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

Inhibition of Lactic Acid Bacteria by ω-Substituted Analogs of Pantothenic Acid[1]

BY WILLIAM DRELL[2] AND MAX S. DUNN

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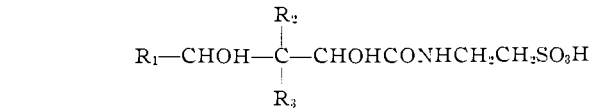
The following ω-substituted analogs of pantolactone have been synthesized, condensed with β-alanine and assayed with lactic acid bacteria: α-hydroxy-β-methyl-γ-valerolactone (ω-methylnorpantolactone), α-hydroxy-β-methyl-γ-caprolactone (ω-ethylnorpantolactone), α-hydroxy-β,β,δ-trimethyl-γ-caprolactone (ω-isopropylpantolactone), α-hydroxy-β,β-dimethyl-γ-(*o*-ethoxyphenyl)-γ-butyrolactone (ω-(*o*-ethoxyphenyl)-pantolactone), α-hydroxy-β-methyl-γ-phenyl-γ-butyrolactone (ω-phenylnorpantolactone). Also condensed with β-alanine and assayed for comparison in these series were α-hydroxy-γ-butyrolactone and α-hydroxy-γ-valerolactone. All analogs inhibited the growth of the lactic acid bacteria, the activity decreasing essentially in the order shown. With one exception, namely, the ω-(*o*-ethoxyphenyl) analog, all compounds exhibited competitive-type inhibition.

Two taurine analogs (ω-methylnorpantoyltaurine and ω-isopropylpantoyltaurine) have been tested and shown to be considerably weaker than either pantoyltaurine or the corresponding ω-substituted pantothenic acids. The lactic acid bacteria have been differentiated on the basis of their susceptibility to the two groups of compounds. The stimulatory response of some bacteria to sub-inhibitory levels of analogs has been discussed.

The relatively high activity of N-(α,γ-dihydroxy-β,β-dimethylvaleryl)-β-alanine (ω-methylpantothenic acid, referred to hereafter as ω-methyl-PA) (II) as a competitive inhibitor of pantothenic acid (PA) (I) in mice[3] as well as lactic acid bacteria[4] suggested a study of other ω-substituted analogs. By this means structure and activity might be correlated. Since it has been reported[5] that N-(α,γ-dihydroxy-β-methylbutyryl)-β-alanine (norPA[6]) (V) was without appreciable PA activity, it was of interest to compare the activity of its methyl homolog, ω-methyl-norPA (VI), with that of ω-methyl-PA. ω-Ethyl-norPA (VII), ω-isopropyl-PA (III), N-(α,γ-dihydroxybutyryl)-β-alanine (IX) and N-(α,γ-dihydroxyvaleryl)-β-alanine (X) were also prepared and tested. ω-Isopropylpantoyltaurine (XIII) and ω-methylnorpantoyltaurine (XIV), the sulfonic acid analogs of ω-isopropyl-PA and ω-methyl-norPA, respectively, were synthesized for comparison with other taurine derivatives, namely, pantoyltaurine (XI) and ω-methylpantoyltaurine (XII). ω-Methyl-norPA, ω-ethyl-norPA, N-(α,γ-dihydroxybutyryl)-β-alanine and N-(α,γ-dihydroxyvaleryl)-β-alanine have been reported previously to be without growth-promoting activity for *Lactobacillus casei*.[5,7]

Two ω-substituted aryl analogs, ω-(*o*-ethoxyphenyl)-PA (IV) and ω-phenyl-norPA (VIII) were synthesized for comparison with the ω-substituted alkyl derivatives. This study seemed of special interest in view of the relatively high inhibitory activity reported for phenylpantothenone in contrast to the small effect of the corresponding methylpantothenone.[8] ω-(*o*-Ethoxyphenyl)-pantolactone was prepared rather than the structurally simpler

	R_1	R_2	R_3
I	H	CH_3	CH_3
II	CH_3	CH_3	CH_3
III	$(CH_3)_2CH$	CH_3	CH_3
IV	*o*-$C_6H_4OCH_2CH_3$	CH_3	CH_3
V	H	CH_3	H
VI	CH_3	CH_3	H
VII	CH_3CH_2	CH_3	H
VIII	C_6H_5	CH_3	H
IX	H	H	H
X	CH_3	H	H



