[CONTRIBUTION FROM THE CHEMICAL LABORATORY, UNIVERSITY OF CALIFORNIA, LOS ANGELES]

Inhibition of Lactic Acid Bacteria by Analogs of Pantothenic Acid[1]

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The synthesis of the sodium salt of N-(α,γ-dihydroxy-β,β-dimethylvaleryl)-β-alanine (Fig. 1, $R_1 = CH_3$, $R_2 = H$), hereafter referred to as ω-methylpantothenic acid, was reported recently.[3]

The study of ω-methylpantothenic acid was undertaken to determine the effect of R_1 substituents on the activity of pantothenic acid toward lactic acid bacteria. Although most analogs of pantothenic acid possess little or no activity, two are partially active as growth promoters. Thus, N-(α-hydroxy-β,β-dimethylolbutyryl)-β-alanine[4] (Fig. 1, $R_1 = H$, $R_2 = OH$) and N-(α-hydroxy-β-methyl-β-methylolvaleryl)-β-alanine[5] (Fig. 1, $R_1 = H$, $R_2 = CH_3$) exhibit growth activity which is significant but less than that of pantothenic acid. Compounds which inhibit competitively or non-competitively the growth of microörganisms include salicylol-β-alanine,[6] mandelyl-β-alanine,[6] analogs of pantothenic acid lacking the α-hydroxy group[7,8] and analogs with different modifications of the β-alanine moiety.[9]

There was no adequate basis on which to predict the type of activity of ω-methylpantothenic acid, although the competitive inhibition exhibited is somewhat analogous to that shown by the methyl homolog (ethionine) of methionine,[10,11] the methyl homolog (β-aminobutyric acid) of β-alanine[12] and the 2-*n*-butylpyrimidine analog of thiamine.[13] However, the lower homologs of thiamine retain growth activity.[13] That ω-methylpantothenic acid is inactive as a growth promoter for *Lactobacillus casei*[5] was not known until the present experiments were completed.

Since pantoyltaurine inhibits the growth of microörganisms,[14] it was considered desirable to test this and other analogs of pantothenic acid and ω-methylpantothenic acid. Pantoyltaurine, pantoylglycine, pantoyl-DL-α-amino-*n*-butyric acid,

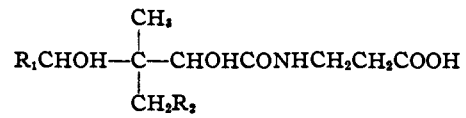

Fig. 1

pantoyl-DL-α-aminoisobutyric acid, pantoyl-DL-α-amino-α-ethyl-*n*-butyric acid, panto-DL-norvaline, ω-methylpantoyl-L-leucine and ω-methylpantoyltaurine were prepared by fusing the *dl*-pantolactones with the sodium salts of these amino acids or by refluxing the lactones and amino acid salts in methanol and precipitating the products with ether. It was found that ω-methylpantoyltaurine inhibited some lactic acid bacteria but that the other analogs were essentially inactive toward *Lactobacillus arabinosus* 17-5. That the α-amino acid analogs were inactive is in agreement with the observations on related α-amino acids reported by earlier workers.[7,15] While this work was in progress, it has been reported that pantoylglycine is inactive toward *Leuconostoc mesenteroides*[16] and that pantoylglycine and related analogs act as antivitamins for *Streptobacterium plantarum*.[17]

Experimental

α,α-Dimethyl-β-hydroxybutyraldehyde.—The method employed is essentially that of Lilienfeld and Tauss.[18] To a cold solution of 102 g. (2.31 moles) of acetaldehyde were added 166 g. (2.31 moles) of isobutyraldehyde and 200 ml. of a saturated aqueous solution of potassium carbonate. The mixture was stirred continuously and maintained below 30°. At the end of an hour the temperature began to rise rapidly and after two hours the reaction appeared to be complete. The viscous mixture was extracted with three 100-ml. portions of ether. The ether solution was washed with *N* acetic acid and saturated sodium bicarbonate solution and dried over sodium sulfate. The ether was removed and the fraction which distilled at 74–76° under 15 mm. (88–90°(22 mm.))[18] was collected in 30% yield.

α-Hydroxy-β,β-dimethyl-γ-valerolactone.—The method employed is essentially that of Stiller, *et al.*[19] To 74.5 g. (0.64 mole) of freshly distilled α,α-dimethyl-β-hydroxybutyraldehyde was added 200 ml. (20% excess) of sodium bisulfite solution. The mixture was stirred and heated on the steam-bath but a small amount of viscous material remained undissolved. The mixture was cooled to 5° and an aqueous solution of potassium cyanide (equivalent to the bisulfite) was added in small portions with stirring while maintaining the mixture between 5 and 10°. Stirring was continued for an hour at 10° and for an additional hour at room temperature. The aqueous and oily (cyanohydrin) layers were separated and the aqueous layer was extracted with three 75-ml. portions of ether. The combined solution of cyanohydrin and ether extracts was added

(1) Paper 44. For Paper 43, see Dunn, Camien, Shankman and Block, *Archiv. Biochem.*, in press. This work was aided by grants from the National Institute of Health of the U. S. Public Health Service and the University of California. Some of the material in this paper was presented before the Division of Biological Chemistry of the American Chemical Society at the New York City meeting in September, 1947.

(2) Junior Fellow, National Institute of Health.

(3) Drell and Dunn, THIS JOURNAL, **68**, 1868 (1946).

