GROWTH FACTORS FOR BACTERIA

VIII. Pantothenic and Nicotinic Acids as Essential Growth Factors for Lactic and Propionic Acid Bacteria[1]

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The preparation of highly active concentrates of a substance essential for the growth of all lactic acid bacteria tested was described by Snell, Strong and Peterson (1937). Further attempts to purify this substance are reported below, together with results which show that the active substance is pantothenic acid (Williams (1938a), cf. Snell *et al.* (1938a)). Nicotinic acid is also shown to be essential for some species of lactic acid bacteria.

EXPERIMENTAL

Methods

The basal media A and B, the cultures used, and the details of carrying out the fermentations have been previously described (Snell *et al.* (1937)). Medium A must be supplemented with tryptophane, and B with riboflavin, for use with organisms which require these substances (Snell and Strong (1938b)).

Bacterial response has been determined in the present work by measuring acid production or in some cases by measuring relative turbidity by the photoelectric colorimeter (Evelyn (1936)). Light transmission of the uninoculated medium was adjusted to 100, and the per cent of this transmitted by the inoculated tubes after 24 to 48 hours incubation was read directly from the gal-

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

vanometer scale. Overhead lights must be off while such readings are being made to prevent reflection of stray light into the photoelectric cell by the turbid suspension. The 540 mμ filter was used with medium B because at this wave-length light absorption of the medium was only slightly greater than that of distilled water, and turbidities could thus be measured with less interference.

Identity of pantothenic acid with the growth factor for lactic acid bacteria

Through the kindness of Professor R. J. Williams it was possible to compare two preparations of pantothenic acid with our liver concentrates. These samples, designated I and II, contained 40 and 83 per cent respectively of calcium pantothenate. Sample I replaced the standard liver extract (Snell *et al.* (1937)), and contained one unit[2] of activity in approximately 0.15 microgram (table 1). The activity of sample I, like that of our liver concentrate, was destroyed by heating in 0.05N NaOH at 100° for one hour.

The effects of I and II in promoting growth and acid production by *Lactobacillus casei* are compared in table 2. The activity of II was almost exactly twice that of I. The latter was easily detectable at 0.001 microgram per ml. One unit of pure pantothenic acid would correspond to approximately 0.06 microgram.

The action of I and II on nine other species of lactic acid bacteria, all of which require our growth factor, is shown in table 3. Sample II completely replaced the liver fractions except for *Lactobacillus delbrückii* and *Lactobacillus mannitopeous*. Some activity was evident in these cases, but growth did not take place on subculture into the same medium, even though a large excess of I (100 micrograms per 10 ml.) was added. Evidently some other growth factor is required by these two species. All of the other species could be subcultured repeatedly in the medium

[2] One unit of activity is defined as that weight of material which must be added to 10 ml. of medium to produce a response equal to that given by 0.05 mgm. of a standard liver preparation. With *L. casei*, 1 unit of the growth factor gives approximately ½ maximum fermentation under the conditions used.

supplemented with I; no growth occurred in its absence. The behaviour of *L. delbrückii* lessened its utility for assays, and *L. casei* was substituted for it in later work. Table 3 again shows sample II to be approximately twice as active as sample I.

The similarity between chemical properties of our growth factor

TABLE 1

*Comparison of activity of 40 per cent calcium pantothenate (sample I) with standard liver preparation**

STANDARD LIVER PREPARATION		40 PER CENT CALCIUM PANTOTHENATE (SAMPLE I)			
		Untreated		Alkali Treated	
Amount added per 10 ml.	Incident light transmitted	Amount added per 10 ml.	Incident light transmitted	Amount added per 10 ml.	Incident light transmitted
micrograms	*per cent*	*micrograms*	*per cent*	*micrograms*	*per cent*
0	93	0	91	0	93
30†	71	0.05	86	1.0	93
60	62	0.10	79	3.0	92
150	46	0.30	52	5.0	93
		0.50	44	10.0	94
		1.0	40		
		5.0	41		

* *Lactobacillus casei* in Medium B. Incubation time, 30 hours at 37°C.
† One unit.