	R_1	R_2	R_3
XI	H	CH_3	CH_3
XII	CH_3	CH_3	CH_3
XIII	$(CH_3)_2CH$	CH_3	CH_3
XIV	CH_3	CH_3	H

ω-phenylpantolactone because of the difficulties described by Stritar[9] in the condensation of benzaldehyde with isobutyraldehyde, compared to the relatively successful condensation of *o*-ethoxybenzaldehyde with isobutyraldehyde observed by Herzog and Kruh.[10] Since Hackhofer[11] reported that benzaldehyde and propionaldehyde react in the expected manner, ω-phenylnorpantolactone was prepared for testing in the norPA series.

Experimental[12]

The lactones were prepared from the corresponding aldols essentially by the method described previously.[4] The pertinent data are summarized in Table I. The aryl analogs required special treatment during their preparation and are therefore described below in detail.

ω-(*o*-**Ethoxyphenyl**)-**pantolactone**.—A mixture of 120 g. (0.80 mole) of *o*-ethoxysalicylaldehyde (prepared from salicylaldehyde in 75% yield,[10] b.p. 92–94° (2 mm.), m.p. 20.0° after recrystallization and drying *in vacuo* over P_2O_5 at 4°) and 58.5 g. (0.82 mole) of freshly distilled isobutyr-

(1) Paper No. 96. For the preceding paper in this series (No. 82) see Drell and Dunn.[3] This work was aided by grants from the National Institutes of Health of the U. S. Public Health Service, the Nutrition Foundation and the University of California. A preliminary report was presented at the 115th Meeting of the Am. Chem. Soc., September, 1947.

(2) Predoctoral Fellow of the National Institutes of Health of the U. S. Public Health Service, 1947–1949.

(3) W. Drell and M. S. Dunn, *Arch. Biochem. Biophys.*, **33**, 110 (1951).

(4) W. Drell and M. S. Dunn, THIS JOURNAL, **70**, 2057 (1948).

(5) H. K. Mitchell, H. H. Weinstock, Jr., E. E. Snell, S. R. Stanbery and R. J. Williams, *ibid.*, **62**, 1776 (1940).

(6) Similar analogs are abbreviated in like manner.

(7) A. H. Nease, Dissertation, University of Texas, 1943.

(8) D. W. Woolley and M. L. Collyer, *J. Biol. Chem.*, **159**, 263 (1945).

(9) M. J. Stritar, *Monatsh.*, **20**, 617 (1899).

(10) O. Herzog and O. Kruh, *ibid.*, **21**, 1095 (1900).

(11) T. Hackhofer, *ibid.*, **22**, 95 (1901).

(12) The authors are grateful to Mr. I. Fatt and Mr. S. Thomas, Jr., for their assistance in preparing ω-methylnorpantolactone and ω-ethylnorpantolactone, respectively, and to Miss R. Malin for aid in many of the microbiological determinations. Melting and boiling points are uncorrected. C, H, Na and N analyses were carried out by Mrs. B. Kent, Mr. J. Murray and Dr. A. Elek, respectively.

TABLE I

ω-SUBSTITUTED ANALOGS OF PANTOLACTONE

Pantolactone	B.p. °C.	Mm.	M.p. or $n^{25}D$[a]	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Yield overall, %	Aldol condensation Precursors		Aldol B.p. °C.	Aldol B.p. Mm.	Aldol Yield, %	Ref.
ω-Methylnor-	110–112	4.5	1.4571	55.37	55.94	7.75	7.88	30	CH_3CHO	CH_3CH_2CHO	73–76	12	58	13
ω-Ethylnor-	120–121	2	1.4575[b]	58.31	58.20	8.39	8.41	25	CH_3CH_2CHO	CH_3CH_2CHO	84–86	12	31	13
ω-Isopropyl-			90.5–92.0	62.76	62.76	9.37	9.37	49	$CH(CH_3)_2CHO$	$CH(CH_3)_2CHO$	...	..	..[c]	..[c]
ω-(o-Ethoxyphenyl)-	194–197	1.5	119.5–120	67.18	67.03	7.25	7.40	54	$C_6H_4(OC_2H_5)CHO$	$CH(CH_3)_2CHO$	...	..	..	10
ω-Phenylnor-	170–174	1	80–81	68.73	68.53	6.30	6.66	34	C_6H_5CHO	CH_3CH_2CHO	...	..	..	11