(4) Mitchell, Snell and Williams, *ibid.*, **62**, 1791 (1940).

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(6) Martin, Lewis and Urist, Abstracts of Papers, 109th Meeting, Amer. Chem. Soc., 21B (1946).

(7) Barnett and Robinson, *Biochem. J.*, **36**, 357 (1942).

(8) McIlwain, *ibid.*, **36**, 417 (1942).

(9) An excellent review article has been presented by Roblin, *Chem. Rev.*, **38**, 255 (1946).

(10) Dyer, *J. Biol. Chem.*, **124**, 519 (1938).

(11) Harris and Kohn, *J. Pharmacol.*, **72**, 383 (1941).

(12) Nielson, *Compt. Rend. Lab. Carlsberg. Ser. physiol.*, **23**, 107 (1940).

(13) Emerson and Southwick, *J. Biol. Chem.*, **160**, 169 (1945).

(14) Snell, *J. Biol. Chem.*, **139**, 975 (1941); **141**, 121 (1941).

(15) Williams, *Advances in Enzymol.*, **3**, 253 (1943).

(16) Snell and Shive, *J. Biol. Chem.*, **158**, 551 (1945).

(17) Nielsen and Roholt, *Acta Pharmacol. Toxicol.* (*Copenhagen*), **1**, 207 (1945); *C. A.*, **40**, 6127 (1946).

(18) Lilienfeld and Tauss, *Monatsh.*, **19**, 77 (1898).

(19) Stiller, Harris, Finkelstein, Keresztesy and Folkers, THIS JOURNAL, **62**, 1785 (1940).

with stirring to 250 ml. of concentrated hydrochloric acid maintained below 15°. The resulting solution was allowed to stand overnight at room temperature, the ether was removed and the acid solution was refluxed for three hours. The acid was neutralized to *p*H 7 and extracted 12 times with ether. The ether solution was evaporated to a volume of one liter and dried over sodium sulfate. The ether was removed and the fraction which distilled at 94–96° under 2 mm. (120–122° (9 mm.))[5] was collected. The yield was 58.5 g. (63%).[20] The lactone was crystallized by dissolving the sirup in dry ether, adding petroleum ether to the point of cloudiness and cooling the solution below 0°. Two recrystallizations from an ether–petroleum ether mixture gave white needles, m. p. 60–60.5°.

Anal. Calcd., for $C_7H_{12}O_3$: C, 58.31; H, 8.39. Found: C, 58.57; H, 8.54.

3,5-Dinitrobenzoate of ω-Methylpantolactone.—This derivative was prepared from approximately equivalent quantities of ω-methylpantolactone and freshly prepared 3,5-dinitrobenzoyl chloride in freshly distilled dry pyridine by the procedure of Stiller, *et al.*[21] After two recrystallizations from ethanol the compound melted at 123–124°.

Anal. Calcd. for $C_{14}H_{14}O_8N_2$: C, 49.71; H, 4.17; N, 8.28. Found: C, 49.83; H, 4.30; N, 8.61.

Sodium Salt of ω-Methylpantothenic Acid.—The sodium salt of β-alanine was prepared by adding an equivalent of base to the amino acid, evaporating the solution to dryness, and powdering the solid. A mixture of 1.58 g. of freshly distilled ω-methylpantolactone and 1.11 g. of the sodium salt of β-alanine was maintained for two hours at 110–120° with occasional stirring. The product was dissolved in 100 ml. of absolute isopropanol, the solution was cooled and the small quantity of white solid which settled was separated.

To about half the isopropanol solution was added 250 ml. of absolute ether. The resulting suspension was filtered and the precipitate was washed with ether and dried for five days at room temperature *in vacuo* over phosphorus pentoxide; yield, 1.3 g. This product contained isopropanol of crystallization.

Anal. Calcd. for $C_{10}H_{18}O_5NNa \cdot C_3H_8O$: N, 4.44. Found: N, 4.43, 4.46.

Two samples (160 and 190 mg.) of this product heated for three weeks at 70° *in vacuo* over phosphorus pentoxide changed from a pure-white to a light-tan color and attained nearly constant weight (loss <1 mg. in four days). The products appeared to be anhydrous and nearly pure.

Anal. Calcd. for $C_{10}H_{18}O_5NNa$: N, 5.49. Found: N, 5.45 (sample 1), 5.43 (sample 2).

It is of interest that Levy, *et al.*,[22] obtained calcium pantothenate with isopropanol of crystallization which could not be removed by drying *in vacuo* at 100°.

The remainder of the isopropanol solution was evaporated to about 15 ml. and preserved at 0°. The almost solid cake of precipitate which formed after two weeks was filtered and the precipitate was washed first with cold isopropanol and then with ether. The product was dried at room temperature *in vacuo* over phosphorus pentoxide. Yield of product, m.p. 160–161.5°, was 0.9 g. It was less hygroscopic and easier to handle than the sodium salts prepared in other ways. It has been observed that crystalline sodium *d*-pantothenate behaves similarly.[23]

Anal. Calcd. for $C_{10}H_{18}O_5NNa$: C, 47.05; H, 7.11; N, 5.49; Na, 9.01. Found: C, 47.65; H, 7.28; N, 5.48, 5.50; Na, 9.02.

Products obtained by fusion of the lactone and the sodium salt of β-alanine were dissolved in water and used directly in determining inhibitory effects of the analog. These materials were from 70 to 85% condensed according to Van Slyke amino nitrogen determinations.

