[CONTRIBUTION FROM THE CLAYTON RESEARCH FOUNDATION AND THE BIOCHEMICAL INSTITUTE, THE UNIVERSITY OF TEXAS]

Growth Effects of α-Methyl Homologs of Pantothenic Acid and β-Alanine

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It has been found that even slight modification of the structure of the molecule of pantothenic acid results in practically complete loss of physiological effect.[2-6] The greatest activity shown by any of its homologs was 1 to 23% by "hydroxypantothenic acid," a compound in which one of the terminal methyl groups of pantothenic acid may be considered to be replaced by a hydroxymethyl group.[7]

It seemed to us that the introduction of a methyl group in the alpha position on the β-alanine part of the molecule might be innocuous enough so that the resulting compound, "α-methylpantothenic acid," might have about the same order of activity as pantothenic acid.

To this end, α-methyl-β-alanine was prepared and condensed in the form of the sodium salt with α-hydroxy-β,β-dimethyl-γ-butyrolactone, thus obtaining the sodium salt of a α-methylpantothenic acid as a very hygroscopic solid.

The amino acid was obtained by condensing α-chloropropionic acid with sodium cyanide, whereby the α-cyanopropionic acid resulted. This was esterified, hydrogenated in the presence of platinum oxide, and finally hydrolyzed to the desired α-methyl-β-alanine. Since β-alanine is a strong growth-promoting principle in its own right,[8] it was also interesting to determine the effects of the α-methyl homolog on the growth of yeast.

Experimental

Ethyl α-Cyanopropionate.—An aqueous solution of 1.06 moles of sodium chloropropionate in 140 ml. of water was prepared using 115 g. of α-chloropropionic acid and 58 g. of sodium carbonate. To this was added carefully a solution of 59 g. (1.16 moles) of sodium cyanide in 150 ml. of water, and the whole was heated with continuous stirring to 85°. At this point, an exothermic reaction occurred which raised the temperature just about to the boiling point where it maintained itself for ten minutes before beginning to cool. A temperature of 100° was maintained by external heating for twenty minutes before cooling. The dark brown reaction mixture was filtered to remove sludge, and the filtrate was acidified by the dropwise addition of 100 ml. of 37% hydrochloric acid.

The reaction mixture was concentrated to a thick sludge by evaporation on a water-bath at 55–65° under reduced pressure, followed by the addition of alcohol and further evaporation to remove water. The residue (wt. 94 g.) was esterified by heating with anhydrous ethanol in the presence of sulfuric acid as catalyst. The acid was then neutralized by aqueous sodium carbonate and the ester was extracted with ether. After drying, the ether was removed by distillation and the residue was distilled under reduced pressure; 25.5 g. of ethyl α-cyanopropionate was obtained with the physical constants: b. p. 77° (9.5 mm.); d^{23}_4 1.006; $n^{28.5}_D$ 1.4104.

α-Methyl-β-alanine.—Twenty-four and seven tenths grams of ethyl α-cyanopropionate (0.194 mole) was mixed with a solution of 7.3 ml. of concd. sulfuric acid in 290 ml. of glacial acetic acid. To this was added 2.2 g. of Adams catalyst (platinum oxide), and the mixture was hydrogenated by shaking in a bomb at room temperature for seventy-five minutes under an initial pressure of 530 lb. of hydrogen.

The spent catalyst was removed by decantation and the acetic acid was removed by distilling as far as possible on a water-bath under the suction of a water pump. The residue was evaporated repeatedly in this fashion after the addition of water in order to effect hydrolysis and to remove ethanol and acetic acid. The sulfuric acid was then removed with barium hydroxide, and the remaining solution was concentrated by evaporation until crystals appeared. These crystals were filtered and recrystallized from water. In this way, large, colorless truncated tetragonal pyramids were obtained which were powdered and dried *in vacuo*. The melting point was 181–182°, and a mixed m. p. with β-alanine (m. p. 198–200°) was 160°. The total yield was 14.6 g. (73%). In a semi-quantitative formol titration, 9.6 ml. of 0.1 *N* sodium hydroxide was required to restore the phenolphthalein end-point to an aqueous solution of 0.1 g. of the amino acid (10 ml. of the alkali is the theoretical). *Elemental Analysis.*[9] Calcd.: C, 46.64; H, 8.81; N, 13.60. Found: C, 45.89; H, 8.26; N, 12.94.