[a] $n^{25}D$ for ω-methylpantolactone is 1.4581. [b] Since this result did not agree with a previously reported value,[7] it was checked with a redistilled sample. Found: C, 58.28; H, 8.43. [c] The aldol cyanohydrin is precipitated (61% yield) by the concerted reaction of KCN on isobutyraldehyde,[14,15] m.p. 155–156°. Calcd. for $C_9H_{17}O_2N$: N, 8.18. Found: N, 8.18. On a small scale when the large temperature rise on initial addition of KCN to the aldehyde could be controlled more readily and maintained below 10°, the yield was 85%.

aldehyde was stirred mechanically with an equal volume of 40% K_2CO_3 solution for 6 days at room temperature. Half of the upper viscous, yellow layer[16] was added to a hot solution (*ca.* 90°) containing 25 g. of $NaHSO_3$ in 50 ml. of water. Water (50 ml.) was added to the resulting thick paste and the mixture was allowed to stand overnight in the refrigerator. Ether (150 ml.) and water (190 ml.) were added to the solidified mixture,[17] which was then stirred mechanically until the precipitate completely dissolved. The lower aqueous phase was extracted twice with 100-ml. portions of ether and the ether extracts were added to the organic phase. After removal of the ether *in vacuo*, 200 ml. of a boiling solution containing 83 g. (0.8 mole) of $NaHSO_3$ was added to the remaining yellow viscous oil. The mixture, while being stirred for 0.5 hr., was heated on a water-bath. The mixture was cooled and allowed to stand overnight in the refrigerator.

To the cold two-phase system was added 95 ml. (0.8 mole) of a solution of KCN with mechanical stirring over a period of 45 min. while maintaining the temperature at 5–10°. Stirring was continued for 2.5 hours at 10° and an additional 2.5 hours at room temperature. The aqueous phase was separated and extracted twice with 125-ml. portions of ether and the ether extracts were added to the organic phase. This solution was added rapidly with mechanical stirring to 300 ml. of 12 *N* HCl maintained below 10°. Stirring was continued for an hour at 5° and 12 hours at room temperature. A cherry-red oil separated and a white solid (NH_4Cl) formed, which was redissolved by adding water (150 ml.). After removal of the ether, the resultant mixture was refluxed for 2.5 hours, neutralized to *p*H 7.2 and extracted 6 times with 150-ml. portions of ether. The ether was removed by evaporation, benzene was added, and the dark oil was dried by azeotropic distillation. The fraction distilling at 194–197° (1.5 mm.) was collected, yield 54 g. (54% based on half the initial amount of *o*-ethoxysalicylaldehyde). The yellow-colored lactone crystallized spontaneously on standing one week. The solid was triturated with a small amount of ether whereby most of the colored impurities dissolved. The undissolved solid was recrystallized twice from benzene. By slow evaporation of the ether solution additional crops were obtained.

From the aqueous phase, α-hydroxyisovaleric acid was recovered by acidification, ether extraction, evaporation and recrystallization from benzene and from ether–petroleum ether, yield 6 g., m.p. 83–84°.

(13) V. Grignard and P. Abelmann, *Bull. soc. chim. France*, [4] **7**, 638 (1910).

(14) L. Kohn, *Monatsh.*, **19**, 519 (1898).

(15) L. Claisen, *Ann.*, **306**, 322 (1899).

(16) Unsuccessful attempts were made to prepare the crystalline aldol reported by Herzog and Kruh.[10] After 12 months a small amount of white rosettes deposited in the oily product. The solid, m.p. 103–105°, obtained by recrystallizing this material from acetic acid–water (5:1) solution containing a trace of isobutyraldehyde, could not be identified. *Anal.* Found: C, 72.56; H, 8.54.

(17) The solid appeared to be the crystalline bisulfite addition product of *o*-ethoxysalicylaldehyde.[18] A small amount of the filtered solid was washed with ether and treated with strong acid whereupon the odor of SO_2 was noted. Extraction of the cloudy acid solution with ether followed by evaporation of the ether yielded a relatively nonvolatile colorless oil with the characteristic odor of the starting aldehyde.

(18) W. H. Perkin, *Ann.*, **145**, 301 (1868).

Anal. Calcd. for $C_5H_{10}O_3$: C, 50.84; H, 8.54. Found: C, 50.61; H, 8.32.

