the rotation of the solution appeared to be constant. One specific example is given (hydrolysis of the polymeric dialdehyde from the buffered oxidation of the polysaccharide from the carob bean): One gram of the polymeric dialdehyde was refluxed with 100 cc. of 2% aqueous sulfuric acid. After eight hours, the reaction mixture had darkened and a small amount of insoluble material was noted. A little norite was added and the mixture was filtered. Refluxing was then continued (total time, fifteen hours) during which the mixture again darkened. It was finally treated with norite and filtered.

Isolation and identification of glyoxal (as pure phenylosazone): The above reaction mixture was neutralized with barium carbonate and the barium sulfate was removed by filtration. A 50-cc. aliquot of the filtrate (corresponding to 0.5 g. of cleavage product) was warmed with sodium acetate (1.0 g.), acetic acid (2 cc.) and phenylhydrazine (2 cc.) for fifteen minutes. After standing at room temperature for three hours, the crude phenylhydrazone was collected by filtration and dried *in vacuo*. The product weighed 0.53 g. and melted at 131–134° with decomposition. The crude product, when recrystallized from benzene, was crystalline; it weighed 0.22 g. (30%) and melted at 162–164°. After crystallization from ethanol, the pure glyoxal phenylosazone weighed 0.16 g. (22%) and melted at 168–170° alone or when mixed with an authentic sample. The yields obtained from the other cleavage products are given in detail in Table III.

Summary

1. The periodate oxidation of the reserve carbohydrates from carob, honey locust and guar seeds has been studied. Each polysaccharide consumed one mole of oxidant per hexose unit and the cleavage products yielded glyoxal on hydrolysis in every case. These results are interpreted as being indicative of the presence of 1,4-linkages. In the case of polymeric carbohydrates from carob and honey locust beans these results are at variance from previously published results.

2. The effects of the temperature and the *p*H on the periodate oxidations of these polysaccharides have been discussed.

3. The sensitivity of these mannogalactans to borax has been explained on the basis of the *cis* glycol group found at the C_2 and C_3 positions of the mannose units.

4. The possibility of branching has not been eliminated and no conclusion in regard to the configuration at the C_1 position has been drawn.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, OREGON STATE COLLEGE]

Pantothenic Acid Studies. I. Growth Effect of Pantoic Acid Analogs[1]

BY VERNON H. CHELDELIN AND CHESTER A. SCHINK

Analogs and derivatives of pantothenic acid have been prepared in a number of laboratories. The earlier ones resulted from studies on the structure of the vitamin,[1a] and from efforts to produce compounds with similar biological activity.[2-7] Following the preparation of pantoyltaurine,[8-10] however, most syntheses have aimed at the development of inhibitors related to pantothenic acid. *In vitro* growth tests with microörganisms have revealed a number of compounds which competitively inhibit the growth-promoting action of pantothenic acid over a wide range of concentrations.

In general, the most successful inhibitors have been those in which the pantoic acid[10a] moiety of the molecule is coupled to a suitable amino acid,[8,10,11,12] amino ketone,[13] amino alcohol[14] or amine.[15] Alterations in the pantoic acid moiety, on the other hand, have with but one exception[16] given rise to inactive or very slightly stimulatory substances, when tested on organisms requiring the preformed vitamin.

It was felt that an organism such as *Acetobacter*, which utilizes pantoic acid as readily as the intact vitamin,[17] might be inhibited by compounds resembling this acid, as well as by their condensation products with β-alanine. We have found two such compounds to effectively inhibit the coupling of pantoic acid to β-alanine. Other analogs have been prepared which possess considerable growth-promoting activity. The details of these studies and some of their implications are presented below.

(1) The data in this paper are taken from the dissertation presented by one of the authors (C. A. S.) for the Ph.D. degree, Oregon State College, 1947. Presented before the Northwest Regional Meeting of the American Chemical Society, Pullman-Moscow, May 2, 1947. Published with the approval of the Monographs Publication Committee, Oregon State College, Research Paper No. 111, School of Science, Department of Chemistry. This work was supported by the Nutrition Foundation, Inc., Research Corporation, Inc., and by the General Research Council, Oregon State System of Higher Education, Corvallis, Oregon.