TABLE 2

*Quantitative comparison of the activity of samples I and II in promoting growth and acid production of Lactobacillus casei**

40 PER CENT CALCIUM PANTOTHENATE (I)			83 PER CENT CALCIUM PANTOTHENATE (II)		
Amount added per 10 ml.	Acid produced per 10 ml.	Visible growth†	Amount added per 10 ml.	Acid produced per 10 ml.	Visible growth
micrograms	*cc. 0. 1 N*		*micrograms*	*cc. 0. 1 N*	
0.00	0.5	−	0.00	0.5	−
0.05	1.2	+	0.025	1.6	+
0.10	3.5	++	0.05	3.3	++
0.30	5.6	+++	0.15	5.8	+++
0.50	6.8	++++	0.25	7.2	++++

* Medium B. Incubation time, 4 days at 37°C.
† − indicates no visible growth; + slight growth; ++ fair growth; +++ heavy growth; ++++ very heavy growth (with sediment).

TABLE 3

*Effect of pantothenic acid concentrates on other species of lactic acid bacteria**

ORGANISM	SUPPLEMENT TO MEDIUM					ACTIVITY							
	Sample	Micrograms per 10 ml.				cc. 0.1 N acid per 10 ml. medium				Visible growth†			
		a	b	c	d	a	b	c	d	a	b	c	d
Lactobacillus arabinosus	I	0	0.3	0.5	1.0	0.8	7.7	8.4	8.4	−	++++	++++	++++
	II	0.05	0.1	0.3	0.5	5.2	6.3	8.5	8.4	+++	++++	++++	++++
Lactobacillus delbrückii	II	0	0.1	0.3	0.5	1.2	2.4	3.4	2.5	+	++	++	++
Lactobacillus mannitopoeus	II	0	0.1	0.3	0.5	0.3	0.5	0.9	1.2	−	−	+	+
Lactobacillus pentosus	II	0	0.1	0.3	0.5	1.2	4.2	7.6	8.3	+	+++	++++	++++
Bacillus brassicae	I	0	0.3	0.5	1.0	0.6	7.4	8.5	8.4	−	++++	++++	++++
	II	0.05	0.1	0.3	0.5	5.9	6.7	8.4	8.4	+++	++++	++++	++++
Bacillus lactis-acidi	I	0	0.3	0.5	1.0	0.8	3.6	4.9	6.8	−	+++	+++	++++
	II	0.05	0.1	0.3	0.5	2.1	3.3	4.8	5.3	++	+++	+++	++++
Leuconostoc mesenteroides	II	0	0.1	0.3	0.5	0.9	2.0	3.4	4.3	+	+++	++++	++++
Streptococcus lactis	II	0	0.1	0.3	0.5	0.3	1.5	2.5	2.7	−	++	+++	+++
Propionibacterium pentosaceum	II	0	0.1	0.3	0.5	0.3	0.9	1.9	2.2	−	++	++++	++++

* Medium A.
Incubation time, 4 days at 37 or 28°C.
† As in table 2.

and those of crude pantothenic acid has been borne out by recent reports of Williams and co-workers (1938a, b). Both substances are inactivated by alkali or acid, by acetylation or esterification, and both are comparatively resistant to oxidation by hydrogen peroxide. Further evidence for the identity of the two consists in the partial synthesis of pantothenic acid described below. The active substance in the liver preparations thus appears to be identical with pantothenic acid, and will be so designated.

TABLE 4

Stability of pantothenic acid in the alkaline range

pH	TIME OF TREATMENT*		ACTIVITY
	At room temperature	At 100°C.	
	hours	*hours*	*micrograms per unit*
7.0	0	0	1
7.0	22	2	1
8.0	22	2	1
9.0	22	2	1
10.0	5	0	1
	24	0	1
	4	1	1.3
	22	2	1
11.0	5	0	1
	24	0	1
	4	1	3.5
	22	2	5.7

* Samples were held first at room temperature, then at 100° for the time indicated.

