THE INFLUENCE OF GLUTAMIC ACID AND PROPIONIC ACID UPON PANTOTHENIC ACID METABOLISM IN MICROORGANISMS

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THE INFLUENCE OF GLUTAMIC ACID AND PROPIONIC ACID UPON PANTOTHENIC ACID METABOLISM IN MICROORGANISMS

Chapter I. Introduction

In 1933 R. J. Williams and coworkers (17), as a result of long, painstaking and intelligent work, found a naturally occurring compound of unknown chemical composition which stimulated the growth of yeast. They called it "pantothenic acid". β-alanine was later shown (1936) to be a nutrilite for yeast by Williams and Rohrmann (16); in 1939 it was found to constitute part of the pantothenic acid molecule (18). The final structure of the compound, which was then recognized to be a vitamin for animals, was announced by Williams and Major in 1940 (19).

Pantothenic acid is required for the growth of many yeasts and may be replaced in most cases by β-alanine (15). Yeast is able to synthesize pantoic acid, which in turn can be coupled with β-alanine forming pantothenic acid (12). However, utilization of the β-alanine moiety for growth is frequently far less efficient than that of pantothenic acid. Indeed, β-alanine is toxic to yeast except when asparagin or aspartic acid is a constituent of the medium (16). α-Aminobutyric acid, β-phenyl-β-alanine and isoserine neutralize the growth promoting action of β-alanine on

yeast, although they have no effect when pantothenic acid is used. Sarett and Cheldelin (9) have shown that several amino acids may retard growth stimulation by β-alanine, although not by pantothenic acid. On the other hand, ammonium ion at proper concentration can enhance the utilization of β-alanine but not pantothenic acid (14).

Wright and Skaggs (20) in a recent publication have shown that propionic acid can inhibit the growth of Escherichia coli. They observed that this inhibition could be reversed in large measure by β-alanine or pantothenic acid, and explained that propionic acid operates to prevent the synthesis of β-alanine by E. coli. This explanation, however, does not eliminate the possibility that (a) propionic acid may combine with β-alanine, forming an inactive or even toxic peptide, or (b) that propionic acid combines with the specific cellular constituent which normally engages β-alanine, thus eventually preventing coupling of the pantothenic acid moieties.

Since the utilization of β-alanine is influenced by other substances in the medium, it was felt that these might be employed to study the biosynthetic processes involving pantothenic acid. In the light of the latter in particular, and in order to obtain further information on the mechanism of inhibition by antimetabolities in general,

the present work was conducted from several angles.

Chapter II. Experimental and results

1. The effect of glutamic acid and pantoic acid on the growth of various yeasts.

The method of determining yeast growth was the same as that used by Sarett and Cheldelin (9). The substances to be tested were measured into 20x150 mm. lipless pyrex test tubes, diluted to a total volume of 2 ml., and 5 ml. of the medium No. 101 (Table 1) were added to each tube. The tubes containing the samples and medium were steamed for 15 minutes at 100°C. and inoculated when cool with 1 ml. of suspension containing 0.024 mg. per ml. of yeast from a freshly grown slant.

The tubes were incubated at 30°C. for 16 - 18 hours, then 2 ml. of a saturated solution of p-chlorothymol were added to each tube. After cooling 30 minutes in a refrigerator, the tubidity of the yeast cultures was measured in an electrophotometer using a 5400Å filter. Turbidities were expressed in term of optical density, which is equal to log 100 minus log per cent transmission (2 - log G).

The effect of various levels of glutamic acid was tested in the presence of 0.1γ pantothenic acid or 0.2γ β-alanine per tube for four yeasts, viz. Saccharomyces

cerevisiae S. C. (ATCC No. 9369), S. cerevisiae 2504 (ATCC No. 9370), S. cerevisiae L. M. (ATCC No. 9371) and S. carlsbergensis 1036 (ATCC No. 9373). All showed enhanced growth when the level of glutamic acid was less than 1 mg. per tube in the presence of 0.1γ of pantothenic acid. Two strains, L. M. and 2504, were stimulated even when glutamic acid was present at a level of 2 mg. per tube, while the other two strains were inhibited at the higher level of glutamic acid.

