Pantothenic Acid Derivatives and Growth of *Acetobacter suboxydans.** (20722)

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Acetobacter suboxydans was the first microorganism(1) to be found responsive to bound forms of pantothenic acid (PA).† The conjugates PAC†(2) and coenzyme A† (Co A)(3,4) were both more active for this organism than is free PA. However, when Co A was filter sterilized, no activity could be observed. This paper will report the behavior of panteth(e)ine† (*Lactobacillus bulgaricus* factor; LBF), LBF-γ-phosphate† and α,γ-diphosphate,† pantothenyl cystine† (PA-cystine) and Co A after heat or filter sterilization, in the growth of this organism.

Experimental. The medium and assay method were the same as previously reported (5), except for the sterilization. Heating of all samples was done by autoclaving at 120° for 10 minutes, whereas filter sterilization was accomplished by use of Seitz or "ultra-fine" sintered glass filters. *A. suboxydans* ATCC strain 621 was used. Co A, approximately 80% pure, was purchased from Pabst Laboratories, Inc. LBF samples, prepared by three different methods, were obtained from 3 laboratories, as follows: sample (a), prepared by condensation of pantothenic azide and aminoethyl mercaptan(6), from Parke, Davis and Co.; sample (b), prepared by the method of Baddiley and Thain(7), from these authors; and sample (c), prepared by the method developed in this laboratory(8). LBF-γ-phosphate was prepared by the method of Baddiley and Thain(9) and furnished by them. Pantothenyl cystine was supplied as the disodium salt by J. F. Cavalla, Parke, Davis and Co., London. Crude LBF-α,γ-diphosphate† was prepared by condensing methyl bis (diphenylphospho)-pantothenate(10) with aminoethyl mercaptan. After reduction, a crude sample of 30-40% purity was obtained by fractionation with $Ba(OH)_2$.

Results. LBF was first tested for its growth-promoting effect in the absence of PA by heat sterilizing the sample with the medium in the usual manner. The results are summarized in Fig. 1-A. It is evident that LBF is more active than free PA. The effect could not be duplicated by supplementing the free vitamin with aminoethyl mercaptan. This fact indicated that the biosynthesis of the amide linkage between PA and aminoethyl mercaptan might be a limiting reaction in the growth of the microorganism. LBF prepared by different methods in different laboratories showed practically the same activity. *LBF-γ-phosphate* has been shown as an intermediate(11) in Co A formation in liver. Accordingly, its stimulatory behavior was tested. When the sample was sterilized along with the medium, as shown in Fig. 1-A, the phosphate was much superior to PA and even slightly more active than Co. A.

However, when LBF-γ-phosphate was filter sterilized or its aqueous solution was heated and then added aseptically to the (heated) growth medium, no activity could be demonstrated (Fig. 1-B). Likewise, *Co A* was inactive after filter sterilization (Fig. 1-C). The activity of LBF was also less after filtering than after heating, whereas PA gave the same response after either treatment (Fig. 1-A).

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† The following abbreviations will be used throughout this paper:

pantothenic acid = PA
" " conjugate = PAC
coenzyme A = Co A
panteth(e)ine = *Lactobacillus bulgaricus* factor = LBF
panteth(e)ine-γ-phosphate = LBF-γ-phosphate
" α, γ-diphosphate = LBF diphosphate
pantothenyl cyst(e)ine = PA-cyst(e)ine