ω-Phenylnorpantolactone.—A mixture of 174 g. (3.0 moles) of redistilled benzaldehyde, 450 ml. of saturated K_2CO_3 solution and 40 ml. of water was stirred mechanically under nitrogen for 9 hr. at 22–24°. The yellow viscous aldol was separated with the aid of ether (200 ml.) from the aqueous layer and the latter extracted again with 100 ml. of ether. The combined aldol–ether solution was washed once with *N* acetic acid solution (125 ml.), and once with saturated $NaHCO_3$ solution (150 ml.). The ether was removed *in vacuo* and the aldol added with mechanical stirring to 650 ml. of a hot solution containing 312 g. (3.0 moles) of $NaHSO_3$. Upon cooling the mixture to 10° a viscous semi-solid mass was obtained, to which was added over a period of an hour 350 ml. of a solution of KCN (3.0 moles) while maintaining the temperature below 15°. Stirring was continued for an hour following complete solution of the solid. The orange-yellow cyanohydrin layer was separated with the aid of ether and the aqueous phase was extracted twice with 150-ml. portions of ether. The combined ether and cyanohydrin solution was added with stirring to 900 ml. of 12 *N* HCl at 5° and allowed to stand overnight. After filtration, to remove NH_4Cl, and evaporation of the organic solvent, the acid solution was refluxed for 3 hours, resulting in a 2-phase system. The mixture was neutralized to *p*H 7.2 and extracted 9 times with 200-ml. portions of benzene.[19] The organic extract, after removal of most of the benzene, was heated in a boiling water-bath 0.5 hr. with 1400 ml. of 1.7 *N* NaOH. The basic solution was extracted with benzene (five 200-ml. portions) until no further color was removed and the benzene extracts were discarded. Upon acidification to *p*H 3, a heavy oil separated. The mixture was heated for one hour on a boiling water-bath (to complete lactonization). The aqueous phase was separated from the oil phase and extracted 5 times with 200-ml. portions of benzene. The extracts were added to the organic layer. The benzene was removed by distillation at atmospheric pressure and the lactone was distilled *in vacuo*. The fraction distilling at 170–174° (1 mm.) was collected, yield 177 g. (34% based on the initial benzaldehyde). The light yellow sirup crystallized spontaneously on standing for about 50 days. It was recrystallized twice from an ether–petroleum ether mixture.

Preparation of the ω-Substituted PA Analogs.[20]—Each crude compound was prepared by fusion of the sodium salt of β-alanine or taurine with the appropriate lactone in an oil-bath at 115–125° for 2–3 hours (method A of Table II). Each analog was purified, where quantities permitted, by dissolving the freshly condensed compound in absolute ethanol, filtering the solution and precipitating the product with absolute ether (method B). All compounds so prepared were quite hygroscopic and in some cases precipitated as gums, solid being obtained only as a second crop on the addition of more ether to the mother liquor. The taurine analogs were more easily isolated than the corresponding β-alanine derivatives. The samples were dried to constant

(19) Mandelic acid (40 g., m.p. 116°) was isolated from the residual aqueous solution after acidification, extraction with ether and crystallization from benzene.

(20) α-Hydroxy-γ-valerolactone and α-hydroxy-γ-butyrolactone were obtained from the collection of Dr. R. J. Williams through the courtesy of Dr. W. Shive of the University of Texas. These compounds were received and preserved in sealed glass ampoules.

TABLE II

ω-SUBSTITUTED ANALOGS AND DERIVATIVES OF PANTOTHENIC ACID

	Analog (sodium salt)	Condensation Method[b]	% [c]
III[a]	ω-Isopropylpantothenic acid	A	36
		B	67
IV	ω-(*o*-Ethoxyphenyl)-pantothenic acid	B	83
VI	ω-Methylnorpantothenic acid	A	72
		B	100[d]
VII	ω-Ethylnorpantothenic acid	A	83
		B	89
VIII	ω-Phenylnorpantothenic acid	B	94
IX	N-(α,γ-dihydroxybutyryl)-β-alanine	A	59
X	N-(α,γ-dihydroxyvaleryl)-β-alanine	A	56
XIII	ω-Isopropylpantoyltaurine	A	45
		B	94
XIV	ω-Methylnorpantoyltaurine	B	90