Sodium Salts of Pantoyltaurine and ω-Methylpantoyltaurine.—These salts were prepared in crude form by fusing equivalent quantities of the lactone and the dry sodium salt of taurine for five hours at 110–120°.[14] According to amino nitrogen analyses, 55 to 85% condensation occurred. The compounds were purified by dissolving the fused products in absolute ethanol, filtering to remove the unreacted amino acid salts, precipitating the analogs with ether, and drying the products *in vacuo* over phosphorus pentoxide.

Anal. Calcd. for $C_8H_{16}O_6NSNa$ (pantoyltaurine): N, 5.05; Na, 8.30. Found: N, 5.12; Na, 8.36. Calcd. for $C_9H_{18}O_6NSNa$ (ω-methylpantoyltaurine): N, 4.81. Found: N, 4.59.

Some of the fusion products were used without purification since the presence of the uncondensed components was found to have relatively little effect on the response of the microörganisms.

Sodium Salt of ω-Methylpantoyl-L-leucine.—The analog was prepared by fusing equivalent amounts of ω-methyl pantolactone and the dry sodium salt of L-leucine for two hours at 110°. Amino nitrogen determinations before and after acid hydrolysis indicated 64% condensation. The product was used without further purification.

Sodium Salts of the α-Amino Acid Analogs of Pantothenic Acid.—These analogs were prepared[7] by refluxing equivalent quantities of *dl*-pantolactone and the amino acid salts in absolute methanol for two hours, filtering the solutions, and precipitating the compounds with ether. The nitrogen (Kjeldahl) of the products, dried *in vacuo* over phosphorus pentoxide, is shown below:

Analog (sodium salt) Name	Formula	Nitrogen, % Calcd.	Found	% Condensation
Pantoylglycine	$C_8H_{14}O_5NNa$	6.16	6.58, 6.61	85
Pantoyl-DL-α-aminobutyric acid	$C_{10}H_{18}O_5NNa$	5.49	5.58, 5.62	98
Pantoyl-DL-α-aminoisobutyric acid	$C_{10}H_{18}O_5NNa$	5.49	5.62, 5.68	97
Pantoyl-DL-norvaline	$C_{11}H_{20}O_5NNa$	5.20	6.40	77
Pantoyl-DL-α-amino-*d*-ethylbutyric acid	$C_{12}H_{22}O_5NNa$	4.95	6.46, 6.41	65

Testing Procedure.—The methods commonly employed in the authors' laboratory were used to determine the microbiological activity of the present compounds. The basal medium was Medium B given in Table I of a previous paper[24] modified in that amino acids, ammonium chloride and pantothenic acid were omitted and the following supplements were added per liter of diluted medium: casein hydrolysate,[25] 7.5 g. (solids); natural asparagine, 100 mg.; L-tryptophan, 50 mg.; L-cysteine hydrochloride, 200 mg.; and xanthine, 12 mg. Four-inch test tubes containing final 3-ml. volumes of solutions were covered with toweling, sterilized, inoculated with a syringe or automatic pipet, and incubated at 35° for seventy-two hours. The acid produced was titrated with standard approximately 0.04 *N* sodium hydroxide using brom thymol blue as indicator.

Results

The growth-promoting activity of ω-methylpantothenic acid for *Lactobacillus casei*, *Lactobacillus arabinosus* 17-5, *Lactobacillus fermenti* 36 and *Leuconostoc mesenteroides* P-60 was investigated in the present experiments but the data have been omitted to conserve space. There was no stimula-

(20) A large scale synthesis (9 moles) was carried out without purification of the intermediate aldol in an over-all yield of 38% based on acetaldehyde.

(21) Stiller, Keresztesy and Finkelstein, THIS JOURNAL, **62**, 1779 (1940).

(22) Levy, Weijlard and Stiller, *ibid.*, **63**, 2846 (1941).

(23) Parke and Lawson, *ibid.*, **63**, 2869 (1941).

(24) Dunn, Shankman, Camien, Frankl and Rockland, *J. Biol. Chem.*, **156**, 703 (1944).

(25) Green, Black and Howland, *Ind. Eng. Chem., Anal. Ed.*, **15**, 77 (1943).