A modified procedure for preparing pantothenic acid concentrates

Assays for pantothenic acid have been made on medium B with *L. casei* because this organism grows well on repeated subculture in medium B plus pantothenic acid, and thus obviates complications arising from lack of unrecognized nutritive factors.

To determine more accurately the alkali stability of the active substance, a liver preparation containing one unit of activity per microgram (approximately six per cent pantothenic acid) was treated with various buffers as indicated in table 4. The activity was retained at pH 11 at room temperature for 24 hours, but was diminished at pH 10 when the mixture was heated to 100°.

Almost none of the active substance was adsorbed from liver extracts by an equal weight of norite at pH 9, although the solution was almost entirely decolorized. At pH 3 or 4 adsorption was almost quantitative. On the basis of these results a modified procedure, avoiding use of lead acetate, was developed.

One hundred fifty grams of the alcohol-soluble liver fraction[3] (1 unit in 0.05 mgm.) was dissolved in 500 ml. of water, filtered, and diluted to seven liters. NaOH was added to pH 9, 120 grams of Pfansteihl Norit A added, the mixture stirred for one hour, and filtered. The filtrate was adjusted to pH 3.8 with H_2SO_4, 95 grams of norite added and the mixture again stirred for one hour. The norite was filtered off, washed once in water, then eluted three times by stirring for 15 minutes with 500-ml. portions of a pyridine-ethanol-water mixture (1:1:2). The solvents were removed below 40° by distillation under reduced pressure. The eluate (about 18 to 20 grams) was dissolved in 100 ml. of water, adjusted to pH 6.3 with NaOH, and extracted continuously with ether for two days. The inactive extract was discarded, the pH of the residue adjusted to 1.0 with H_2SO_4, and ether extraction continued for 48 hours. The extract, approximately 3.3 grams., contained one unit of activity in 0.8 microgram (approximately 6.5 per cent pantothenic acid), which accounts for about 90 per cent of the original activity. Attempts to carry out this procedure on kilogram batches of material resulted in much poorer yields, probably because of destruction during ether extraction.

The ether extract may be further purified by reëxtraction with ether, or by salt fractionation.

Fractionation of the sodium salts of the ether extract

To 1.3 grams of an ether extract in water solution, NaOH was added to pH 7.5. The solution was concentrated to dryness, and the sodium salts were extracted with two 25 ml. portions of absolute alcohol, which removed 1.02 grams. To 200 mgm. of this preparation dissolved in 15 ml. of absolute alcohol, was added

[3] That portion of an aqueous extract of liver soluble in 92 per cent alcohol. The authors wish to thank Dr. David Klein of Wilson and Company, Chicago, for supplying this material.

15 ml. of acetone, and the resulting precipitate (A) centrifuged out. The supernatant liquid was concentrated to dryness, the residue dissolved in 7 ml. of absolute alcohol, and 18 ml. of acetone added. The precipitate (B) was removed, the supernatant liquid concentrated to dryness, the residue dissolved in 5 ml. absolute alcohol, and 50 ml. of acetone added. The precipitate (C) was centrifuged out, and the mother liquors concentrated to dryness (D).

	WEIGHT	ONE UNIT	TOTAL UNITS	ACTIVITY RECOVERED
	mgm.	*micrograms*		*per cent*
Starting material	200	0.83	241,000	
A	32.6	3.0	10,900	4.5
B	40.0	0.9	44,400	18.4
C	45.0	0.4	112,500	46.7
D	67.0	1.5	44,700	18.6

Eighty-eight per cent of the original activity was recovered in the four fractions. The most active fraction was not precipitated from absolute alcohol by 72 per cent acetone, but was precipitated by 91 per cent acetone.