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Experimental

α-Hydroxy-β,β-dimethylbutyric Acid.—Pinacol hydrate and pinacolone were prepared from acetone in the customary manner.[18] Forty grams of pinacolone was oxidized with potassium permanganate according to the method of Glücksmann.[19] The product was then reduced in the prescribed manner. However, in isolating the product, the aqueous solution was distilled to about 100 ml. and then extracted with ether. The ether solution was concentrated and the concentrated solution was placed in a vacuum desiccator over sulfuric acid. A yield of 11.4 g. (21.6%) of white crystals was obtained, m. p. 87.0–87.5°.

Sodium N-(α-Hydroxy-β,β-dimethyl-butyro)-β-alanine.—The coupling of α-hydroxy-β,β-dimethylbutyric acid was carried out according to the procedure of Parke and Lawson.[20] To 18 ml. of refluxing isopropyl alcohol was added 0.52 g. of sodium through the condenser. After all of the sodium had gone into solution, 2.00 g. of β-alanine was added and the mixture was allowed to reflux for fifteen minutes. Two and ninety-seven hundredths grams of α-hydroxy-β,β-dimethylbutyric acid was then added and the refluxing was continued. Within fifteen minutes a white curdy precipitate appeared. After three hours 15 ml. of isopropyl alcohol was added and the mixture was allowed to cool in the refrigerator. The cold mixture was filtered and the residue was washed with cold isopropyl alcohol; yield was 4.5 g. (82%).

Anal. Calcd. for $C_9H_{16}O_4NNa$: N, 6.21; Na, 10.20. Found: N, 6.09; Na, 10.35.

Methallyl Cyanide.—The procedure described in "Organic Syntheses"[21] for allyl cyanide was found to be applicable for the preparation of methallyl cyanide. However, two to five hours of heating on the water-bath was required to start the reaction. Cooling was not necessary at any point during the reaction. The fraction[21a] boiling at 133–135° was collected from the redistillation of the crude product. Yield was 84%.

Oxidation of Methallyl Cyanide with Peracetic Acid.—Peracetic acid was prepared by adding 56 g. of 30% hydrogen peroxide to 115 ml. (120 g.) glacial acetic acid and warming the mixture at 80° for forty-five minutes. To the cooled solution was added a 20-g. sample of methallyl cyanide. This solution was allowed to stand at room temperature for one week.

The volume of the solution was reduced to about one fourth by distillation *in vacuo*. Twenty-five ml. of 6 *N* hydrochloric acid was added to the residue and the mixture was refluxed for one hour, after which it was distilled at reduced pressure to a small volume. The pasty residue was extracted with ether. The oil obtained upon evaporation of the ether was dissolved in 25 ml. of water and 25 ml. of concentrated hydrochloric acid was added. The mixture was refluxed for two hours. The hydrochloric acid solution was distilled *in vacuo* until a sticky solid residue remained. This residue was extracted with several portions of acetone. The acetone solution was dried on sodium sulfate, after which the acetone was stripped off, leaving a brown viscous oil. The oil was partially purified by dissolving it several times in ether–acetone mixtures, removing the insoluble portions and evaporating the solvent.

Sodium N-(β-Methyl-β,γ-dihydroxy butyro)-β-alanine.—Five grams of partially purified β-methyl-β-hydroxy-γ-butyro lactone was coupled with β-alanine as previously described. The coupled product was purified by repeatedly dissolving it in methanol and precipitating it in a large excess of dry acetone. The product was a light brown, amorphous, deliquescent solid which was not decolorized by charcoal. Yield was 20%.

Anal. Calcd. for $C_8H_{14}O_5NNa$: N, 6.16; Na, 10.12. Found: N, 6.33; Na, 10.32.

β,β-Dimethyl-γ-butyrolactone.—β,β-Dimethylglutaric acid, prepared from malonic ester and mesityl oxide according to the method of Komppa,[23] was oxidized with iodine to give the desired β,β-dimethyl-γ-butyrolactone,[24] m. p. 56°.[24a]

Sodium N-(γ-Hydroxy-β,β-dimethyl-butyro)-β-alanine.—This coupling was carried out as described above using 0.043 g. of sodium, 2 ml. of isopropyl alcohol, 0.166 g. of β-alanine, and 0.213 g. of β,β-dimethyl-γ-butyrolactone. Yield was 245 mg. (58%).

