheine (mg. per tube)	β-alanine (g. per tube)				
	0	0.2	0.5	2.0	5.0
	Optical Density				
0	0.04	0.12	0.18	0.58	0.75
0.50	0.06	0.13	0.19	0.65	0.77
0.75	0.08	0.14	0.22	0.66	0.80
1.00	0.09	0.16	0.24	0.68	0.83

reaction mixture. The response of the undigested sample to the Lactobacillus arabinosus 17-5 was 5-6% of the total pantothenic acid activity after enzymatic treatment. MacRorie and Williams (9) reported that pantetheine only exhibited a growth response equal to 8% of its pantothenate content for L. arabinosus 17-5. This suggested that the crude preparations derived their growth promoting activity from LBF rather than from free pantothenic acid. Had there been formation of pantothenic acid per se, during the attempted condensation, the percentage of activity before digestion with respect to the total pantothenic acid content after digestion would have been much greater than 8%. This view was further confirmed by the fact that both the preparations were inactive for Lactobacillus fermenti in a pantothenic acid free medium. This organism as reported by Craig and Snell (5) can utilize pantothenic acid but not pantetheine for supporting growth. The results from studies with Lactobacillus acidopholus 832, Lactobacillus delbruckii 72, and Lactobacillus delbruckii A.T.C.C. 3 also showed that the condensation mixture contained LBF. Its content was practically the same as that obtained from the bound pantothenic acid assay.

All the results demonstrated the method of synthesis being possible. Further investigation, however, will be done to improve the yield of the last condensation and to

Table II

Effect of Autoclaving on Growth
Promotion by β-Aletheine

Filter Sterilized		Autoclaved*	
mg. β-aletheine	optical density	mg. β-aletheine	optical density
0	0.04	0	0.01
0.50	0.06	0.62	0.48
1.00	0.09	1.24	0.84

* Autoclaved at 15 p.s.i. for 10 minutes.

facilitate the isolation of the product. Since β-aletheine is unstable at high temperatures the traditional condensation methods used in the preparation of pantothenic acid (16) must be modified. The other steps in the synthesis showed good yields and will be suitable for adaption to any size.

Table III

Release of Bound Pantothenic Acid by Enzymatic Digestion

Sample	Pantothenic Acid per Tube	
	before digestion	after digestion
1 mg. prepared by direct fusion	2.1 mμg.	36 mμg.
1 mg. prepared by coupling in methanol	1.8 mμg.	38 mμg.

SUMMARY

(1) β-aletheine ($H_2NCH_2CH_2CONHCH_2CH_2SH$) is prepared by the condensation of β-alanine with 2-aminoethylmercaptan. It is neither active nor inhibitory for yeast.

(2) The attempt to prepare pantetheine by the condensation of β-aletheine and pantoyl lactone has been made. The microbial studies show that the condensation is possible.

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