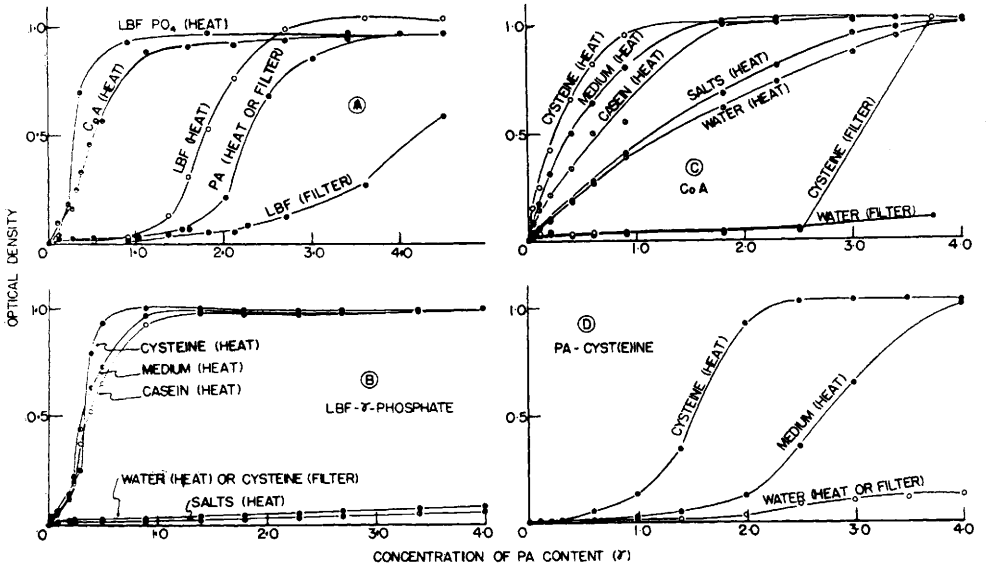

FIG. 1. Growth promoting effects of various bound forms of pantothenic acid (PA†) for *A. suboxydans*. The concentration of each PA derivative is given in terms of its PA content. 1-A: "Heat" = compound autoclaved with basal medium; "filter" = compound sterilized by filtration, added aseptically to heat-sterilized medium. 1-B, 1-C and 1-D: Growth factor mixed with each listed component (concentration equal to that present in medium), heated or filtered as indicated, then combined with remaining ingredients of medium; the latter were heat sterilized. L-Cysteine was added at 2 mg/10 ml without adjustment of amino acids in medium.

The component(s) in the medium responsible for the activation of these compounds were then sought. The results are shown in Fig. 1-B and 1-C. Co A could be partially activated by heating in water and almost fully by heating with casein hydrolysate. High activity could also be produced for LBF-γ-phosphate by heating with casein hydrolysate. When various amino acids were tested for activating ability, it was found that cysteine could replace casein hydrolysate. However, when the samples were filter sterilized with cysteine, the growth stimulation was much lower. Ascorbic acid and thioglycolic acid were much less effective than cysteine, either in a heated or filtered mixture. Hydrogen sulfide-treated Co A or LBF-γ-phosphate were stimulatory, but became less so as complete removal of hydrogen sulfide was achieved. The reactivation of Co A or LBF-γ-phosphate by heating with cysteine (medium autoclaved separately) suggests that only the reduced forms are utilizable. The reduction is obviously more favorable at an elevated temperature. The somewhat greater ease of reactivation of Co A over LBF-γ-phosphate in various heat treatments indicates a difference in their redox potentials, and of activation energies in the reduction. This non-utilizability of the oxidized forms is in line with the fact that a reduced compound, either cysteine or glutathione, is required in all enzymic experiments with Co A.

Recently Brown and Snell(12) reported that PA-cysteine was more active than the free vitamin for *A. suboxydans,* and that the reduced form was also more active than the oxidized. They concluded that PA-cysteine was a precursor of LBF and Co A. It was therefore of interest to compare whether PA-cyst(e)ine showed the same trend of behavior as LBF-γ-phosphate and Co A under various conditions of sterilization. The results, which are summarized in Fig. 1-D, indicate a pattern similar to those of Co A and LBF-γ-phosphate, although with PA-cystine the basal medium was much less effective than cysteine as an activating agent. This may have been due either to the difference of redox potentials of PA-cystine, LBF-γ-phosphate and Co A, or to insufficient reducing agent in the medium. PA-cysteine was less active for growth than

LBF-γ-phosphate in these experiments.

Other derivatives. Pantothenic acid phosphates(13,14) are inactive for *A. suboxydans*, possibly due to cell impermeability. The phosphates of LBF, on the other hand, appear to be freely permeable in view of the foregoing experiments with LBF-γ-phosphate and with *LBF-α,γ-diphosphate*. The latter compound was found in a heated medium (data not shown) to possess 30 to 50% of the equivalent activity of pantothenic acid, at concentrations of the diphosphate ranging from 20 to 120 γ of the preparation per 10 ml culture.

The order of effectiveness of PA derivatives for *A. suboxydans* growth is thus, roughly, Co A = LBF-γ-phosphate >LBF = PA-cysteine (approx.) >PA = pantoic acid(15) >LBF-diphosphate >PA-α- or γ-phosphate (→0), with Co A and LBF-γ-phosphate about 10 to 20 times as active as PA. The parallelism between activity and increasing structural complexity of the PA derivative, up to the level of LBF-γ-phosphate, points to a restricted synthetic ability of the organism for 1) the amide linkage between aminoethyl mercaptan with the carboxyl group of PA, and for 2) the phosphorylation of the γ-hydroxy group. If the assumption is valid that Co A is the only physiologically active form of the vitamin, the syntheses of the other moieties of Co A from LBF-γ-phosphate are apparently accomplished easily in this organism.

Summary. 1. Various derivatives of pantothenic acid have been compared for growth promoting activity in *A. suboxydans*. The order of effectiveness is roughly Co A† = LBF-γ-phosphate >LBF = PA-cysteine (approx.) >PA = pantoic acid >LBF-diphosphate >PA phosphates, with Co A and LBF-γ-phosphate about 10 to 20 times as active as PA. The derivatives containing –SH groups appear to be active principally, or even exclusively, in the reduced state. 2. It is concluded that the "*A. suboxydans* stimulatory factor" is multiple in nature, but is not identical with pantothenic acid phosphates.

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