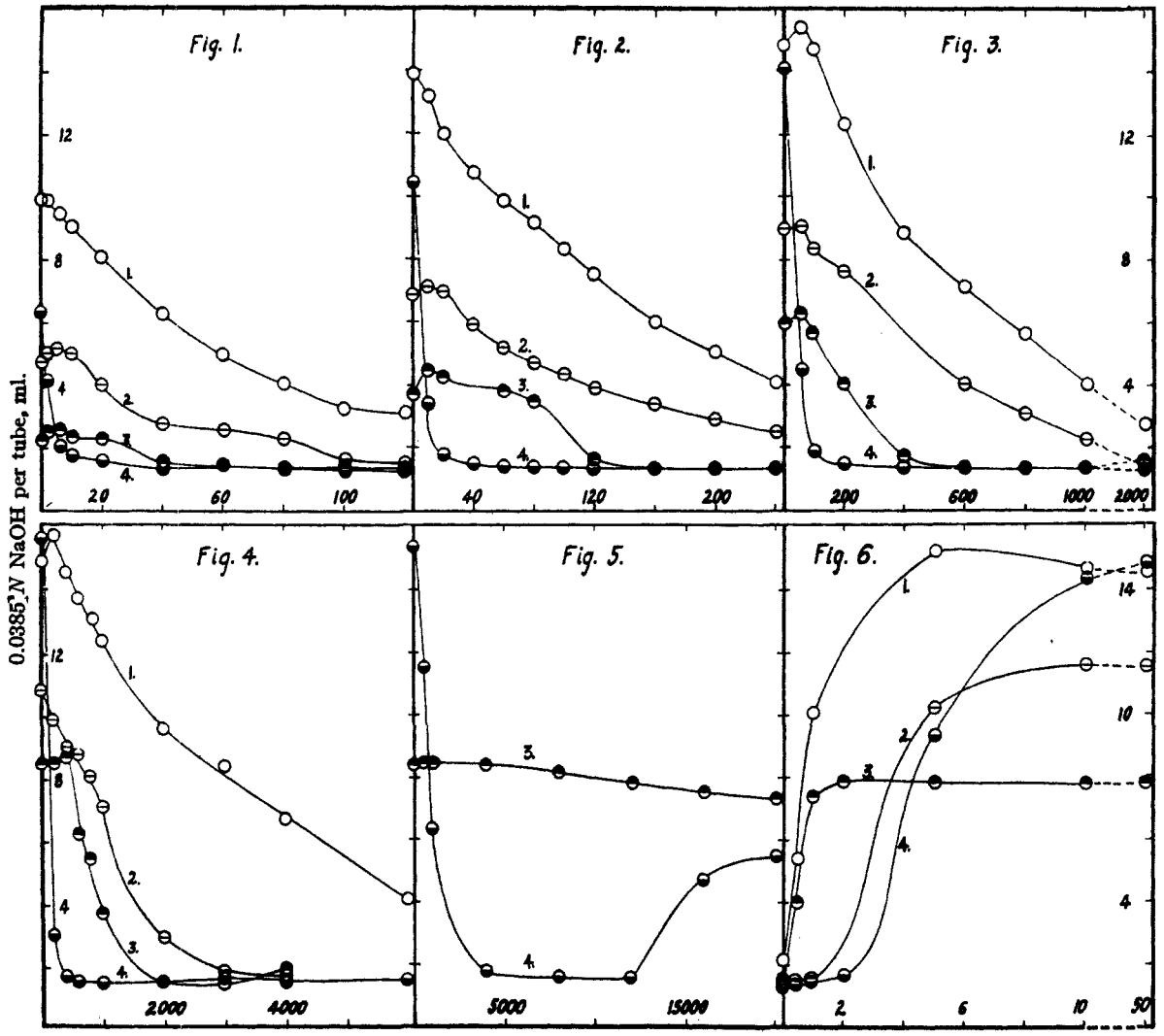

Plate I: Figs. 1–6.—The volumes of base consumed are plotted against γ of sodium ω-methylpantothenate added per tube in the presence of the following amounts of calcium *d*-pantothenate: 0.02γ (Fig. 1), 0.06γ (Fig. 2), 0.2γ (Fig. 3), 0.6γ (Fig. 4), and 2.0γ (Fig. 5). Figure 6 shows the response to increasing concentrations (γ) of pantothenic acid in the presence of 10,000 γ of sodium ω-methylpantothenate. The values at zero γ of pantothenic acid were obtained by extrapolation from other (not shown) data. The responses of four lactic acid bacteria are plotted as: (1) *Lactobacillus arabinosus* 17–5, (2) *Leuconostoc mesenteroides*, P-60, (3) *Lactobacillus fermenti* 36, and (4) *Lactobacillus casei*. Their "blank" titrations were 4.05, 2.33. 1.55 and 1.80 ml., respectively. The uninoculated blank was 1.15 ml. Points are averages of duplicate tubes.

tion at any level (0.01 to 1000 γ per tube) of analog and the "blank" acid production was suppressed. ω-Methylpantoyl-L-leucine was inactive except that at high levels (above 4000 γ) it diminished the blank titration values.

As shown in plate I, ω-methylpantothenic acid repressed the growth of the four lactic acid bacteria. The concentrations of analog required to inhibit the organisms were proportional to the concentrations of pantothenic acid in the medium, and the inhibition was competitive over a wide range in concentrations both of inhibitor and nutrilite. The direct reversal by pantothenic acid of the effects of ω-methylpantothenic acid is shown in Fig. 6, Plate I. Since growth of the organisms was normal in the presence of sufficient pantothenic acid, even at the highest level (20 mg./3 ml.) of inhibitor tested, it appears that ω-methylpantothenic acid is non-toxic for the bacteria investigated.

That the analog is stimulatory for some organisms under certain conditions is indicated by the data in Plate I. Examples of this effect at concentrations below the inhibition range are the stimulation of *L. fermenti* and *L. mesenteroides* (at 0.02, 0.06, and 0.2 γ of pantothenic acid) and *L.*

