

PANTOTHENIC ACID CONJUGATES AND GROWTH OF ACETOBACTER SUBOXYDANS¹

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Underkofler *et al.* (1943) first reported that *Acetobacter suboxydans* required pantothenic acid (III, figure 1) or pantolactone (I) for growth. Pantoic acid (II) was reported subsequently (Sarett and Cheldelin, 1945) to be 4 to 5 times more active on the weight basis than its lactone and to equal pantothenic acid in activity.

Attempts to apply the acetobacter test as an assay method to natural materials showed these to contain a conjugate of pantothenic acid (PAC) that was considerably more active in promoting growth than would have been predicted from its pantothenate content, and which had no coenzyme A activity (King *et al.*, 1948, 1949). Novelli *et al.* (1949) reported that coenzyme A (CoA, VIII) and a coenzymatically inactive phosphorylated product formed from it by action of a pigeon liver enzyme had activity in the acetobacter tests similar to that of pantothenic acid conjugate, thus clarifying in part the nature of the latter product. Inactivation of coenzyme A with alkaline phosphatase yields pantetheine (VI) or related disulfides (Brown *et al.*, 1950; Brown and Snell, 1951, 1952); the corresponding disulfide, pantethine, greatly surpasses pantothenate in activity for many lactic acid bacteria (Snell *et al.*, 1950; Craig and Snell, 1951; Snell and Brown, 1953) but was reported without details to be no more active than pantothenate for *A. suboxydans* (Novelli *et al.*, 1949; Novelli, 1952, 1953a).

Of other known cleavage products of coenzyme A, 4'-phosphopantetheine (VII) is reported without details (Baddiley *et al.*, 1953; Baddiley and Thain, 1953) to possess high acetobacter activity. This product or its disulfide appears identical with that formed from coenzyme A by action of a potato dinucleotidase, and was reported earlier (Novelli *et al.*, 1950; Novelli,

1952, 1953a) to equal or surpass coenzyme A in activity for *A. suboxydans*, and to be about three times as active as the dephosphorylated compound, pantethine. Removal of the β -mercaptoethylamine residue from 4'-phosphopantetheine yields 4'-phosphopantothenic acid (IV); synthetic samples of the latter have no activity for *A. suboxydans* (King and Strong, 1951a,b; Baddiley and Thain, 1951a,b), a finding that leaves unexplained the earlier report (Novelli *et al.*, 1949) that coenzyme A treated with pigeon liver enzyme (a treatment which should remove the β -mercaptoethylamine residue (Brown *et al.*, 1950; Novelli, 1952)) retained high activity for *A. suboxydans*.

Most of the reports dealing with the activity of coenzyme A fragments for this organism contain no experimental details. A reassessment of their activities therefore appears desirable, especially in view of the finding (Brown and Snell, 1953) that pantothenylcystine, the disulfide of pantothenylcysteine (V), serves as a precursor of pantetheine and possesses high activity for *A. suboxydans* that is increased greatly by addition of sulfhydryl compounds to the medium. This suggests that activity of these sulfur containing compounds may vary greatly with the proportion present in the reduced ($-SH$) form, a suggestion amply confirmed by findings reported below.²

MATERIALS AND METHODS

Strain 621 (ATCC) of *Acetobacter suboxydans* and the pantothenate-free medium of Underkofler *et al.* (1943) were used. Samples for testing were added to 5 ml of the double-strength medium in 25 by 200 mm pyrex culture tubes and diluted to 10 ml with distilled water. Sterilization was by

² Since our initial report (Brown and Snell, 1953), a preliminary abstract by King and Cheldelin (1953) also has indicated that the activity of pantethine and related compounds for *Acetobacter suboxydans* varies with the technique of testing.