Sodium α-Methylpantothenate.—The sodium salt of α-methyl-β-alanine was prepared by mixing 2.5 g. of the amino acid with a solution of an equimolar amount of sodium hydroxide in 8 ml. of water. Complete solution occurred on heating and this solution was evaporated nearly to dryness on a steam-bath in a stream of air. Drying was completed *in vacuo* over concentrated sulfuric acid, during which the salt was powdered.

One-hundredth mole of this salt was mixed with 1.30 g.

(1) Present address: E. F. Drew and Co., Inc., Boonton, N. J.

(2) (a) Mitchell, Weinstock, Snell, Stanberry and Williams, THIS JOURNAL, **62**, 1776 (1940); (b) Subbarow and Rane, *ibid.*, **61**, 1616 (1939).

(3) Reichstein and Grüssner, *Helv. Chim. Acta*, **23**, 650 (1940).

(4) Weinstock, *et al.*, *J. Biol. Chem.*, **135**, 343 (1940).

(5) Woolley and Hutchings, *J. Bact.*, **38**, 285 (1939).

(6) McIlwain, *Biochem. J.*, **36**, 417–427 (1942).

(7) Mitchell, Snell and Williams, THIS JOURNAL, **62**, 1791 (1940).

(8) Williams and Rohrmann, *ibid.*, **58**, 695 (1936).

(9) By C. Tiedcke, 366 Fifth Avenue, New York, N. Y.

TABLE I

STIMULATORY GROWTH EFFECTS

Compound	Amount added, γ	Cell production[a]					Acid production[b]		
		Lactobacillus casei[c]	*Streptococcus lactis R*[c]	*Lactobacillus arabinosus* 17.5[c]	Gebrüder Mayer yeast[d]	Fleischmann's Baker yeast[d]	*Lactobacillus casei*[c]	*Streptococcus lactis R*[c]	*Lactobacillus arabinosus* 17.5[c]
Blank	0	3.0	12.0	8.4	9.5	16.2	0.96	0.71	0.74
α-Methylpantothenic acid	0.1		11.8	7.8		16.5	0.97	.96	.84
	1.0		11.8	8.8	11.9	16.8	1.03	.83	.93
	10	5.4	17.2	11.3	21.0	26.6	1.11	1.08	1.51
	100	7.2	38.2	16.0	90.4	91.6	1.94	2.14	7.88
	500	8.7							
	1000	9.6			96.8				
Pantothenic acid	0.01	6.7	29.1	13.0	12.9	19.3	1.24	1.69	1.33
	.02		30.4	18.8			1.30	2.01	2.52
	.05	25.8	38.0	32.2	33.6	38.3	2.10	2.53	4.96
	.10	59.6	59.7	55.5	58.2	68.2	4.40	3.92	8.53
	.20	70.2			89.4	93.7			
α-Methyl-β-alanine	1.0				10.7				
	10	2.0			10.3				
	100	2.2			12.3				
	500	2.3							
	1000	2.2			62.4				
β-Alanine	0.1				16.8				
	.3				25.4				
	.5				45.6				
	1.0				83.6				

[a] Readings of the turbidimeter galvanometer. [b] Ml. of 0.1 *N* NaOH required to neutralize the acid produced (brom thymol blue end-point). [c] The medium used was that of Pennington, Snell and Williams, *J. Biol. Chem.*, **135**, 213 (1940). [d] The medium used was that of Snell, Eakin and Williams, THIS JOURNAL, **62**, 175 (1940), except that the β-alanine was omitted, and 0.2 γ of biotin was added per liter of medium.

(0.01 mole) of levo-α-hydroxy-β,β-dimethyl-γ-butyrolactone (Merck), and 8.0 ml. of isopropyl alcohol was added. The mixture was brought to reflux and, since complete solution did not occur, 4.0 ml. of ethanol was added. On gentle refluxing for three hours, a small amount of powder was still insoluble. The mixture was filtered hot, and the residue was washed with 4 ml. of isopropyl alcohol, which was added to the filtrate, and the solution was allowed to stand at room temperature. No precipitate appeared, and sodium α-methylpantothenate was finally thrown out of solution by the addition of 3–4 times the volume of petroleum ether. The precipitate was redissolved in isopropyl alcohol and reprecipitated with petroleum ether. The solvents were decanted and the residue was dried *in vacuo* over concentrated sulfuric acid. The dried salt (2.3 g.) was powdered and analyzed.[9] The compound was very hygroscopic and the analyst found it extremely difficult to handle. Nevertheless, the analytical results indicate that the material was essentially the desired sodium α-methylpantothenate. Calcd.: Na, 9.01; N, 5.49; C, 47.06; H, 7.11. Found: Na, 8.36; N, 4.73; C, 46.30; H, 5.43.