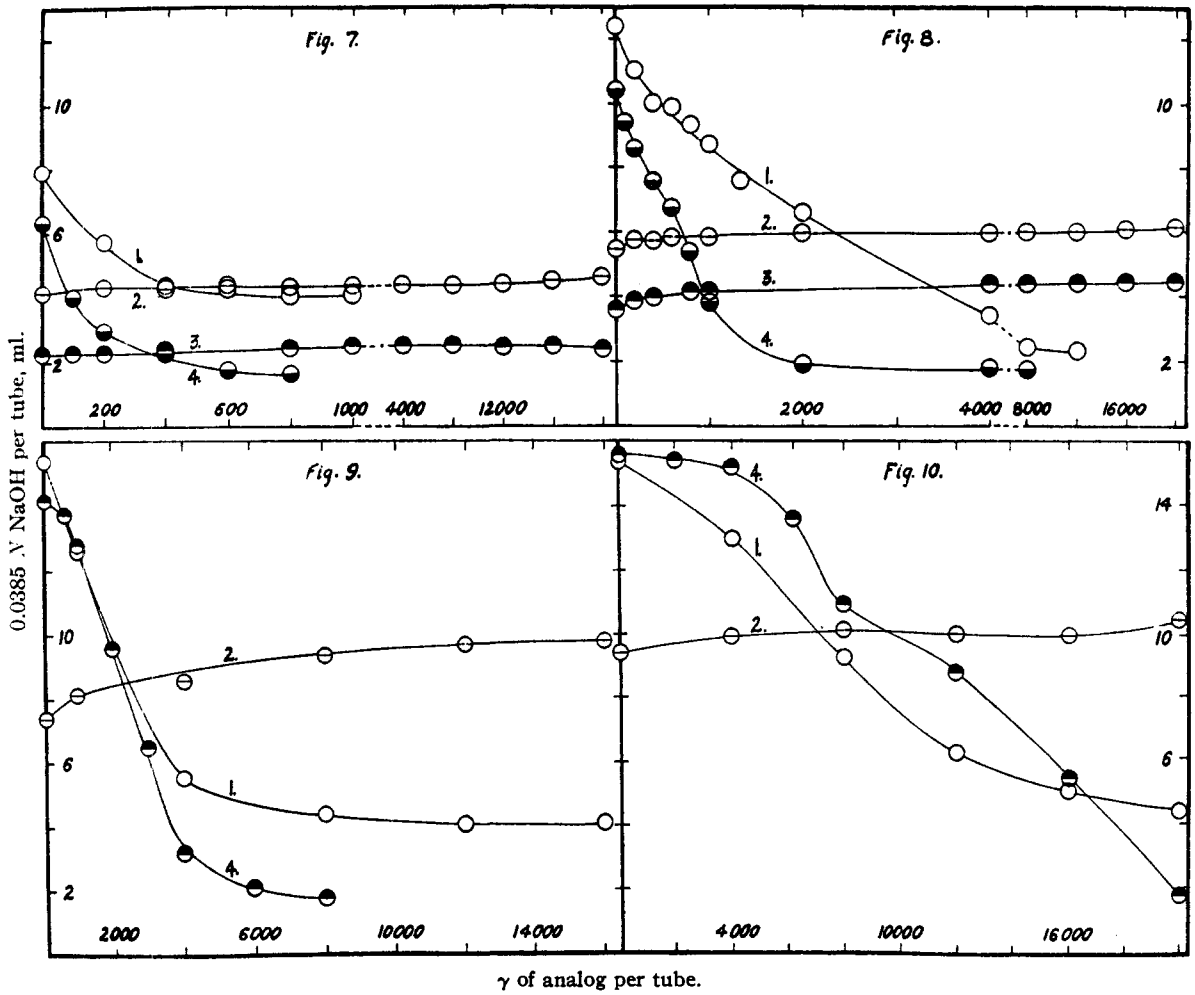

Plate II: Figs. 7–10.—The volumes of base consumed are plotted against γ of the sodium salt of ω-methylpantoyltaurine added per tube in the presence of the following amounts of calcium *d*-pantothenate: 0.02γ (Fig. 7). 00.6γ (Fig. 8). 0.2 γ (Fig. 9). and 0.6 γ (Fig. 10). The lactic acid bacteria are numbered as in Plate I and the blank titration values are the same except for *L. mesenteroides* P-60 (3.70 ml.). The values for *L. mesenteroides* and *L. fermenti* beyond 1000 γ and 8000 γ (Figs. 7 and 8, respectively) were calculated from data obtained in a separate experiment. Points are averages of duplicate tubes.

arabinosus (at 0.2 and 0.6 γ). After complete inhibition has been reached stimulation is observed in the cases of *L. casei* (at 2 γ) and *L. fermenti* (at 0.2 and 0.6 γ). That *L. arabinosus* and *L. fermenti* are not completely inhibited at 0.5 γ of pantothenic acid and 10000 γ of analog (Fig. 6), as would be expected, may be explained by this effect.

ω-Methylpantoyltaurine was found (Plate II) to inhibit the growth of only two of the four lactic acid bacteria. In both cases (*L. casei* and *L. arabinosus*) it was considerably less active than ω-methylpantothenic acid. *L. fermenti* and *L. mesenteroides* were stimulated by the analog, with growth increasing as the level was raised. No inhibition was observed at any concentration tested.

A study of pantoyltaurine under the same conditions was carried out for comparative purposes. It was observed (unpublished work[26]) that both *L. fermenti* and *L. mesenteroides* were stimulated up to high levels (2000–4000 γ) by the inhibitor. Stimulation was maximum at 1000 and 600 γ, respectively, and decreased thereafter as the level was raised leading to inhibition and complete cessation of growth. The degree of stimulation exerted by pantoyltaurine was nearly approached but not exceeded by its homolog.