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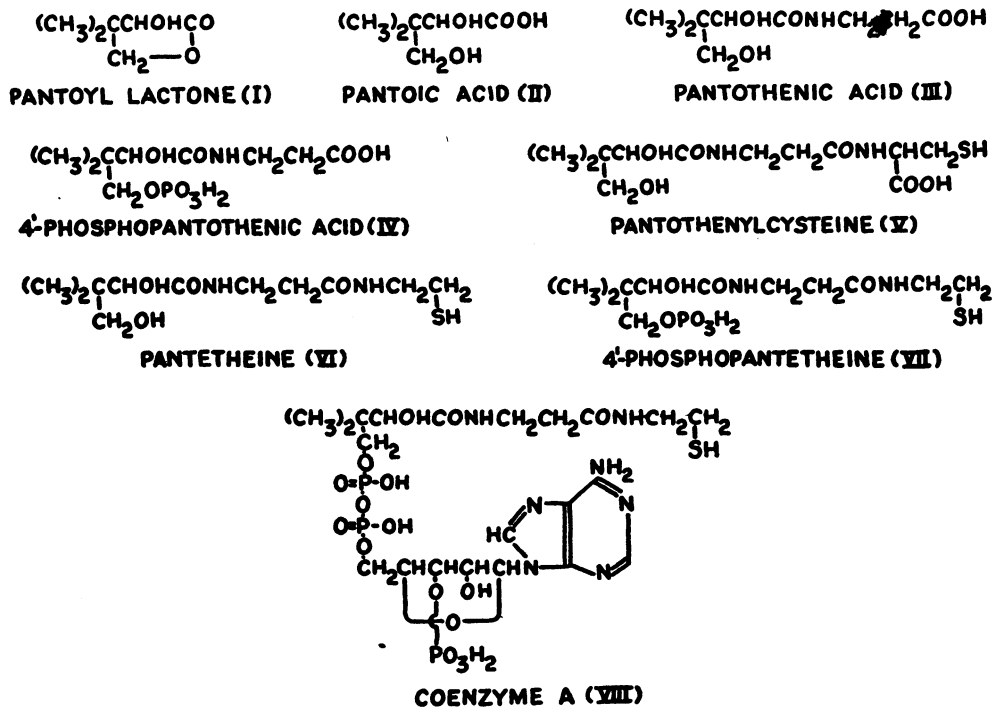


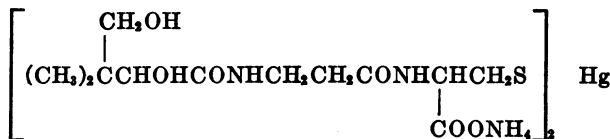
Figure 1. Pantoic acid and its derivatives.

autoclaving at 15 lb pressure for 10 minutes. Samples tested without autoclaving were filtered through UF bacterial sintered-glass filters and added aseptically to the previously autoclaved medium.

Cells for inocula were grown in 50 ml Erlenmeyer flasks containing 10 ml of the basal medium supplemented with 5 micrograms of calcium pantothenate. After 40 hours' incubation at 30 C on the rotary shaker, the culture was centrifuged, the cells washed once with sterile distilled water, and resuspended in 10 ml of sterile water. One drop of this suspension was used for each assay tube. The tubes were incubated in a slanted position on a reciprocal shaker for 40 to 48 hours at 30 C; growth was estimated then turbidimetrically with the Evelyn colorimeter. Under our conditions, consistent results were obtained if inocula no older than 48 hours were used, and assays were incubated for at least 40 hours but no longer than 48 hours.

D-Pantoylactone, a gift from Merck and Co., Inc., was converted to sodium D-pantoate without racemization by allowing it to stand with 0.1 N NaOH for one hour at room temperature (Stansly