Sodium α-methylpantothenate was also prepared by fusing the proper proportions of the lactone and the sodium salt of α-methyl-β-alanine without the use of solvents. Heating was carried out at 108° for one and one-half hours, and the product was a colorless glassy mass, which dissolved rapidly in water and produced the same growth effects as the product obtained above.

Results

1. Growth-Promoting Effects.—To determine whether α-methylpantothenic acid had the growth-promoting action of pantothenic acid, graded amounts of the former were added to growth media which were complete except for the absence of pantothenic acid, and these media were then inoculated with different bacteria and yeasts. The results are shown in Table I. In all cases, blanks and standards, in which graded amounts of pure pantothenic acid were added to the media, also were run. In most cases, the results were evaluated by comparing the turbidities of the cultures quantitatively[10] after fifteen to twenty-four hours of growth, but in the instances of the acid-producing bacteria, the total acid productions were also determined after a three-day growth period.

It can be seen from Table I that α-methylpantothenic acid does possess the ability to replace pantothenic acid to some extent in every instance tested, but that much larger doses are

(10) Williams, McAlister and Roehm, *J. Biol. Chem.*, **83**, 315 (1929).

necessary. In terms of relative potencies, it may be stated that for the acid-producing bacteria, α-methylpantothenic acid was only 0.0001–0.001 as potent as pantothenic acid. This was true whether the results were compared on the turbidimetric or titrimetric bases. The compound appeared more powerful in the case of the yeasts, the figures being 0.002–0.003 for relative potency in both cases.

The possibility that this growth effect could be due to hydrolysis of α-methylpantothenic acid by the organisms followed by utilization of the lactone moiety was tested by determining the growth effect of the pure lactone on *Lactobacillus casei* and Gebrüder Mayer yeast. Some growth promotion was observed, but the effects were too small to account for the results obtained with α-methylpantothenic acid. The relative potencies of the lactone for the bacteria and the yeast were, respectively, 0.000007 and 0.00001.

Table I also shows the comparative growth-promoting action of β-alanine and its α-methyl homolog for Gebrüder Mayer yeast. Again, the α-methyl homolog yielded a growth effect, and again the effect was much smaller than realized with the unsubstituted growth principle. The relative potency of α-methyl-β-alanine in terms of β-alanine was 0.0006.

2. Antagonistic Growth Effects.—Since α-methylpantothenic acid and α-methyl-β-alanine were not particularly powerful growth-promoting principles, it was decided to determine whether or not they would interfere with the stimulating actions of the unsubstituted homologs. To this end, increasing doses of the substituted compounds were added to media containing pantothenic acid or β-alanine, and the results are shown in Table II.

It is at once clear that the presence of large amounts of α-methylpantothenic acid in the medium represses the growth-promoting action of pantothenic acid for *Lactobacillus casei*. Furthermore, it appears that this repression increases with concentration of the α-methyl homolog only up to a point, beyond which further addition leads to somewhat increased growth and acid production.

In the case of Gebrüder Mayer yeast, the addition of α-methylpantothenic acid to a medium containing pantothenic acid produced no regression, but instead a regularly increased stimulation.

TABLE II

ANTAGONISTIC GROWTH EFFECTS

Pantothenic acid, γ	α-Methylpantothenic acid, γ	Growth of *Lactobacillus casei* Cell production	Growth of *Lactobacillus casei* Acid production	Growth of G.M. yeast Cell production
0.05	0	25.8		
	1	24.8		
	5	19.3		
	10	17.7		
	50	11.2		
	100	8.2		
	200	10.6		
	500	11.3		
	1000	11.8		
0.20	0	75.2	10.16	
	1			82.2
	5			82.1
	10	78.3	9.84	83.8
	25			84.9
	50	44.3	8.38	85.1
	100	32.0	7.74	85.3
	500	23.0	7.34	
	1000	20.8	6.69	
	5000	20.1	7.02	
	10,000	22.0	7.63	
β-Alanine (γ)	α-Methyl-β-alanine (γ)			
1.0	1			84.2
	5			78.2
	10			76.4
	50			82.4
	100			82.5
	200			86.4
	500			87.7
	1000			88.1
	10,000			

On the other hand, another minimum growth point was shown by cultures of Gebrüder Mayer yeast containing increasing amounts of α-methyl-β-alanine and a constant amount of β-alanine.