Fractionation of the barium salts of the ether extract

To a solution of 2.65 grams of ether extract (1 unit in 2.0 micrograms; 3.1 per cent pantothenic acid) $Ba(OH)_2$ was added to pH 7.5, the solution concentrated to 25 ml., and 300 ml. of alcohol added slowly with stirring. The insoluble material was centrifuged out, washed with alcohol, and discarded. The supernatant liquid and washings were concentrated to dryness, the residue was dissolved in 10 ml. of water and barium removed exactly by adding H_2SO_4 and centrifuging. The solution of free acids was concentrated to dryness under reduced pressure and gave 259.2 mgm. of a light yellow syrup, which contained one unit of activity in 0.25 microgram (25 per cent pantothenic acid). An almost 10-fold concentration and 78 per cent recovery of activity was obtained. This procedure regularly gave concentrates containing 10 to 15 per cent of pantothenic acid, and often

as above, better. This fraction represents the purest preparation of pantothenic acid obtained in this work.

Organic derivatives of pantothenic acid

(*a*) *Acetyl derivatives.* To 78.8 mgm. of ether extract was added 0.5 ml. acetic anhydride and 1 ml. of dry pyridine. The mixture was heated one hour at 100°, and evaporated to dryness under reduced pressure. The residue weighed 99.2 mgm. and was completely inactive. The acetyl derivative was hydrolyzed by heating in a sealed tube at 100° for one hour with a methyl alcohol solution of NH_3 (saturated at 0°). Some of the unacetylated material was treated in the same way as a control.

	WEIGHT	ONE UNIT	TOTAL UNITS	ACTIVITY RECOVERED
	mgm.	*micrograms*		*per cent*
Starting material	77.8	1.2	65,600	
Acetylated material	99.2	Inactive		
Hydrolyzed acetylated material		1.4*	70,800	107
Hydrolyzed starting material	78.8	1.4	56,200	86

* Expressed in terms of acetylated material.

Although ether solubility was greatly increased by acetylation, no fractionation was effected by use of this property.

(*b*) *Methyl ester.* 110 mgm. of ether extract were dissolved in 5 ml. of methanol, cooled to 0° and an excess of diazomethane in ether added. Nitrogen evolution was slow. After one-half hour evolution of gas ceased and the solution was concentrated to dryness.

Hydrolysis with alcoholic ammonia gave inconsistent results, but the ester was successfully hydrolyzed as follows: to a solution of 9.5 mgm. in 5 ml. of methanol was added 5 ml. of 2.4 N KOH in methanol. One aliquot was allowed to stand at 25° for one hour, another was heated at 100° for 40 minutes. Each was neutralized with HCl in methanol, and assayed. Suitable controls assayed at the same time showed that the KCl or methanol in the concentrations necessary for the assay had no inhibitory effect on growth.

	WEIGHT	ONE UNIT	TOTAL UNITS	ACTIVITY RECOVERED
	mgm.	*micrograms*		*per cent*
Starting material	110	1.7	64,700	
Esterified material	95.1	Inactive		
Esters hydrolyzed at 25°		1.7*	55,900	87
Esters hydrolyzed at 100°		Inactive		0

* Expressed in terms of esterified material.

This hydrolysis procedure was also successfully applied to the acetyl derivatives.

(*c*) *Methyl ester of the acetyl derivative.* 1.16 grams of ether extract acetylated as above were dissolved in a mixture of water and ether, and the ether layer shaken five times with equal volumes of water. Evaporation of the ether left 0.252 gram of a transparent, red-brown, oily liquid. The aqueous solution was concentrated to dryness at reduced pressure, the residue dissolved in 60 ml. of absolute methanol, and 345 mgm. of diazomethane in ether solution added. The vigorous initial effervescence slowed and stopped after 45 minutes. The solution was concentrated to a syrup, which was taken up in water and ether and shaken out three times with equal volumes of ether. The yellow ether solution, after removal of the solvent, left 0.766 gram of a red-brown liquid. Evaporation of the aqueous solution yielded 0.433 gram of red-brown glassy solid.