and Schlosser, 1945). Pantetheine and the mercuric mercaptide of pantetheine were synthetic products described by Wittle *et al.* (1953). Pantothenylcysteine was kindly supplied by Drs. J. F. Cavalla and R. E. Bowman of Parke, Davis and Co., Ltd., Hounslow, England. The sample upon acid hydrolysis yielded the theoretical amount of β -alanine. Pantothenylcysteine was prepared by reduction of the sodium salt of pantothenylcysteine with sodium in liquid ammonia by the procedure previously used to prepare pantetheine from pantethine (Wittle *et al.*, 1953). The diammonium salt of pantothenylcysteine thus obtained was converted to its mercuric mercaptide by treating it in 80 per cent ethanol with an excess of mercuric oxide, filtering, and concentrating the filtrate to dryness in vacuum. The residue was dissolved in a minimum of methanol and poured into 100 volumes of acetone. The white precipitate was dried at room temperature over P_2O_5 to a hygroscopic white powder. Analytical data indicated that this compound was the diammonium salt of the mercuric dimercaptide of pantothenylcysteine:



Analysis. Calculated percentages for $\text{C}_{24}\text{H}_{48}\text{O}_{12}\text{N}_6\text{S}_2\text{Hg}$: total N, 9.59; ammonia N, 3.20; β -alanine after acid hydrolysis, 21.5. Found percentages: total N, 9.02, ammonia N, 3.20; β -alanine, 20.1.

The β -alanine was determined microbiologically with *Saccharomyces carlsbergensis*. Ammonia nitrogen was determined colorimetrically by direct nesslerization. Total nitrogen was by the Dumas method.

4'-Phosphopantethine (barium salt), prepared by Baddiley and Thain (1953), and samples 1 and 2 of coenzyme A were gifts from Dr. G. D. Novelli; coenzyme A samples 3 and 4 were commercially available products obtained from Pabst Laboratories and Armour and Co., respectively.

Each of the active compounds contains a pantoic acid residue; activities are compared therefore on the basis of the equivalents of pantoic acid added.

RESULTS

Effect of reduction on the activity of sulfur compounds. The activities of both pantethine and pantothenylcystine are increased greatly by autoclaving with the medium (figure 2). The latter compound is essentially inactive unless autoclaved. Similarly, 4'-phosphopantethine and coenzyme A are less active when filter sterilized than when autoclaved with the medium; all samples of these two compounds tested, however, showed some growth promoting activity when filter sterilized.

The simplest explanation of these activity changes is to assume that the $-\text{SH}$ forms of these compounds are utilized more readily than the $-\text{S}-\text{S}-$ forms and that partial reduction of the latter is effected by autoclaving with the medium. To test this assumption, alternative methods of reduction were employed. After standing briefly with a 400-fold excess of cysteine or of sodium sulfide, solutions of pantothenylcystine were sterilized by filtration and added aseptically to the previously autoclaved medium. Results (figure 3) showed great increases in activity, the sodium sulfide treatment being especially effective. Several other reducing

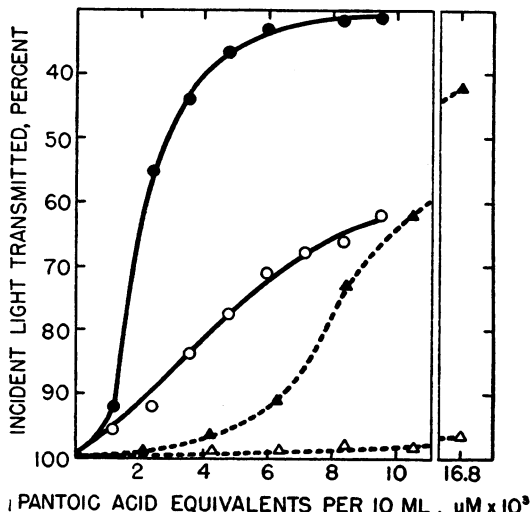


Figure 2. The comparative activities of pantethine (●, ○) and pantothenylcystine (▲, △) in promoting growth of *Acetobacter suboxydans* when autoclaved with the medium (●, ▲) or when filter sterilized and added aseptically (○, △).