3. Reversal of Antagonistic Growth Effect.—It was further found that the antagonistic action of α-methylpantothenic acid on the stimulatory effect of pantothenic acid for *Lactobacillus casei* could be overcome by adding larger amounts of pantothenic acid to the cultures. This was

TABLE III

REVERSAL OF ANTAGONISTIC GROWTH EFFECT

α-Methylpantothenic acid, γ	Pantothenic acid, γ	Growth of *Lactobacillus casei* Cell production	Growth of *Lactobacillus casei* Acid production
10,000	0	18.5	5.48
	0.05	21.2	6.83
	.10	20.7	7.07
	.20	20.2	7.94
	.50	19.6	6.92
	1.0	22.7	6.81
	5.0	27.0	7.93
	10.0	31.0	8.29
	25.0	47.9	9.68

true for both cell growth and acid production, as shown in Table III. It will be noted that there was a small increase in growth with the addition of the first small quantities of pantothenic acid, followed by a slight decrease, and then by a much larger increase. Since this same effect was also observed in the three-day acid production test, it is probably significant, although difficult to interpret at present.

Discussion

The results show that α-methylpantothenic acid and α-methyl-β-alanine are growth stimulants, but that, under certain conditions, their presence may lead to growth inhibitory effects. In the absence of pantothenic acid or β-alanine, the α-methyl homologs are capable of acting as substitutes, but much larger amounts are necessary than would be required of the unsubstituted growth factors. However, in the presence of pantothenic acid and β-alanine, it appears that large amounts of the α-methyl compounds may produce serious disturbances in certain cases, leading to actual repressions of growth. It was shown in the case of *Lactobacillus casei* that this inhibitory effect could be overcome by the addition of larger doses of pantothenic acid. This indicates that the determining factor in such systems is the ratio of substituted to unsubstituted growth principles.

In the case of Gebrüder Mayer yeast, it is very interesting to note that whereas α-methylpantothenic acid showed no antagonistic action toward the growth effect of pantothenic acid, there is evidence of an antagonism in the addition of increasing amounts of α-methyl-β-alanine to a medium containing β-alanine. Since β-alanine is capable of replacing pantothenic acid as a growth factor for yeasts, it is commonly thought that the yeasts utilize the amino acid by building it into pantothenic acid. If, therefore, β-alanine and its α-methyl homolog are considered to exert their growth effects via synthesis through the corresponding pantothenic compounds, it seems surprising that α-methyl-β-alanine should yield an antagonism when α-methylpantothenic acid does not. However, the answer may lie in the fact that α-methylpantothenic acid is a much stronger growth stimulant for the yeast than is α-methyl-β-alanine, and that antagonistic effects will only be produced by substances which are very weak growth stimulants, if at all. Further studies may show that in general the antagonistic action of any compound toward the growth effect of a homolog will decrease with increasing growth-promoting ability of the former. In the present instance, α-methylpantothenic acid showed a strong repressive effect on *Lactobacillus casei*, where it had a relative potency of less than 0.001 as compared with pantothenic acid, but no repressive action on Gebrüder Mayer yeast, where its relative potency was greater (0.002–0.003).

Whether acting as stimulant or inhibitor, the α-methyl homolog of pantothenic acid is a much less effective substance in regard to direct action on the microörganisms than is the vitamin. This is another case where a slight modification of the structure voided most of the physiological "punch" of the compound. It would be interesting to determine the effect of such slight modifications as the introduction of isotopes on the potency of pantothenic acid and β-alanine. The enzyme system in which pantothenic acid undoubtedly functions appears to be a highly particular one. There is so little difference in so far as chemical reactivity is concerned between pantothenic acid and α-methylpantothenic acid that it would seem that the inefficient utilization of the latter is probably due to physical reasons, such as a poor "fit" into the enzyme pattern.

Summary

1. α-Methylpantothenic acid and α-methyl-β-alanine were synthesized and tested for growth activity on some bacteria and yeasts.

2. α-Methylpantothenic acid can replace pantothenic acid to a limited extent as a growth stimulant for *Lactobacillus casei*, *Lactobacillus arabinosus* 17.5, *Streptococcus lactis R*, Gebrüder Mayer yeast and Fleischmann baker's yeast, but the former compound is only about 0.0001–0.003 as potent as pantothenic acid.

3. α-Methyl-β-alanine can replace β-alanine as a growth stimulant for Gebrüder Mayer yeast, but it is about 0.0006 as potent as β-alanine.