[a] See Fig. 1 for structures. [b] See Experimental for conditions. [c] Method A: based on Van Slyke amino nitrogen determinations. The percentage of amino nitrogen is a measure of the uncondensed amino acid. The values represent maximum impurities since some hydrolysis occurs during such analyses. Method B: calcd. from the N values (Kjeldahl) corrected for the difference in molecular weight between the compound and the principal impurity (sodium salt of β-alanine). See Barnett and Robinson[21] for method and justification. [d] Calcd. for $C_9H_{16}O_5NNa$: N, 5.81; Na, 9.54. Found: N, 5.80; Na, 9.48.

weight *in vacuo* over P_2O_5 at about 70° and were analyzed for N and Na (Table II). In one case (ω-ethyl-nor PA, dried for 5 days), the analyses suggested that a compound containing ethanol of crystallization had been isolated.[22]

The condensation by method A of ω-isopropylpantolactone with β-alanine was investigated under varying conditions of temperature, time and excess of lactone without improving the purity of the resulting product. While β-alanine was non-toxic to the organisms studied at concentrations as high as 10 mg. per tube, ω-isopropylpantolactone was slightly inhibitory at levels of 5 mg. per tube and much more so at 10 mg. per tube. Therefore, in the assay of ω-isopropyl-PA prepared by method A, the excess and unreacted lactone was first extracted from the neutralized aqueous solution with ether.

Testing Procedure.—The method has been described previously.[4]

Results

The response of the four lactic acid bacteria, previously studied in detail with ω-methyl-PA,[4] to the described analogs is summarized in Table III. At least two levels of PA were used with each compound. ω-Isopropyl-PA, ω-methyl-norPA, ω-ethyl-norPA and ω-phenyl-norPA ranked well with ω-methyl-PA in inhibitory activity toward *L. casei*. However, ω-(*o*-ethoxyphenyl)-PA was considerably less active. ω-Methyl-norPA retained a high order of activity against *L. arabinosus* but was much less inhibitory toward the other two organisms. The other analogs possessed substantially less activity in all other instances. Stimulation[24] at concentrations below the inhibitory level was noted for *L. fermenti* and *L. mesenteroides* in almost all instances, but none was observed with the other two organisms.

TABLE III

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

Analog[b]	Ca *d*-pantothenate, γ per tube	Antibacterial index[a] *L. casei* (7469[c])	*L. arabinosus* 17-5 (8014[c])	*L. fermenti* 36 (9338[c])	*L. mesenteroides* P-60 (8042[c])
III	0.02	1600	32000[f]	190000[d,f]	>100000[d]
	.06	2100	45000[f]	70000[e]	
	.20	1600	34000[f]		
IV	.02	18000[e]	300000	330000[f]	330000
	.06	18000[e]	.....	170000[e]	110000
	.20	15000[e]	38000		33000
VI	.02	1000	9000	28000[d,f]	40000[d,f]
	.06	1000	7000	60000[d,f]	100000[d,f]
	.20	1400	15000	45000[d,f]	70000[d,f]
VII	.02	3300	120000	370000[d,f]	>800000[d]
	.06	4200	110000	270000[d,f]	
	.20	3300	100000		
VIII	.02	750	600000[g]	900000[e]	>750000[d]
	.06	750	210000[e]	>300000[d]	
	.20	750			
IX	.02	53000	17000	200000[f]	330000[f]
	.06	85000	18000		
X	.02	35000	60000	600000[d,f]	>600000[d]
	.06	40000	60000		
XIII	.02	700000[e]	>700000	>700000[d]	>700000[d]
XIV	.02	200000[e]	300000[e]	>450000[d]	>450000[d]

[a] Based on average values for duplicate tubes corrected for percentage condensation of the *dl*-analogs. [b] See Table II for corresponding compounds. [c] American Type Culture Collection number. [d] Stimulatory[24] at concentrations below the inhibitory range. [e] Half-maximum inhibition. [f] The half-maximum point was achieved at a concentration of analog approximately half that required for complete inhibition. [g] The corrected ratio at half-maximum inhibition was 210000.

All compounds with one exception exhibited essentially constant antibacterial indices with increasing PA concentrations indicating competitive inhibition. However, ω-(*o*-ethoxyphenyl)-PA, when assayed with either *L. arabinosus* or *L. mesenteroides* but not the other two bacteria elicited an inhibitory response which was dependent on the concentration of analog and independent of the PA level. This non-competitive response was evident whether results were evaluated on the basis of half-maximum inhibition, or complete inhibition.