In the presence of 0.2 γ β-alanine, glutamic acid showed increased growth in strains L. M. and 2504 at all levels of glutamic acid. Five-tenths mg. per tube appeared to be optimum. Growth in these tubes was nearly as effective as an equivalent amount of pantothenic acid. Table II illustrates the effects of different glutamic acid levels at suboptimum levels of β-alanine and pantothenic acid, whereas Table III summarizes the effect of 0.5 mg. glutamic acid per tube on different levels of β-alanine or pantothenic acid.

When glutamic acid was fixed at 0.5 mg. per tube its stimulatory effect on yeast was exhibited at any level of pantothenic acid, but was most distinctive at the lower concentrations. On the other hand, except with strain L. M., 0.5 mg. of glutamic acid suppressed the growth

promoting property of lower levels of β-alanine, as shown in Table II.

In order to determine whether the inhibitory action of glutamic acid on S. carlsbergensis 1036 (as well as several strains of S. cerevisiae tested) might be due to competition between pantoic acid and glutamic acid for conjugation with β-alanine, growth was noted with varying amounts of pantoic acid. This is summarized in Table IV for two yeasts.

In the absence of glutamic acid, pantoic acid in moderate concentration stimulated strain 1036 slightly, but had no effect on the growth of L. M. This can be explained by assuming that the rate of synthesis of pantoic acid by 1036 is not as rapid as the coupling of pantoic acid with β-alanine. However, an excess of pantoic acid inhibited growth at all levels of β-alanine and the inhibitory action of glutamic acid on 1036 could not be released by the addition of a ten fold excess of pantoic acid. This inhibition, therefore, cannot be due to conjugation of glutamic acid with pantoic acid. Further, the stimulatory action of glutamic acid on L. M. yeast was not enhanced by the addition of pantoic acid to the medium; instead a high concentration of pantoic acid caused an 80% reduction in growth.

Glutamic acid and pantoic acid were both found (Table IV) to be without activity when tested in the absence of β-alanine or pantothenic acid. The stimulatory effect of these substances, therefore cannot be due to their activity per se.

The experiments in Table II were repeated in a medium which has been sterilized by filtration. The results were the same as noted above, so it may be concluded that the effect of glutamic acid is not due to the formation of other growth substances during heating.

2. Propionic acid and β-alanine utilization in microorganisms.

In order to study the effect of each pantothenic acid moiety upon propionic acid inhibition, two microorganisms were chosen in which the ability to synthesize the vitamin is more restricted than in E. coli. These were Acetobacter suboxydans, which requires pantoic acid for growth, and Saccharomyces cerevisiae L. M., which requires β-alanine.

The testing methods for yeast and acetic acid bacteria were the same as reported by Sarett and Cheldelin (8,9). The media (medium No. 102 for yeast and No. 103 for A. suboxydans)were adapted to the needs of the present study so

that the effect of propionic and other acids could be observed. Their compositions are shown in Table I.

The sodium propionate solution was prepared by dissolving propionic acid in water and adjusting the pH to that of the medium used. Sodium acetate solution was made similarly from the anhydrous salt. In order to avoid volatilization of fatty acids during steaming, the solutions were sterilized by filtration and transferred aseptically to the flasks or tubes immediately prior to inoculation with organisms.

Propionyl-β-alanine was prepared by condensing propionyl chloride with β-alanine in dilute sodium hydroxide solution. After acidification the resulting mixture was evaporated in <u>vacuo</u> to dryness. The residue was exhaustively extracted with anhydrous petroleum ether in a Soxhlet extractor. The propionyl-β-alanine was isolated by extraction with dry ethyl ether. It was obtained as a light yellow syrup. Calculated for $C_6H_{11}O_3N$: N, 9.65%; N. E., 145.6. Found: N (micro Kjeldahl), 9.50%; N. E., 149.