	WEIGHT	ONE UNIT	TOTAL UNITS	ACTIVITY RECOVERED
	grams	*micrograms*		*per cent*
Starting material	1.16	2.3	505,000	
Ether soluble acetylated material	0.252	9.5*	26,500	5
Water soluble acetylated material	1.27	Not assayed		
Ether soluble esterified material	0.766	2.4*	319,000	63
Water soluble esterified material	0.433	3.6*	120,000	24

* Hydrolyzed with CH_3OH—KOH in each case before assaying; weights expressed in terms of unhydrolyzed material.

Distillation of pantothenic acid derivatives

Preliminary determinations of the heat stability of pantothenic acid, its methyl ester and its acetyl derivative showed that stabil-

ity to heat was markedly increased by esterification, especially acetylation. The samples were placed in test tubes, heated in an oil bath, and hydrolyzed where necessary before being assayed.

MATERIAL	HEAT TREATMENT		ONE UNIT	DESTRUCTION
	Time	Temperature		
	min.	*°C.*	*micrograms*	*per cent*
Ether extract			1.7	
Ether extract	30	111–117	2.3	29.5
Ether extract	30	145–155	29.0	94.2
Acetylated material			1.4	
Acetylated material	30	145–155	1.4	0.0
Esterified material			1.7	
Esterified material	30	111–117	2.3	29.5
Esterified material	30	145–155	3.2	48.5

In an attempt to distill the free acid 129 mgm. of an ether extract was placed in a molecular still[4] and held at 72 to 75° and 10^{-3} mm. for 2.5 hours. The colorless, oily distillate (I) was removed from the condenser and the residue held at 85° and 2×10^{-5} mm. for 1.25 hours. The distillate (II), and final residue (III) were collected separately and assayed.

	WEIGHT	ONE UNITS	TOTAL UNITS	ACTIVITY RECOVERED
	mgm.	*micrograms*		*per cent*
Starting material	129	0.5	358,000	
Distillate (I)	36	10.0	3,600	1.0
Distillate (II)	10	5.0	2,000	0.6
Residue (III)	71	0.45	165,000	46.1

The large loss of activity in the distillation of the free acid and the increased heat stability of the methyl ester and acetyl derivative suggested use of such derivatives for distillation. Since the methyl ester of the acetyl derivative should be most stable and volatile, it was selected for further trials.

Although distillation of some acetylated esterified material

[4] In this still the distance between the condensing surface, which was kept cold with dry ice and acetone, and the material to be distilled was approximately 0.4 cm.

in the molecular still showed that the active compound distilled without destruction, no appreciable fractionation resulted; a still with greater fractionating powers was necessary. A second sample was therefore distilled from an apparatus similar to that used by Almquist (1937). It consisted of a glass tube sealed at one end and divided by constrictions into equal compartments, A, B, and C, A being at the closed end. The sample was placed in A, and the tube evacuated to 0.1 mm. A was heated electrically to 100 to 130°, B to 60 to 70°, and C left at room temperature. After one hour, the tube was broken at the constrictions, the fractions in the compartments were removed and aliquots hydrolyzed for assay.

	WEIGHT	ONE UNIT	TOTAL UNITS	ACTIVITY RECOVERED
	mgm.	*micrograms*		*per cent*
Starting material	553	2.1	264,000	
Fraction C	200	Inactive		
Fraction B	74.7	2.5	30,000	11
Fraction A (residue)	235.6	0.9	261,000	99

At the lower pressure, the undistilled material was most active, and considerable purification resulted. Subsequent distillation of Fraction A in the molecular still effected no increase in activity.