Anal. Calcd. for $C_9H_{16}O_4NNa$: N, 6.21. Found: N, 5.90.

Sodium N-(α-Hydroxy-β,β-dimethylbutyro)-taurine.—This condensation was carried out as described above using 0.35 g. of sodium, 15 ml. of isopropyl alcohol, 1.89 g. of taurine and 2.00 g. of α-hydroxy-β,β-dimethylbutyric acid. The product melted at 190–195°; yield was approximately 70%.

Anal. Calcd. for $C_8H_{16}O_5NSNa$: N, 5.36. Found: N, 5.21.

Pantoyltaurine.—This compound was prepared according to the method of Snell,[8] from *dl*-α-hydroxy-β,β-dimethyl-γ-butyrolactone and taurine.

N-Pantoyl-*n*-butylamine.—This compound was prepared as prescribed by Shive and Snell,[15] using *dl*-α-hydroxy-β,β-dimethyl-γ-butyrolactone and *n*-butylamine.

Organisms and Testing.—The organisms used for testing the various compounds were *Acetobacter suboxydans*, A. T. C. C. No. 621; *Lactobacillus arabinosus* 17-5; and *Saccharomyces cerevisiae*, Gebrüder Mayer strain. All tests were performed by using previously published methods.[17,25,26] β-Alanine was omitted from the *A. suboxydans* medium when pantothenic acid was employed as the growth factor.

Results

Preliminary experiments showed that with the exception of *A. suboxydans*, organisms were relatively unaffected by several of the new analogs. Since complete inhibition of growth often requires much higher concentration of the analog than is needed for partial inhibition, it was decided to express molar analog:growth factor ratios at 50% inhibition, *i. e.*, where growth is equivalent to that produced by one-half of the growth factor present.

In Table I, the inhibitory action of three analogs of *pantoic* acid is compared, for three organisms. None of these compounds is able to counteract the growth-promoting effect of pantothenic acid in *L. arabinosus*, which utilizes only the intact vitamin molecule, or in G. M. yeast, which can synthesize the correct hydroxy acid (pantoic acid) readily. In *A. suboxydans*, on the other hand, where growth is dependent upon pantoic acid, a competitive inhibition is observed between each antimetabolite and the growth fac-

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(21a) Tamele, *et al.* (ref. 22), prepared this compound using nitrobenzene as a solvent for the reaction. They give 136.2–136.4° as the boiling point with a yield of 58%.

(22) M. Tamele, C. J. Ott, K. E. Marple and G. Hearne, *Ind. Eng. Chem.*, **33**, 115 (1941).

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(24) A. Windaus and F. Klönhardt, *ibid.*, **54B**, 581 (1921).

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(25) E. H. Hoag, H. P. Sarett and V. H. Cheldelin, *Ind. Eng. Chem. Anal. Ed.*, **17**, 60 (1945).

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TABLE I

EFFECT OF PANTOIC ACID ANALOGS UPON VARIOUS ORGANISMS

Analog: Growth Factor Ratio at 50% Inhibition

		Growth factor				
		Lactobacillus arabinosus 17-5	Acetobacter suboxydans		G. M. Yeast	
Compound	Formula	Pantothenic acid	Pantoic acid	Pantothenic acid	β-Alanine	Pantothenic acid
1	$HOCH_2C(CH_3)_2CH_2COOH$	No inhibition	10	300	100,000	100,000
2	$CH_3C(CH_3)_2CH(OH)COOH$	No inhibition	1000		25,000	No inhibition
3	$HOCH_2C(CH_3)(OH)CH_2COOH$	No inhibition	12,000	10[illegible],000	40,000	No inhibition