Propyl propionate was prepared by refluxing propyl alcoholaand propionic acid in the presence of 10% sulfuric acid. The ester was separated, dried and redistilled, and the fraction boiling at 122-4° C. was collected.

Table V shows the effects of sodium propionate and sodium acetate on the growth of yeast. Inhibition at low levels of propionate (0.0012 M), whereas acetate was much less potent. This is in keeping with Wright and Skeggs' observations with E. coli (20). At low levels of β-alanine the ratio of propionate to β-alanine to produce 50% inhibition is roughly 100 to 1; for complete inhibition, a ratio of about 10,000 to 1 is required. The ratio is not constant, since relatively more β-alanine is necessary to overcome increasing doses of propionate, and at higher levels of the latter compound growth cannot be completely restored even by 160 γ of β-alanine.

It would appear, as had been shown previously (20), that propionic acid inhibits at least two systems, only one of which involves β-alanine. Tubes containing 0.5 mg. of glutamic acid gave better growth but this was considered to be due to the stimulating effect of the amino acid as reported in Chapter II, and presumably is not directly related to propionate.

The methyl and propyl esters of propionic acid showed feeble inhibition, but propionyl-β-alanine stimulated growth slightly. Both effects may be due to hydrolysis by the yeast, or possibly (in the latter case) to the contamination with traces of β-alanine. The results are summarized in Table VI.

In other experiments, (data not shown) the reversing power of β-alanine and pantothenic acid in yeast was compared to that of other vitamins, in the presence of various levels of propionic acid. An increase of two- or six-fold in the concentrations of the vitamins present in the basal medium had no effect on propionate inhibition. Five-tenths of pantothenic acid, on the other hand, completely reversed the effect of 0.25 mg. propionate (0.0003 M). The intact vitamin was thus about 40 times as effective as β-alanine in this respect, whereas pantoic acid had no reversing power whatever, even in doses as large as 40 mg. per tube. It would appear, therefore, that the propionate effect is related to β-alanine utilization, even though β-alanine cannot completely overcome high levels of propionate.

The effects of sodium propionate on A. suboxydans are summarized in Table VII. This organism, like E. coli (20) synthesizes β-alanine, and like the latter organism, requires much higher levels of propionate for inhibition than does yeast. Likewise, relatively large amounts of extra β-alanine are needed to relieve the inhibition. It is again evident that the inhibition is not due to pantoic acid, as was shown for yeast above and for E. coli by Wright and Skeggs (20).

Chapter III. Discussion

The literature dealing with yeast nutrition during the past thirty years has often contained contradictory material. This is probably due mainly to differences in the strains of yeast employed. Two such observations have been made by workers at the Carlsberg Laboratories which do not agree with these presented here. Nielson and Johansen (6) from an experiment of "Hefestamm C. L. 1" concluded that when either or both glutamic acid and thiamin are added to the medium, growth promoting action is increased and that of β-alanine becomes as great as that of pantothenic acid. Similar experiments in this laboratory, however, indicate that thiamin is not directly concerned with the β-alanine -- pantothenic acid system. Growth in the absence of thiamin is attained equally by β-alanine and pantothenic acid, and an additional growth effect is produced by thiamin which is of equal magnitude for both β-alanine and pantothenic acid.

Hartelius and Johansen (3) observed further that in the presence of suboptimum amounts of β-alanine, excess pantoic acid approximately doubled yeast growth, and provided a possible stimulation even with optimum levels of β-alanine and all levels of pantothenic acid. The present

findings, as already noted, do not agree with these.

The stimulatory effect of glutamic acid (as well as aspartic acid and asparagin) in the presence of suboptimal amounts of pantothenic acid or β-alanine does not seem to occur commonly among yeasts. Previously (9) asparagin had been found to curtail growth in 14 of 16 strains of S. cerevisiae and one strain of S. carlsbergensis whereas only strains 2504 and L. M. resisted curtailment. The reasons for the inhibitory effects are difficult to evaluate, since several other amino acids also exhibit this property, but the utilization of β-alanine is undoubtedly involved inasmuch as these effects are not observed when pantothenic acid is the growth factor. This inhibitory effect can, however, be explained by the hypothesis which will be described later.