Some of these analogs[25] were also investigated with the nineteen lactic acid bacteria used in the previous study (Table II of ref. 4). For ease of comparison to this study the organisms are listed in Table IV in the same order. As noted above, ω-methyl-norPA was the most active analog of the group. Stimulation was produced by these analogs at levels below the inhibitory range with twelve organisms.[26] Three organisms, *L. dextranicum* 8086, *L. pentosus* and *L. brassicae* were not stimulated at lower levels of these analogs; the

(21) J. W. Barnett and F. A. Robinson, *Biochem. J.*, **36**, 357, 364 (1942).

(22) The isolation of sodium ω-methylpantothenate with 2-propanol of crystallization has been described previously.[4] The alcohol was removed completely only after drying *in vacuo* at 70° for two weeks. Levy, *et al.*,[23] reported the isolation of calcium pantothenate with 2-propanol of crystallization which could not be removed by drying at 100°.

(23) H. Levy, J. Weijlard and E. Stiller, THIS JOURNAL, **63**, 2846 (1941).

(24) A stimulatory response was considered significant if the average base consumption was increased 10% or more above that of the control (without added analog) at two or more consecutively increasing levels of analog.

(25) The analogs used for testing in this group were, with the exception of ω-methylnorpantoyltaurine, prepared by method A, whereas in testing the four organisms of Table III compounds (with the exception of ω-isopropyl-PA) prepared by method B were used. ω-Methyl-norPa has been assayed as a crude (method A) as well as a purified preparation and its corrected activities agree satisfactorily in such experiments.

(26) Five of these organisms have previously exhibited stimulatory behavior.[4]

TABLE IV

ACTIVITY OF ANALOGS OF PANTOTHENIC ACID AGAINST LACTIC ACID BACTERIA

Organism		Antibacterial index[a] III[c]	VI[c]	VII[c]	XIV[c]
Leuconostoc citrovorum	8082[b]	900[f]	150[d,f]	1200[d,f]	250000[d,f]
Lactobacillus fermentatus	4006	300	150	1600	220000[e]
Lactobacillus pentoaceticus	367	4000	1200	7700	[h]
Lactobacillus brevis	8287	1500	150	2300	150000[e]
Leuconostoc citrovorum	797	60000[d,f]	500[d,f]	30000[d,f]	[d,h]
Leuconostoc citrovorum	7013	50000	500[i]	30000	150000[d,e]
Streptococcus faecalis R.	8043	30000	600	11500	170000[e]
Lactobacillus helveticus	335	2000[d]	75	5100	270000[d,e]
Lactobacillus helveticus	6345	5000	600	7700[d]	[d,h]
Lactobacillus lycopersici	4005	[g]	20000[d,f]	180000[e]	[h]
Leuconostoc dextranicum	8358	65000[d]	400	51500[d,f]	12500[d]
Leuconostoc dextranicum	8086	50000	2200	25000	75000
Leuconostoc mesenteroides	9135	75000[d,f]	13000[d,f]	40000[d,f]	35000[d]
Leuconostoc mesenteroides	8293	60000[d,f]	700	30000[d,f]	250000[d,e]
Lactobacillus gayonii	8289	110000[d,f]	10000[d,f]	205000[d,f]	[d,h]
Leuconostoc dextranicum	8359	100000[d,f]	1300[d]	100000[d,f]	23000[d]
Lactobacillus pentosus 124-2	[i]	[g]	10000[f]	180000[e]	[h]
Lactobacillus brassicae	8041	[g]	45000	220000	[h]
Lactobacillus mannitopoeus	[j]	100000[d,e]	12000[d,f]	140000[d,e]	[d,h]

[a] Based on average values of duplicate tubes corrected for percentage condensations of the *dl*-analogs. Each tube contained 0.06 μg. of calcium *d*-pantothenate. [b] American Type Culture Collection number. [c] See Table II for corresponding compounds. [d] Stimulatory[24] at concentrations below the inhibitory range. [e] Half-maximum inhibition. [f] The half-maximum inhibition point was achieved at a concentration of analog approximately half that required for complete inhibition. [g] The half-maximum inhibition point had not been reached at a corrected analog–metabolite ratio of 100,000. [h] The half-maximum inhibition point had not been reached at a corrected analog–metabolite ratio of 250,000. [i] Obtained through the courtesy of Dr. E. E. Snell. [j] Obtained through the courtesy of Dr. V. H. Cheldelin.

remaining organisms' responses could not be evaluated because of lack of data.