The activities of the analogs described above are compared in Table I expressed in terms of the antibacterial index[8] (the molar ratio of inhibitor to growth promoter which inhibits completely the growth of the organisms.). It may be noted that the antibacterial indices of the analogs remained essentially constant over a wide range in

(26) Some of the data are summarized in the Thesis by W. Drell submitted in partial fulfillment of the requirements for the Master of Arts in Chemistry, June, 1946.

concentration of pantothenic acid in almost all cases. That the values rose significantly at the highest levels of nutrilite particularly at 2.0 γ with *L. casei* and *L. fermenti* probably was not due to differences in assay conditions.[27]

TABLE I

ANTIBACTERIAL INDICES OF PANTOTHENIC ACID ANALOGS AT VARYING CONCENTRATIONS OF PANTOTHENIC ACID

Analog	Ca *d*-pantothenate γ per tube	Antibacterial index[a] *Lactobacillus casei* 7469[c]	*Lactobacillus rabinosus* 17-5 8014[c]	*Lactobacillus fermenti* 36 9338[c]	*Leuconostoc mesenteroides* P-60 8042[c]
ω-Methylpantothenic acid	0.02	350	3000	1500	3000
	.06	260	3000	1600	3300
	.20	450	3900	1600	3900
	.60	450	5200	2200	3300
	2.0	950		>7500	
ω-Methylpantoyltaurine	0.02	12500	16500	[b]	[b]
	.06	16500	22000	[b]	[b]
	.20	14500	25000		[b]
	.60	16500	22000		[b]
Pantoyltaurine	0.02	13000	4000	150000	300000
	.06	15000	4700	130000	250000
	.20	15000	6200		

[a] Corrected for the per cent. condensation of the *dl*-analogs. [b] Stimulation observed. [c] American Type Culture Collection Number.

The relative concentrations of the analogs required for half-maximum inhibition and for complete inhibition were compared. In the case of ω-methylpantothenic acid, the ratio was approximately two for *L. fermenti* and *L. mesenteroides* and between two and three for *L. arabinosus*. With pantoyltaurine this ratio was about two for all three organisms. ω-Methylpantoyltaurine was required in larger than twofold amounts to achieve complete inhibition with *L. arabinosus*. *L. casei* required threefold or higher concentrations of all analogs.

In view of the results obtained, it was of interest to investigate further the comparative activity of these analogs against a large number of lactic acid bacteria particularly with reference to the mutual influence of the two combined inhibitory groups (ω-methyl and sulfonic acid). The responses of 19 organisms are summarized in Table II. All lactic acid bacteria tested were susceptible to inhibition by ω-methylpantothenic acid. The antibacterial indices ranged from 80 to 13000. Stimulation was observed in those cases (five) where concentrations of analog were sufficiently small to lie below the inhibitory level.

(27) Shive and Snell[28,29] have showed that time of incubation and concentration of inocula modify the responses of organisms to inhibitors of pantothenic acid. An example of the latter effect is illustrated by *L. arabinosus*. When, under otherwise uniform assay conditions, the density of the washed inoculum was increased markedly (from a blank titration value of 4.05 to 6.08 ml. of 0.0385 *N* sodium hydroxide) the antibacterial index of ω-methylpantothenic acid increased from 3000 to 12000. This difference cannot be due to an increase in nutrilite concentration alone. The synthetic ability of *L. arabinosus* (Shankman, Camien, Block, Merrifield and Dunn, *J. Biol. Chem.*, **168**, 23 (1947)) may play a more prominent role under these conditions.

(28) Shive and Snell, *Science*, **102**, 401 (1945).

(29) Shive and Snell, *J. Biol. Chem.*, **160**, 287 (1945).

TABLE II

ACTIVITY OF ANALOGS OF PANTOTHENIC ACID AGAINST LACTIC ACID BACTERIA

Organism	Antibacterial index[a] ω-Methylpantothenic acid	ω-Methylpantoyltaurine	Pantoyltaurine
Leuconostoc citrovorum 8082[b]	80[c]	2400[c]	4200[c,d]
Lactobacillus fermentatus 4006	150	51000[d]	113000
Lactobacillus pentoaceticus 367	270	175000	85000[g]
Lactobacillus brevis 8257	270	75000	140000
Leuconostoc citrovorum 797	330	7300[c]	8500[c,d]
Leuconostoc citrovorum 7013	330	6000[c,d]	5100[c,d]
Streptococcus faecalis R 8043	330	26000	35000
Lactobacillus helveticus 335	500[d]	51000[d]	42500[d]
Lactobacillus helveticus 6345	550[d]	44000[d]	57000[d]
Lactobacillus lycopersici 4005	800	[e]	51000
Leuconostoc dextranicum 8358	900	2200[c]	850
Leuconostoc dextranicum 8086	900	5000[c]	1350[c]
Leuconostoc mesenteroides 9135	900[c]	[e]	1350[c]
Leuconostoc mesenteroides 8293	1100	4400	7000[c,d]
Lactobacillus gayoni 8289	2200[d]	[e]	225000[d]
Leuconostoc dextranicum 8359	2700[d]	3500[c]	1400[c]
Lactobacillus pentosus 124.2	4000	[f]	[f]
Lactobacillus brassicae 8041	7500	[f]	[f]
Lactobacillus mannitopoeus	13000[c,d]	[e]	225000[d]

[a] Based on average values of duplicate tubes. Corrected for the per cent. condensation of the *dl*-analogs. [b] American Type Culture Collection Number. [c] The half-maximum point was achieved at a concentration of analog approximately half that required for complete inhibition of growth. [d] Stimulation was observed at concentrations below the inhibitory range. [e] Stimulation only was observed. Levels up to 20,000 γ in the presence of 0.06 γ of calcium *d*-pantothenate were tested. [f] Relatively little effect was observed at levels up to 20,000 γ in the presence of 0.06 γ of calcium *d*-pantothenate. [g] Half-maximum inhibition; growth was not completely repressed at the analog–metabolite molar ratio of 280,000.