The stimulatory action of 0.5 mg. of glutamic acid on the growth of yeasts L. M. and 2504 cannot be due to the extra nitrogen added, nor can it be explained by simple decarboxylation, since aspartic acid is less effective (unpublished observation of V. H. Cheldelin and T. E. King). If glutamic acid were oxidized by β - or ω - oxidation to form β-alanine which in turn could couple with pantoic acid to form more pantothenic acid, then glutamic acid should show some stimulatory action in the absence of pantothenic acid or β-alanine.

Autocatalysis also seems unlikely, since very small amounts (0.01γ) of pantothenic acid or β-alanine were ineffective in the presence of glutamic acid. Moreover, after 96 hours incubation glutamic acid improved the growth in the presence of either nutrilite (unpublished observation of V. H. Cheldelin and T. E. King).

The results expressed in Table IV have shown that glutamic acid probably does not combine with pantoic acid, nor is it a growth factor for yeast itself. The most favorable explanation of the role of glutamic acid seems at present to be that under the influence of yeast cells it combines with pantothenic acid or β-alanine (and possibly with pantoic acid in the proper linkage) to produce a substance which is more active for the growth of yeast. A recent experiment (R. Lindsay, personal communication) indicates that γ-glutamyl-β-alanine is more active for the growth of L. M. yeast than is β-alanine alone or in a mixture with glutamic acid. This supports the concept that glutamic acid may combine with β-alanine or pantothenic acid to form a more active metabolite. Studies of such peptides are in progress.

Concerning propionate inhibition, the first alternative mechanism proposed, namely that β-alanine combines with propionic acid, now seems improbable on the basis of the results presented above. The great superiority of panto-

thenic acid over β-alanine in reversing the inhibition is difficult to explain on this basis, as is the fact that propionate is several hundred times as effective as acetate, and is correspondingly more easily counteracted by β-alanine.

The final question of whether propionate inhibits β-alanine utilization directly can be answered by the experiments with yeast. Since this organism cannot synthesize the nutrilite, it should be relatively insensitive to propionate if the latter were merely capable of blocking synthesis (20). The observed great sensitivity to propionate and ready reversal by β-alanine, on the other hand, indicates that the utilization of β-alanine is impaired by this inhibitor.

On the basis of present knowledge, it appears likely that the formation of pantothenic acid by E. coli, A. suboxydans, or yeast may take place as follows: pantoic acid first combines with an enzyme, presumably through one or both of the hydroxyl groups (1) as in Figure 1.

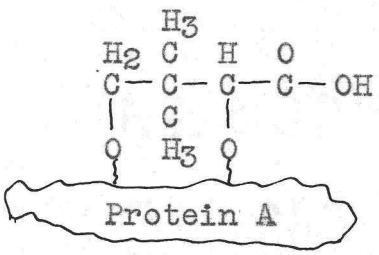

Fig. 1.

Meanwhile, β-alanine may become attached to another enzyme, as in Figure 2.

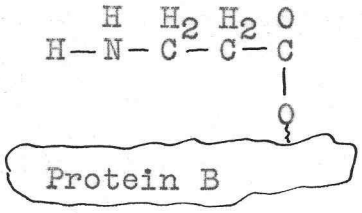

Fig. 2

Such a combination would presumably be a loose one, probably of the type formed by the acid group in cocarboxylase (7, 13). This should dissociate readily, permitting competitive linkage of propionic acid to protein B. Moreover, the ready dissociability would permit disengagement of this protein after the β-alanine -- protein complex (Fig. 2) becomes conjugated with the pantoic acid -- protein complex (Fig. 1). Protein B could thus function in a catalytic capacity, bringing about the coupling of the pantothenic acid moieties.