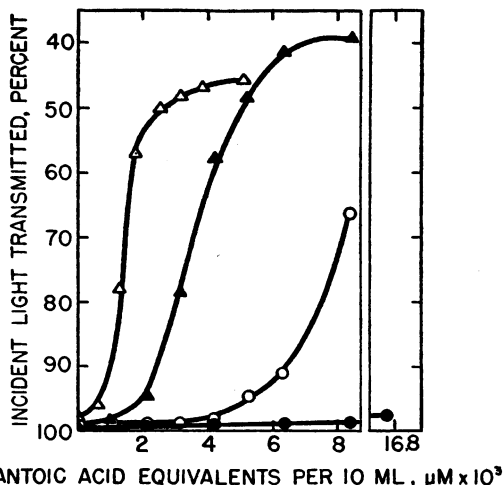


Figure 3. The effect of reduction on the activity of pantothenylcystine. All samples were sterilized by filtration and added aseptically to the medium. ●, Pantothenylcystine; ○, Pantothenylcystine + cysteine; ▲, Pantothenylcystine + $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$; △, Mercuric mercaptide of pantothenylcystine.

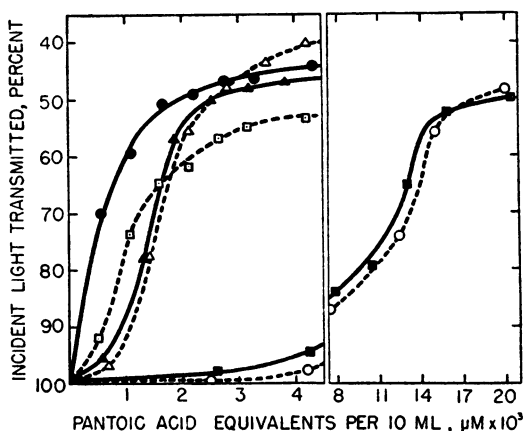


Figure 4. The comparative activities of pantoic acid conjugates in supporting growth of *Acetobacter suboxydans* under conditions such that each compound shows the maximum activity observed.

- , Pantoic acid, autoclaved with medium;
- , Pantothenic acid, autoclaved with medium;
- △, Mercuric mercaptide of pantetheine, filter sterilized and added to previously autoclaved medium;
- ▲, Mercuric mercaptide of pantothenylcysteine, filter sterilized and added to previously autoclaved medium;
- , 4'-Phosphopantetheine + Na₂S, autoclaved with medium;
- , Coenzyme A (Pabst) + Na₂S, filter sterilized and added to previously autoclaved medium.

agents were used in a similar fashion. In order of effectiveness they were Na₂S > β-mercaptoethylamine > cysteine = thiomalic acid = thioglycolic acid > ascorbic acid. The activity of unheated pantothenylcysteine treated with reducing agents in this fashion frequently surpassed that following autoclaving with the medium (*cf* figures 2 and 3); the combined treatment was usually somewhat more effective than either separately.

Because aeration of the medium is necessary for rapid and uniform growth, the fraction of the added growth factor actually available as —SH compound is uncertain. To minimize this difficulty, the mercuric mercaptide of pantothenylcysteine, in which the —SH group is

³ In the amounts necessary for growth, these mercuric compounds do not supply toxic amounts of mercury.

TABLE 1

The comparative activities of various preparations of coenzyme A for *Acetobacter suboxydans* before and after treatment with sodium sulfide

COENZYME SAMPLE	LIPMANN UNITS PER MG	CALCULATED PURITY*	PANTOIC ACID EQUIVALENTS (μM × 10 ³) PER 10 ML REQUIRED FOR HALF-MAXIMUM GROWTH*	
			Untreated†	After Na ₂ S‡
1	170	41.0	5.0	1.20
2	184	44.5	1.8	1.17
3	300	73.0	1.6	1.15
4	13	3.1	1.2	1.15

* Calculated on the assumption that pure coenzyme A contains 413 units per mg and 28.9 per cent of pantothenic acid (Gregory *et al.*, 1952).

† Filtered and added aseptically to previously autoclaved medium.