Pantoyltaurine inhibited the growth of all but two (*L. pentosus* and *L. brassicae*) of the lactic acid bacteria tested. In most cases the activity was considerably less than that of ω-methylpantothenic acid. Two organisms (*Leuconostoc dextranicum* 8358 and 8359) were more susceptible to the latter than to pantoyltaurine. At levels below the inhibitory range, eight organisms were found to be stimulated and five (*L. fermentatus*, *L. pentoaceticus*, *L. brevis*, *L. pentosus*, and *L. brassicae*) appeared unaffected by pantoyltaurine. The responses of the remaining organisms were not determined in the experiment.

ω-Methylpantoyltaurine was less active than the β-alanine analog against all organisms tested, but in many cases it was more inhibitory than pantoyltaurine. Four organisms (*L. lycopersici*, *L. mesenteroides* 9135, *L. gayoni*, and *L. mannitopoeus*) exhibited stimulation with the analog of the type described previously for *L. fermenti* and *L. mesenteroides* P-60. With these bacteria inhibition was not observed at the levels tested (up to 20 mg. in the presence of 0.06 γ of pantothenic acid). In the cases of *L. pentosus* and *L. brassicae* neither stimulatory nor strong inhibitory effects were noted. At concentrations below the inhibitory level, four lactic acid bacteria were stimulated and one, *L. pentoaceticus*, was not. The responses of the other organisms inhibited by ω-methylpan-

toyltaurine were not determined at these low levels.

The relative concentrations of analogs required for half-maximum and complete inhibition were calculated for these organisms. Of the fourteen bacteria for which data at the half-maximum inhibition level were obtained with ω-methylpantothenic acid, a ratio of two was observed with three organisms. In the case of pantoyltaurine, seven of the sixteen organisms observed were of this type. With ω-methylpantoyltaurine, seven of thirteen organisms showed this behavior. The responses of these bacteria to pantoyltaurine had a high correlation to those obtained with ω-methylpantoyltaurine.

ω-Methylpantoylleucine showed relatively little inhibitory activity against four lactic acid bacteria (those listed in Table I were used) at levels as high as 20,000 γ in the presence of 0.02 γ of pantothenic acid. The uncondensed amino acids, β-alanine, L-leucine and taurine were inactive except for a slight inhibition of *L. casei* and *L. mesenteroides* by leucine (4000 γ) and of *L. arabinosus* by taurine (8000 γ). Somewhat greater inhibition, reversed by the nutrilite, was observed for ω-methylpantolactone in the absence, or at low levels, of pantothenic acid. The behavior of mixtures of the lactone and an amino acid resembled that of the lactone alone.

Discussion

That substitution at the ω-hydroxymethyl group of pantothenic acid leads to compound possessing inhibitory properties has been shown by the results given above and by investigations with other analogs.[30] This is in contrast to substitution at one of the ω-methyl groups whereby significant growth promoting ability is retained. It would seem therefore that methyl substitution in the former case produces a more significant change in the steric configuration, interfering in some manner with the normal functioning of the ω-hydroxy group. The relatively high activity of ω-methylpantothenic acid toward all lactic acid bacteria studied in comparison with the varying effectiveness of inhibitors such as pantoyltaurine and others is noteworthy. Of these β-methylpantothenic acid (N-pantoyl-β-aminobutyric acid)[28] although it shows a somewhat different bacterial spectrum, is the most similar to ω-methylpantothenic acid. The two inhibitors resemble each other further in that both contain methyl groups substituted in positions adjacent to functional groups.[31]

Lipmann and co-workers[32,33] have clearly demonstrated that the pantothenic acid in living organisms is largely bound in the form of a coenzyme. They have shown further that two distinct enzymes, phosphatase and liver enzyme, are required to liberate pantothenic acid for bacterial use, indicating that at least two groups of the growth factor are tied up. The work of Williams[34] has indicated that a point of attachment in the coenzyme is probably at the carboxyl group through an amide linkage, while the activity of phosphatase denotes phosphorylation of a hydroxy group. From the inhibitory activity of ω-methylpantothenic acid it might appear that the ω-hydroxy is the group so concerned. However, it is of interest that methyl substitution at the 2-hydroxyethyl group of thiamine, which is the site of phosphorylation, results in an analog which is entirely active upon the pea root, slightly so upon *Phycomyces blakesleeanus* and inactive upon the rat.[35]

It appears that ω-methylpantothenic acid and pantoyltaurine may interfere with the metabolism of pantothenic acid at different loci either in the same or in different reaction steps. This could account for their greatly different inhibitory activities as well as for the results observed with the hybrid, ω-methylpantoyltaurine. With the latter no synergism was encountered and its observed activity could be correlated in general with the relative inhibitory powers of its two parent compounds. In those cases where ω-methylpantothenic acid was considerably more active than pantoyltaurine, the hybrid was intermediate in strength between the two. If the parent analogs were of approximately equal activity the hybrid was weaker than either. In instances where pantoyltaurine was very weakly inhibitory and (very frequently) stimulatory at concentrations below the range of inhibition, the hybrid was found to exhibit only stimulatory activity. The effect might be one of further weakening in activity, in which case inhibitory effects would be encountered at much higher concentrations of inhibitor than those tested. The hybrid appears to be more sensitive to the variations induced by the sulfonic acid than by the ω-methyl group. In comparing the relative concentrations of inhibitor to produce half-maximum or complete inhibition with these organisms, greater correlation was obtained between pantoyltaurine and ω-methylpantoyltaurine than between the latter and ω-methylpantothenic acid. Against *L. brassicae* and *L. pentosus*, pantoyltaurine and likewise ω-methylpantoyltaurine were almost completely without effect. Further, neither analog was stimulatory at any of the levels tested. This response is similar to that observed with inactive pantoylleucine and ω-methylpantoylleucine.