TABLE II

EFFECT OF PANTOTHENIC ACID ANALOGS UPON VARIOUS ORGANISMS

Analog:Growth Factor Ratio at 50% Inhibition

		Lactobacillus arabinosus 17-5	Acetobacter suboxydans		G. M. Yeast	
		Growth factor				
Compound	Formula	Pantothenic acid	Pantoic acid	Pantothenic acid	β-Alanine	Pantothenic acid
4	$HOCH_2C(CH_3)_2CH_2CONHCH_2CH_2COOH$	0.02% act.[a]	0.05% act.	0.03% act.	8% act.	3% act.
5	$CH_3C(CH_3)_2CH(OH)CONHCH_2CH_2COOH$	.004% act.	.02% act.	.01% act.	90% act.	30% act.
6	$HOCH_2C(CH_3)(OH)CH_2CONHCH_2CH_2COOH$	No inhibition	.01% act.		15% act.	3% act.
7	$CH_3C(CH_3)_2CH(OH)CONHCH_2CH_2SO_3OH$	No inhibition	800	2000	No inhibition	No inhibition
8	$HOCH_2C(CH_3)_2CH(OH)CONHCH_2CH_2SO_3OH$	1000	10% act.	10% act.	No inhibition	10,000
9	$HOCH_2C(CH_3)_2CH(OH)CONHCH_2CH_2CH_2CH_3$	12,500	0.04% act.	0.04% act.	30,000	25,000

[a] All compounds showing activity were without inhibition at any concentration.

tor. The effective ratios remain fairly constant over wide concentration ranges (data not shown).

The three antimetabolites are most effective when the vitamin moieties are used to promote growth. This is especially true in *A. suboxydans*, where a low analog/growth factor ratio of 10 is observed for β,β-dimethyl-γ-hydroxybutyric acid against pantoic acid. It would appear, therefore, that these hydroxy acids are able to prevent the coupling of β-alanine to pantoic acid which normally occurs in *A. suboxydans* or yeast when the intact vitamin is not available. As might be expected, yeast is inhibited less by these acids than is *A. suboxydans*, since pantoic acid is normally produced by yeast. Organisms which synthesize pantothenic acid[13,14] are not in general affected by pantothenic acid analogs.

Table II outlines the effect of six analogs of *pantothenic acid* upon the three test organisms. Compounds 4, 5 and 6 are the β-alanides of nos. 1, 2 and 3, respectively. Compound 7 is obtained by condensing taurine with no. 2. No. 8 is pantoyltaurine,[8] which is included for comparison. No. 9 is N-pantoyl-*n*-butylamine.[15]

The results obtained with compounds 4, 5 and 6 are in marked contrast to those obtained with 1, 2 and 3. The pantothenic acid analogs are of no value as growth inhibitors, and in fact stimulate growth to some extent in all of the test organisms. The growth stimulation is greatest for yeast, where Compound 5 has virtually the same activity as β-alanine, expressed on a molecular basis. This may be due to hydrolysis by the yeast, since β-alanine would be formed in each case. The relative activities of the three compounds seem to be in accord with their supposed ease of hydrolysis; compound 5, with a hydroxy group alpha to the amide, should hydrolyze most readily.

The differences between the effect of compound 7 and pantoyltaurine are striking. The removal of the γ-hydroxy group changes this potent inhibitor of *L. arabinosus* and yeast growth into a substance which is completely inert. Conversely, whereas pantoyltaurine promotes the growth of *A. suboxydans*, the desoxy compound is a good inhibitor. The last observations may be due to the ability of the organism to hydrolyze pantoyltaurine to pantoic acid. A similar hydrolysis of compound 7 would yield compound 2, which was shown in Table I to inhibit growth.

Although in our hands compound 9 is a poorer inhibitor of *L. arabinosus* growth than previously reported,[15] it does compete with pantothenic acid in this organism. It is also effective in yeast against either the vitamin or β-alanine. Its stimulatory action on *A. suboxydans* is probably due to pantoic acid, which may be present either as a contaminant in the preparation or as a result of hydrolysis of the analog by the organism.

It is interesting to note that *A. suboxydans* apparently does not hydrolyze compounds 4, 5 and 6, for the cleavage products (compounds 1, 2 and 3, respectively), would be inhibitors.

Discussion

With each organism studied, growth has been influenced in three ways by the various analogs. As pointed out previously,[14] some are inert, others possess vitamin activity, while still others appear to compete with the growth factor for attachment within the cell, presumably at the surface of an enzyme.