A partial synthesis of pantothenic acid

Lability of pantothenic acid to alkali and acid treatment is ascribed by Williams (1938a) to hydrolysis into β-alanine and a dihydroxy valeric acid. If this explanation is correct, reactivation of alkali-inactivated concentrates should follow recombination of the two components. This was accomplished as follows:

An active concentrate (38 mgm., 5.1 per cent pantothenic acid, one unit in 1.2 micrograms) was completely inactivated by heating at 100° with 10 ml. of N NaOH for one hour. After neutralization with HCl, the hydrolysate was concentrated to dryness and acetylated by heating for one hour at 100° with 3 ml. of pyridine and 10 ml. of acetic anhydride. After concentrating to dryness, 10 ml. of water was added, the mixture acidified with

H_2SO_4 (pH 2.0), and extracted continuously with ether for 2 hours. The extract was concentrated to dryness, and the acetylated acids redissolved in absolute ether. The acid chlorides of the mixture were formed by allowing the solution to stand (with occasional shaking) at room temperature for 3 hours with 0.2 gram PCl_5. Three cubic centimeters of anhydrous acetic acid were added to react with excess PCl_5, and the resulting solution concentrated to dryness *in vacuo*. To a solution of the resulting acid chlorides in absolute ether was added an ether solution containing 0.5 gram of β-alanine ethyl ester. The mixture was concentrated to dryness. The residue, which should contain the acetyl derivative of the ethyl ester of pantothenic acid, was dissolved in methanol and hydrolyzed with methyl-alcoholic KOH as described above. Assay showed one unit of activity in 1.8 micrograms (3.3 per cent pantothenic acid). Therefore, 65 per cent of the original activity was recovered by the resynthesis. In conjunction with data given above, this experiment furnishes conclusive evidence of the identity of the active factor in our liver concentrates with pantothenic acid.

Nicotinic acid as a growth factor for lactic acid bacteria

The identification of pantothenic acid as essential for growth of lactic acid bacteria made it desirable to test the ability of these organisms to grow on a more highly purified medium containing this substance. The basal medium consisted of acid-hydrolyzed, purified casein,[5] 0.5 per cent; tryptophane, 0.01 per cent; cystine, 0.01 per cent; mineral salts;[6] glucose, 1 per cent; sodium acetate, 0.6 per cent; riboflavin, 0.01 mgm. per 100 ml. The medium was tubed in 10 ml. lots, sterilized for 15 minutes at 15 pounds pressure and inoculated with the test organism. For inoculum, cells grown for 24 hours in medium B supplemented with pantothenic acid concentrates were centrifuged out and resuspended in an equal volume of 0.9 per cent NaCl solution. Each assay tube was

[5] Labco vitamin-free casein was hydrolyzed with 20 per cent H_2SO_4 at 120° for eight hours. H_2SO_4 was quantitatively removed with $Ba(OH)_2$.

[6] K_2HPO_4, 0.5 gram; $MgSO_4 \cdot 7H_2O$, 0.2 gram; NaCl, 0.01 gram; $FeSO_4 \cdot 7H_2O$, 0.01 gram; $MnSO_4 \cdot 3H_2O$, 0.01 gram; in 1000 ml. of medium.

inoculated with 0.05 ml. of this suspension. Of the organisms tested none made more than slight growth and this only occasionally. Such growth was probably due to carry-over of essential substances with the heavy inoculum, because it did not continue on subculture. Evidently essential substances, present in the hydrolyzed peptone of medium A, and the sodium-hydroxide-treated peptone of medium B, were lacking. That nicotinic acid is one such substance is evident from table 5. Nicotinic acid

TABLE 5

*Nicotinic acid as a growth factor for certain lactic acid bacteria**

ORGANISM	MICROGRAMS NICOTINIC ACID ADDED PER 10 ML.					ACTIVITY†				
	a	b	c	d	e	a	b	c	d	e
Lactobacillus arabinosus	0	0.1	0.5	1	3	+ 1.7	+++ 4.7	+++ 5.2	+++ 5.3	+++ 5.2
Lactobacillus casei	0	0.1	0.3	0.5	1	+ 2.2	++ 4.1	+++ 5.4	+++ 6.6	+++ 5.4
Leuconostoc mesenteroides	0	0.1	0.5	1	3	+ 1.0	+ 1.6	+ 1.2	+ 1.4	+ 1.3
Propionibacterium pentosaceum	0	0.1	0.5	1	3	+ 0.9	+ 1.2	+ 1.2	+ 1.1	+ 1.0
Streptococcus lactis	0	0.1	0.5	1	3	+ 1.0	+ 1.2	+ 1.2	+ 1.1	+ 1.1

* Incubation time, 5 days. *L. casei* at 37°C.; all other organisms at 28°C.