4. In large doses, α-methylpantothenic acid repressed the growth effect of pantothenic acid for *Lactobacillus casei*, but not for Gebrüder Mayer yeast, while α-methyl-β-alanine showed a slight repressing action on the growth effect of β-alanine for Gebrüder Mayer yeast. There appeared to be minimum growth points in both instances of repression.

5. The repressive action of α-methylpanto-

thenic acid for the growth effect of pantothenic acid for *Lactobacillus casei* could be overcome by the addition of more pantothenic acid.

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Condensations by Sodium. XXVI. Metalation of Benzene, Toluene and Xylene. The Orienting Influence of Sodium and the Influence of Alkyl Groups on Metalation

BY AVERY A. MORTON, ERNEST L. LITTLE, JR., AND WILLIAM O. STRONG, JR.

The purpose of this paper is to show (a) that benzene and toluene can be dimetalated so that the products after carbonation are exclusively iso- and homoisophthalic acids, (b) that *m*- and *p*-xylene can be dimetalated so that one sodium atom is introduced into each methyl group but that *o*-xylene is substituted to some degree in the nucleus and (c) that the three xylenes can be alkylated by the sodium-alkyl halide method, albeit with more difficulty than is met in the case of toluene. Formation of iso- and homoisophthalic acids will be interpreted from the viewpoint that sodium and, for that matter, other alkali metal substituents are *m*-orienting, and that the organometallic reagent behaves as an ion pair. The principles developed in connection with this orientating influence will be applied to interpretations of the reactions of amylsodium and like reagents. It will be shown that such agents can be classed as electrophilic. Finally, the decreased activity of the xylenes and of *o*-xylene in particular will be discussed in terms of the retarding influence which alkyl groups in general exert on metalation.

Dimetalations and Alkylation.—Previous work[1] has shown that benzene is readily monometalated and can be dimetalated to some extent by refluxing with amylsodium to give a mixture, the disodium content of which consists of 80% meta and 20% para phenylenedisodium. The present study shows that dimetalation can be made the principal reaction and can be caused to take place *exclusively* in the meta position. The change is brought about by restricting the amount of benzene used and by improving the stirring. Carbonation gives isophthalic acid in a yield of 51% based on the amyl chloride used in preparing amylsodium. The amount of monometalated product represented by benzoic acid is only 4.5%. Since these two products are the only ones obtained which are derived from benzene, it is apparent that about 85% of the benzene consumed in the reaction is dimetalated. The low yields calculated from the amyl chloride are due to the fact that there is about a 40% loss in preparing the amylsodium from the chloride and there is a small amount (about 2%) of amylsodium which does not participate in the reaction. As a preparative method the process is probably not as convenient as the permanganate oxidation of *m*-xylene[2] (95% yield of the hydrocarbon consumed), although that method has been reported[3] as being apt to give a contaminated product because of impurities in the *m*-xylene.

Toluene has previously[4] been found to be converted easily to benzylsodium with no trace of dimetalation. Under the improved conditions used in this study a second sodium atom is readily introduced. The dicarboxylic acid which results from carbonation is exclusively homoisophthalic acid and the yield is 40% based on the amyl chloride. The procedure is an excellent preparative one compared with the previous method[5] which requires six steps from 2-nitro-4-toluidine, itself produced from toluene, in three operations.

The three xylenes, which hitherto have not been studied in this series, undergo lateral metalation preferentially. The alkylation[6] reaction (amyl chloride dropped on sodium in hot xylene) shows that in all cases monosubstitution takes place exclusively in a methyl group giving the methylhexylbenzene in yields of 54, 32 and 22% (amyl chloride basis) for *p*-, *m*- and *o*-xylene, respectively. Dimetalation, by the method used with benzene and toluene, occurs on the two methyl groups exclusively in the case of *p*- and *m*-xylene

(1) Morton and Fallwell, THIS JOURNAL, **60**, 1924 (1938).

(2) Ullmann and Uzbachian, *Ber.*, **36**, 1797 (1903).

(3) Schlenk and Brauns, *ibid.*, **48**, 661 (1915).

(4) Morton and Fallwell, THIS JOURNAL, **60**, 1426 (1938).

(5) Komppa and Hirn, *Ber.*, **36**, 3611 (1903); Komppa, Hirn, Rohrmann and Beckmann, *Ann.*, **521**, 242 (1936).

(6) (a) Morton and Fallwell, THIS JOURNAL, **60**, 1429 (1938); (b) Morton, Richardson and Hallowell, *ibid.*, **63**, 327 (1941).