If the action of different analogs is viewed in the light of the Woods–Fildes theory, it must be assumed that inert compounds are incapable of attachment within the cell, at least in the normal manner. Inert analogs of pantothenic acid are seen to differ from the vitamin in the pantoic acid

portion of the molecule. This suggests that the vitamin is normally attached through the pantoic acid portion, since changes in the β-alanine structure seldom, if ever, produce inert analogs. Both hydroxy groups appear important; the removal of either one greatly reduces the activity, and further changes, as in compound 6, remove the activity altogether. Similar losses in activity have been recorded by other workers,[1a,6,8,27] although Barnett and Robinson[6,10] noted some inhibition of growth of *E. coli* with compound 4 and with the β-alanides of γ-hydroxybutyric and γ-hydroxyvaleric acid. However, the effects were not reversed by pantothenic acid, and it would seem that these analogs do not directly involve the utilization of the vitamin.

The behavior of stimulatory and inhibitory analogs is also compatible with the assumption that they are attached through the hydroxy acid moiety, although the picture is somewhat complicated for *A. suboxydans* and yeast wherever hydrolysis may produce the proper growth factor. For example, a weakened or restricted attachment of compound 4, 5 or 6 in *L. arabinosus* or *A. suboxydans* would still permit the β-alanine structure to engage in metabolic activities. Likewise, pantoyltaurine and other pantoyl amides have the correct configuration in the hydroxy acid moiety, and should attach to the enzyme; but here the alteration or removal of the carboxyl group presumably prevents the analog from entering into further reactions in the cell.

A similar situation exists with pyrithiamine.[28] Here the amino and hydroxyethyl groups of thiamine are duplicated in the analog, so that attachment may take place,[29] but the absence of the sulfur atom prevents further action, and the compound becomes a competitive inhibitor.

One of the best lines of evidence for attachment of pantothenic acid through the pantoic acid moiety in yeast comes from the behavior of pantoyltaurine. Although this compound effectively inhibits growth produced by pantothenic acid, it has been shown[3,26] that it has no influence on growth when β-alanine is supplied. If both the growth factor and the analog were attached by means of the carboxyl group (or the β-amino group), competition should be found in any case. However, if we regard pantoic acid (produced by the cells) as being combined with the enzyme, β-alanine would be free to couple without interference from pantoyltaurine.

Inhibition of microbial growth may in some cases be due to prevention of proper attachment of the vitamin to the enzyme. This is probably true of pyridine-3-sulfonic acid,[30] 3-acetylpyridine[31] or lumiflavin,[32] as well as the β-alanide of α,γ-dihydroxy-β,β-dimethylvaleric acid.[16] So far as we have been able to determine, the latter is the only inhibitory analog of pantothenic acid which exhibits alterations in the pantoic acid moiety.

It is not yet possible to form a detailed picture of the combination of pantothenic acid to cellular enzymes. Organisms differ widely in their resistance to inhibitors, and the different ability of yeast and *A. suboxydans* to hydrolyze various analogs suggests that the vitamin may be oriented differently toward its respective points of attachment in the two types of cells. Moreover, it is possible that the substituted amide group in pantothenic acid may take part in some of its reactions. We are attempting to settle this point through the preparation of suitable analogs. The scope of possible reactions which such a group may undergo is limited, however, and in any case it appears difficult on the basis of present evidence to postulate unstable linkages which would permit pantothenic acid to take part *per se* in catalytic reactions. Such speculation favors the existence of catalytically active conjugates of the vitamin, a point which is being pursued at the present time.

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Summary

1. Several new analogs and derivatives of pantoic acid have been prepared. These are: β-methyl-β,γ-dihydroxybutyric acid, sodium N-(α-hydroxy-β,β-dimethylbutyro)-β-alanine, sodium N-(β-methyl-β,γ-dihydroxybutyro)-β-alanine and sodium N-(α-hydroxy-β,β-dimethylbutyro)-taurine. The above compounds, together with several others previously described, were tested for their growth effects upon *A. suboxydans*, *L. arabinosus* and *S. cerevisiae*.

2. Three analogs of pantoic acid were observed to competitively inhibit the coupling of β-alanine to pantoic acid in *A. suboxydans*.

3. Analogs of pantothenic acid possessing alterations in the β-alanine structure were found to be generally good growth inhibitors. Changes in the pantoic acid moiety produced slightly stimulatory or inert compounds.

4. On the basis of the evidence obtained, attachment of pantothenic acid to cellular enzymes is postulated, through groups in the pantoic acid moiety.

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