‡ Autoclaved with medium.

protected from oxidation, was supplied (figure 3) and proved highly active³; furthermore, its activity was not increased by autoclaving with the medium or by treatment with reducing agents. The failure of cysteine and sodium sulfide treated samples to equal the activity of the mercuric mercaptide is undoubtedly due to incomplete reduction or, once reduced, to the difficulty of keeping the compound in the reduced state during the incubation period.

Experiments conducted with pantetheine gave entirely similar results. Its activity was increased greatly by the addition of reducing agents, but the mercuric mercaptide of pantetheine exhibited the greatest activity which equaled almost exactly that of the mercuric mercaptide of pantothenylcysteine (figure 4). The mercaptides of 4'-phosphopantetheine and of coenzyme A were not available; however, treatment with sodium sulfide considerably increased the activity of each of these compounds, indicating that *A. suboxydans* utilizes their —SH forms more effectively than their —SS— forms. The comparative activities of the available compounds under conditions such that each shows the maximum activity observed are shown in figure 4. The activities of pantothenic acid and of pantoic acid were unchanged by autoclaving with the medium, by reducing agents, or by addition of up to 5×10^{-3} μM of Hg⁺⁺ per 10 ml, thus confirming the interpretation that the

TABLE 2

The comparative activities of various compounds in supporting growth of *Acetobacter suboxydans*

COMPOUND	PANTOIC ACID EQUIVALENTS ($\mu\text{M} \times 10^3$) PER 10 ML. REQUIRED FOR HALF-MAXIMUM GROWTH		COMPARATIVE ACTIVITIES
	Before reduction*	Na_2S treated†	
D-Pantoyllactone.....	> 120		0
D-Pantoic acid.....	13		1.0
D-Pantothenic acid.....	12.6		1.0
Pantethine.....	4.5	2.3	
Pantetheine Hg mercaptide.....	1.8		7.2
Pantothencycystine.....	120	2.8	
Pantothencycystine Hg mercaptide.....	1.7		7.6
4'-Phosphopantethine...	4.7	1.1	11.8
Coenzyme A.....	2.4	1.2	10.8

* Sterilized by filtration and added aseptically to the previously autoclaved medium.

† Autoclaved with medium.

observed changes with the other compounds are due to the $-\text{S}-\text{S}- \rightarrow -\text{SH}$ transformation.

In contrast to other compounds of figure 4, coenzyme A is not pure, and various preparations were compared therefore on the basis of their enzymatic standardization (table 1). Different preparations showed significantly different activities before reduction but were equally active following treatment with sodium sulfide. The result is consistent with the presence of variable $-\text{SH}$ to $-\text{S}-\text{S}-$ ratios in the various preparations and emphasizes once again the higher availability of the $-\text{SH}$ form of the coenzyme for growth.

A summary of the amounts of the various compounds required before and after reduction with sodium sulfide to permit half-maximum growth of *A. suboxydans* is given in table 2. These amounts vary slightly from one assay to another; the figure given for coenzyme A is an average of the various preparations tested. Filter sterilized pantoyllactone was inactive even at high levels. Previous reports (Underkofler *et al.*, 1943; Sarett and Cheldelin, 1945) attributing activity to this compound undoubtedly result from its partial conversion to pantoic acid during heat sterilization with the medium.

DISCUSSION

Pantethine and a series of mixed disulfides of pantetheine were found equally active on the molar basis in supporting growth of *Lactobacillus helveticus*, strain 80 (Brown and Snell, 1951, 1952). Because of this, it was postulated that the actual growth factor was the $-\text{SH}$ compound, pantetheine, formed by reduction of whatever disulfide was furnished. This view is confirmed by the results with *Acetobacter* species. Unlike *L. helveticus*, this organism appears to have little ability to reduce the disulfide linkage of these compounds, and, consequently, pantethine and pantothencycystine have very much lower activities than the reduced compounds, pantetheine and pantothencycystine. However, pantethine supplied entirely as the disulfide has higher activity than pantothencycystine, an indication that cellular reduction of the former, even though slow and incomplete, takes place to a greater extent than does reduction of the latter. Pantothencycystine also appears more difficult to reduce than pantethine by chemical procedures since it is less active following autoclaving (figure 2) or treatment with Na_2S (table 2) although the completely reduced mercaptides are equally active (figure 4). Since active reduction by the growing cells and chemical reduction through interaction with ingredients of the medium (especially during autoclaving) can occur, it may be expected that the activities of these several compounds in their disulfide forms will vary not only with the strain of acetobacter tested but also with the basal medium used, the incubation time, etc.