The stimulation effects observed above have been reported with other inhibitory analogs of

(30) Drell and Dunn. Abstracts of Papers, 112th Meeting, American Chemical Society, 5C (1947).

(31) The inhibition of the growth of yeast by β-amino-*n*-butyric acid[12] presumably through interference in the utilization of β-alanine for the synthesis of pantothenic acid, may be considered a more direct analogy.

(32) Lipmann, Kaplan, Novelli, Tuttle and Guirard, *J. Biol. Chem.*, **167**, 869 (1947).

(33) Novelli and Lipmann, Abstracts Amer. Soc. Bact., Philadelphia, G-43 (1947); Lipmann, Kaplan and Novelli, *Fed. Proc.*, Part II, **5**, 272 (1947).

(34) Williams, in Evans, "Biological Action of the Vitamins," University of Chicago Press, Chicago, 1942, p. 122.

(35) Bonner and Erickson, *Amer. J. Bot.*, **25**, 685 (1938); Buchman and Richardson, THIS JOURNAL, **67**, 395 (1945).

pantothenic acid including phenylpantothenone,[36] N-pantoyl-β-aminoisobutyric acid[28,37] and N-pantoylisoserine.[28] The latter two compounds possessed weak growth-promoting properties in the absence of pantothenic acid, and in the presence of the nutrilite were inhibitory, but only to the level at which they had been stimulatory. Higher levels of the analog diminished the inhibitory effect. This type of behavior is comparable to that observed for ω-methylpantothenic acid particularly with *L. casei* at the 2 γ level of pantothenic acid. However, *L. casei* was not stimulated by the inhibitor in the absence of the nutrilite at any level tested.

Investigations of the pantothenic acid requirements and of the responses to ω-methylpantothenic acid for a large number of microorganisms (unpublished experiments) have shown that the analog inhibits only those bacteria which require the preformed nutrilite or are stimulated by it. Similar observations have been reported with other pantothenic acid analogs in cases of reversible inhibitions. It has been suggested[38] that the ineffectiveness of pantoyltaurine and related analogs in inhibiting microörganisms which might utilize pantoic acid or lactone may be due to the availability of these components in solution. This explanation is not tenable for ω-methylpantothenic acid although the possibility of inactivation of the analog through hydrolysis cannot yet be ruled out. However, it appears more likely that, as indicated by McIlwain,[8] pantothenic acid may be produced and utilized by these organisms in a form with which these analogs cannot compete.

Streptococci are susceptible to ω-methylpantothenic acid both *in vitro* and *in vivo*. In preliminary experiments it was found that mice were protected from an 80% fatal infection of a β-hemolytic streptococcus (Group A, type 23, no. 1072)[39] when the inhibitor was incorporated in a characterized diet at a molar analog–pantothenic acid ratio of 200 for four days prior to inoculation. Substituted amides of pantoyltaurine have been prepared which are effective against Group A *Streptococcus hemolyticus in vivo*.[40,41]

In view of the stereochemical specificity of the pantolactone moiety in pantothenic acid required for growth,[19] or in analogs which exhibit inhibitory activity,[14,16,41,42,43] it would be of interest to investigate the relative activities of the resolved isomers of ω-methylpantothenic acid. Presumably, at least one of the four possible isomers will show greater activity than the *dl*-compound.

(36) Woolley and Collyer, *J. Biol. Chem.*, **159**, 263 (1945).

(37) Pollack, THIS JOURNAL, **65**, 1335 (1943).

(38) Stansly and Alverson, *Science*, **103**, 398 (1946).

(39) Obtained from the collection of Dr. Alice C. Evans, National Institute of Health, through the courtesy of Dr. M. V. Veldee, Chief of the Biologics Control Laboratory (see Evans, *J. Immun.*, **46**, 399 (1943)).

(40) White, Lee, Jackson, Himes and Alverson, *Fed. Proc.*, Part II, **5**, 214 (1946).

(41) Winterbottom, Clapp, Miller, English and Roblin, THIS JOURNAL, **69**, 1393 (1947).

(42) Kuhn, Wieland and Moller, *Ber.*, **74**, 1605 (1941).

(43) Luts, Wilson, Deinet, Harnest, Martin and Freek, *J. Org. Chem.*, **12**, 96 (1947).

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Summary

ω-Methylpantolactone (α-hydroxy-β,β-dimethyl-γ-valerolactone) has been synthesized and condensed with β-alanine, taurine and L-leucine. It has been found that ω-methylpantothenic acid inhibits the growth of twenty-three strains of lactic acid bacteria which require pantothenic acid. That the inhibitory action is reversed competitively by pantothenic acid over wide ranges in concentrations has been shown with four lactic acid bacteria. The taurine analog has been shown to be less active than the β-alanine derivative, but more inhibitory than that containing L-leucine. The comparative activity of pantoyltaurine has been determined. The contributions of ω-methylpantothenic acid and pantoyltaurine to the activity of ω-methylpantoyltaurine have been discussed.