† −, + etc. as in table 2. Figures are ml. of 0.1 N acid produced per 10 ml. of medium.

greatly stimulated growth of *L. casei* and *Lactobacillus arabinosus* in the first culture, but growth failed on subculture, probably because of a lack of other essential growth factors. The other organisms, data for three of which are given in table 5, failed to show significantly increased growth in the presence of nicotinic acid. All grew well when small amounts of yeast, liver or malt extract were added to the basal medium. The nature of the additional factor or factors necessary for growth is being investigated. The effect of such extracts cannot be duplicated by

pimelic acid, β-alanine, uracil, pyruvic acid, vitamin B_1, vitamin B_6,[7] inositol, betaine, nicotinic acid or glutathione, alone or in various combinations.

DISCUSSION

Four lines of evidence point to the identity of the growth factor for lactic acid bacteria (Snell *et al.* (1937)) with pantothenic acid. (1) Purified preparations of pantothenic acid completely replace growth factor preparations for a variety of lactic acid bacteria. (2) 83 per cent calcium pantothenate is almost exactly twice as active as 40 per cent calcium pantothenate.[8] This relationship would be very improbable if an impurity were responsible for the effect. (3) The formula for pantothenic acid, proposed by Williams (1938a) explains the known properties of the growth-promoting factor for lactic acid bacteria. The proposed formula is that of a substituted amide, formed from β-alanine and a dihydroxy-valeric acid of unknown configuration. Hydrolysis of the amide linkage explains lability of the growth factor to acid and alkali. The presence of hydroxyl and carboxyl groups explains inactivation by acetylation and esterification. Marked water solubility and limited solubility in ether would also be expected in a compound of this type. (4) The active substance in our concentrates has been destroyed by alkaline hydrolysis, and an active compound resynthesized on the basis of the formula for pantothenic acid suggested by Williams.

The assay which has been developed for the present work appears to be a specific and quantitative method for determining pantothenic acid, and may prove useful in view of the growing biological importance of this substance.

The essential nature of pantothenic acid for *P. pentosaceum* is not in agreement with the reports from this laboratory by Wood *et al.* (1937, 1938), although the same strain of organism (no. 11, Hitchner (1934)) was used. The failure of pantothenic acid

[7] We wish to thank Dr. S. Lepkovsky for a sample of crystalline vitamin B_6.

[8] The figures given are those kindly furnished by Professor Williams. Wherever the pantothenic acid content of a fraction is given in this paper it has been referred to these values as standard.

preparations, alone or in combination with other known growth factors to replace ether extract of yeast, must be attributed to some unrecognized deficiency in the basal medium used.

Möller (1938) reported that vitamin B_6 is required by certain lactic acid bacteria. We have been unable to detect any clear-cut stimulating action of this vitamin on *L. casei*, but this result may be due to the lack of a suitable basal medium.

The number of known accessory factors for the lactic acid organisms is now raised to four (riboflavin, pantothenic acid, nicotinic acid, and vitamin B_6). It is also evident that others remain to be discovered.

SUMMARY

The factor previously described as necessary for growth of all lactic acid bacteria tested has been identified with pantothenic acid. Convenient methods for the preparation of highly active concentrates of this substance are described, together with experiments which give further information concerning its properties.

Nicotinic acid greatly stimulates growth and acid production by some but not all lactic acid bacteria, and is regarded as essential for certain of these organisms. Other unidentified factors are also required by these bacteria for growth on highly purified media.

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