The inhibitory response of these bacteria to ω-methylpantoyltaurine was, with the exception of four organisms, considerably less than to any of the other analogs. ω-Isopropylpantoyltaurine was even less inhibitory. Half-maximum inhibition ratios under 100,000 (corrected) were obtained for the compound with only two of the nineteen organisms of Table IV, namely, *L. citrovorum* 8082 (70,000) and *S. faecalis* R. (90,000). In all cases where stimulation was noted for ω-methylnorpantoyltaurine (footnote *d* of Table IV) it was observed for the ω-isopropyl derivative. Conversely, lack of stimulation with the former correlated with lack of stimulation with the latter.

Discussion

It is evident from the results of the present investigation and a previous study[4] that ω-methyl-PA was the most inhibitory analog of pantothenic acid. This finding was not unexpected since ω-methyl-PA is most closely related structurally to the vitamin. However, it was somewhat surprising that analogs lacking a β-methyl group in the lactone moiety had high inhibitory activity since norPA lacks growth-promoting ability.[5] It appears that the effect of β-substitution is less profound than that of other structural changes since N-(β-hydroxypantoyl)-β-alanine[27,28] and N-(β-methylpantoyl)-β-alanine,[29] compounds with modified β-methyl groups, are the only known analogs with significant growth-promoting properties. This view appears to be supported by the observation that N-(α,γ-dihydroxyvaleryl)-β-alanine and N-(α,γ-dihydroxybutyryl)-β-alanine, compounds lacking both β-carbon substitutents, possess inhibitory activity.

The high inhibitory activity of ω-substituted norPA's, compounds with an additional center of asymmetry, indicates that the configuration about the lactone β-carbon may be relatively unimportant compared to the γ-carbon.[30] Wieland and Möller[29] reported only small differences in growth-promoting properties between the (−)-N-(β-methylpantoyl)-β-alanine and the (−)-*allo* compound as well as in growth-inhibiting properties between the corresponding taurine analogs.

The non-competitive inhibition of *L. arabinosus* and *L. mesenteroides* by ω-(*o*-ethoxyphenyl)-PA is of interest in view of the competitive response of *L. arabinosus* to ω-phenyl-norPA.

Classification of the lactic acid bacteria which have been studied reveals that the most clearly delineated group is comprised of those organisms (*L. dextranicum* 8358, 8086 and 8359; *L. mesenteroides* 9135 and 8293) which are readily susceptible to pantoyltaurine[4] and to taurine analogs with a modified pantoyl moiety. In contrast, there is a group of organisms (*L. mesenteroides* P-60, *L. fermenti, L. gayoni, L. pentosus, L. brassicae* and *L. mannitopoeus*) which is relatively indifferent to pantoyltaurine and is unaffected by ω-substituted analogs of pantoyltaurine. Of the 23 bacteria in-

(27) H. K. Mitchell, E. E. Snell and R. J. Williams, THIS JOURNAL, **62**, 1791 (1940).

(28) E. Zachiesche and H. K. Mitchell, *Proc. Soc. Exp. Biol. Med.*, **45**, 565 (1940).

(29) T. Wieland and E. F. Möller, *Ber.*, **81**, 316 (1948).

(30) In preliminary experiments in this Laboratory Mr. Richard Zuckerman found that ω-methylpantoic acid, prepared from freshly-distilled (but uncrystallized) ω-methylpantoyl lactone, exhibited more inhibition toward *Acetobacter suboxydans* than that prepared from the crystalline lactone, m.p. 60°.[4]

vestigated, seven (*L. casei, L. arabinosus, L. pentosus, L. brassicae, L. fermentatus, L. brevis* and *L. pentoaceticus*) showed no stimulation at concentrations of analogs below the inhibitory range. In almost all of the other cases stimulation was noted with all of the analogs.[31]

(31) The data were insufficient to classify *S. faecalis* R. while *L. dextranicum* 8086 gave only a slight stimulation. *L. lycopersici* was the only organism which was stimulated by some but not all analogs.