It is clear from these results that the substances present in natural materials that have activity greater than pantothenic acid for *A. suboxydans* include coenzyme A and its several pantetheine containing degradation products down to and including pantetheine itself. To the extent that it occurs naturally, pantothencycystine, which has been shown to be a precursor of pantetheine (Brown and Snell, 1953), also contributes to this activity. The metabolic defect in *A. suboxydans* responsible for the decreased activity of pantothenate, as compared with these other compounds, appears most likely to be a decreased activity of the enzyme system that condenses pantothenate with cysteine to form pantothencycystine (Brown and Snell, 1953) although a lowered capacity to concen-

trate pantothenate from the medium has not been excluded. All of the compounds showing this enhanced activity for *A. suboxydans* contain potential —SH groups and hence can form mixed disulfides with other thiols, e.g., glutathione (cf Brown and Snell, 1951, 1952). It appears probable that the occurrence of such mixed disulfides explains the reported occurrence of large amounts of glutamic acid in concentrates of pantothenic acid conjugate (King *et al.*, 1949).

Previous reports (cf Novelli, 1953a; Baddiley and Thain, 1953) that 4'-phosphopantetheine but not pantetheine had high acetobacter activity, coupled with the finding of high acetobacter activity in pantothenylcystine, led to the suggestion (Brown and Snell, 1953) that during synthesis of coenzyme A, pantothenylcystine might be phosphorylated and decarboxylated directly to phosphopantetheine, thus by-passing pantetheine as a direct intermediate. The current finding that pantetheine and pantothenylcystine have equal activities for this organism removes the basis for this suggestion. Activities of the several products found herein are consistent with the view that biosynthesis of coenzyme A occurs by the reactions: (1) Pantoic acid (II) + β -alanine \rightarrow Pantothenic acid (III). (2) Pantothenic acid + cysteine \rightarrow Pantothenylcystine (V). (3) Pantothenylcystine \rightarrow Pantetheine (VI). (4) Pantetheine \rightarrow Coenzyme A (VIII). Reaction (1) has been studied in cell-free systems by Maas (1952); reactions (2) and (3) by Brown and Snell (1953), and the several reactions included in (4) by Novelli and co-workers (Novelli, 1953b).

SUMMARY

The activity of pantothenylcystine and pantetheine but not that of pantothenic or pantoic acids in supporting growth of *Acetobacter suboxydans*, strain 621, in pantothenic acid-free medium is increased greatly by autoclaving with the medium or by treatment with reducing agents. The maximum observed activity is displayed by the mercuric mercaptides of pantothenylcystine and of pantetheine. It is concluded that only the —SH compounds, and not the —S—S— compounds, serve as growth factors. The activity of 4'-phosphopantetheine and of coenzyme A also is increased greatly by reducing agents; under optimal conditions coenzyme A, 4'-phosphopantetheine, pante-

theine, and pantothenylcystine show similar activities for this organism that are 7 to 12 times those of pantothenic or pantoic acids.

The activities are thus in accord with the view that biosynthesis of coenzyme A proceeds successively through pantoate, pantothenate, pantothenylcystine, pantetheine, and 4'-phosphopantetheine; all of the intermediates from pantothenylcystine on show high activity for *A. suboxydans* when supplied in the reduced form or as appropriate disulfides that can be reduced by the organism. It is concluded that the *A. suboxydans* stimulatory factor of natural materials (pantothenic acid conjugate) is a mixture of such compounds.

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