LOS ANGELES, CALIFORNIA

[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, UNIVERSITY OF UTAH]

The Binding of Uncharged Molecules to Proteins. II. Testosterone and Bovine Serum Albumin[1,2]

BY JOHN A. SCHELLMAN,[3] RUFUS LUMRY[4] AND LEO T. SAMUELS

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The binding reaction between bovine serum albumin and testosterone has been investigated as a function of steroid concentration, protein preparation, temperature and hydrogen-ion concentration by means of dialysis and partition techniques. The data are interpreted in terms of a linear relation between the reciprocal of the average number of molecules of substrate bound per protein molecule and the reciprocal of substrate concentration. This relation, which introduced the average association constant and a measure of the heterogeneity of binding sites, is shown to apply generally in cases of low substrate concentration. Total binding increases with purity of preparation and with temperature. The amount of binding increases with *p*H and is reduced in the presence of thiocyanate ion, methyl orange anions and zinc cations. The free energy of binding is the same as observed with charged and uncharged organic molecules. The enthalpy change is positive so that binding depends on a large positive entropy change. An explanation for this fact, and in general for "configurational adaptability," is discussed as the result of local relaxation in the "tertiary" folding of the protein. Rough data for the binding of cortisone and estradiol are given and a tentative correlation of the various observations is presented.

In another publication we have shown that a large variety of steroids form stable complexes with serum albumin.[5] These studies plus those of Klotz, *et al.*, on substituted azo benzenes,[6] adenosine[7] and sulfonamides[8]; Bischoff and co-workers on steroids[9]; and the work of Carsten and Eisen[10] on neutral dinitrophenyl compounds have established the binding of uncharged organic molecules to serum albumin as a quite general phenomenon. In the present paper we attempt to correlate the binding of steroids with that of anions to determine the mode of interaction, and to indicate the significance of the empirical isotherm. Since it is known that commercial serum albumin contains traces of strongly bound small molecules varying with the method of preparation,[11] the results reported here may not necessarily be compared with those obtained using other preparations. To some extent the studies reported here are of but relative quantitative significance just as are most other studies with serum albumins.

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(3) United States Public Health Service Fellow.

(4) Merck Fellow in the Natural Sciences during part of this investigation. School of Chemistry, University of Minnesota, Minneapolis, Minn.

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Experimental

The determination of an accurate isotherm in these binding studies, as in other adsorption studies, requires the determination of free and combined substrate over a considerable range of concentration. For this reason two experimental techniques have been developed for use with steroids and similar molecules: dialysis equilibrium such as employed by other investigators in protein binding[12]; and partition analysis, a technique previously employed by Karush[13] in dye-binding studies.

Dialysis Equilibrium.—The procedure is similar to that described by Klotz, Walker and Pivan.[12] A solution of the steroid in Locke buffer[5] (ionic strength 0.155 *M*) was placed outside the dialysis bag (Visking) and a solution of albumin in the same buffer was placed within. Equilibrium was secured by rotating the sample tubes, which were completely filled to prevent protein denaturation at the air interface, in a constant temperature water-bath. Experiments demonstrated that equilibration was complete in 24 hours. Aliquots of the solutions from inside and outside the bag were extracted five times with ethyl ether. The ether was evaporated under an air jet, the steroid taken up in absolute ethanol and the ultraviolet absorption measured on a Cary spectrophotometer over the range of 2200–3000 Å. The non-linear background spectrum, probably the result of extracted phosphate, could be eliminated by the empirical functions

$$\Delta_{2400} = 2.14D_{2400\text{ Å.}} - (D_{2300\text{ Å.}} + D_{2500\text{ Å.}}) \text{ for testosterone}$$

$$\Delta_{2360} = 2.0D_{2360\text{ Å.}} - (D_{2260\text{ Å.}} + D_{2460\text{ Å.}}) \text{ for cortisone}$$

The D's are the optical densities at the indicated wave lengths and Δ is a factor proportionate to the concentration of the steroid at the wave length of maximum absorption in ethanol. Assuming that the protein does not affect the activity of the free steroid, the free steroid in the bag may be equated to the steroid concentration outside and the bound steroid to the difference between this and the total concentration inside the bag. All dialysis experiments were performed on 1% protein solutions. No appreciable changes in the volume contained in the dialysis bag occurred during the experiments as judged by experiments with partially filled dialysis tubes.

Partition Analysis.—The substrate was equilibrated in a two-phase system consisting of the protein solution and an

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