The above scheme explains satisfactorily all of the recorded observations regarding the formation and utilization of pantothenic acid which have come to the author's attention. β-Alanine is considered to be combined with an enzyme primarily because of its presence in very low concentrations and its great activity therein. The superiority of pantothenic acid over β-alanine in reversing growth inhibition, becomes apparent, if it is assumed that these compete with β-alanine for attachment to protein B above. Such competition would, of course, be impossible wherever pantothenic acid serves as the growth promoter.

The above hypothesis can also be applied to explain not only the inhibitory effect of glutamic acid in certain yeasts but also α-amino butyric acid, β-phenyl-β-alanine and isoerine (5), which neutralize the growth promoting action of β-alanine on yeast, although they have no effect when pantothenic acid is used. Although high concentration of glutamic acid or pantoic acid retard the growth of yeast even in the presence of pantothenic acid, this inhibition is evidently due to the interference of other enzyme systems.

Chapter IV. Summary

The effect of glutamic acid on the growth of four strains of yeast has been studied. Growth of all strains was enhanced by 1 mg. or less of glutamic acid in suboptimum concentrations of pantothenic acid. In the presence of suboptimum concentrations of β-alanine, S. cerevisiae S. C. and S. carlsbergensis 1036 were inhibited by glutamic acid, whereas strains L. M. and 2504 of S. cerevisiae were enhanced. Pantoic acid exhibited a slight growth effect upon strain 1036 at suboptimum levels of β-alanine, but not in presence of adequate amounts of β-alanine or at any level of pantothenic acid. Excess pantoic acid inhibited growth of all yeasts. The mechan-

ism of glutamic acid action has been discussed briefly.

The inhibition of growth of yeast and A. suboxydans by propionate has been observed, and the relation of β-alanine to this inhibition has been studied. On the basis of the results obtained, it appears that propionate inhibits growth by competing with β-alanine for attachment to a specific cell constituent, thereby preventing the coupling of the pantothenic acid moieties.

The preparation of propionyl —β-alanine is described.

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TABLE I

Basal Media

	No. 101	No. 102	No. 103
glycerol	0	0	100 g.
glucose	20 g.	20 g.	5 g.
casein hydrolysate, vitamin free	0	0	10 g.
norite treated peptone	0	0	5 g.
norite treated liver concentrate	0	0	2 g.
tryptophane	0	0	200 g.
cystine	0	0	150 g.
ammonium sulfate	3 g.	3 g.	0
potassium dihydrogen sulfate	2 g.	2 g.	0
salt solution 1 and 2 (10)	1 ml. each	1 ml. each	0
salt solution A and B (11)	0	0	1 ml. each
inositol	15 mg.	15 mg.	0
riboflavin and pyridoxin	200 γ each	200 γ each	0
nicotinic acid and p-amino-benzoic acid	200 γ each	200 γ each	200 each
biotin	1 γ	1 γ	0
folic acid	2 γ	2 γ	0
thiamin	100 γ	100 γ	100 γ
adenine sulfate	20 mg.	20 mg.	0
water to	1 liter	1 liter	1 liter
pH	4.8 - 5.0	4.8 - 5.0	6.0

TABLE II

Growth response of yeasts to different levels of glutamic acid at suboptimum levels of β-alanine or pantothenic acid.*

yeast strain / constituents per tube	turbidimetric readings**			
	S. C.	1036	L. M.	2504
β-alanine (standard)				
0.0 γ	0.015	0.015	0.008	0.030
0.2	0.335	0.235	0.055	0.190
2.0	0.480	0.285	0.260	0.240
0.2 γ β-alanine + glutamic acid				
0.5 mg.	0.050	0.030	0.280	0.280
1.0	0.030	0.050	0.250	0.270
2.0	0.030	0.025	0.238	0.260
5.0	0.030	0.010	0.100	0.210
pantothenic acid (standard)				
0.1 γ	0.175	0.115	0.100	0.235
0.2	0.350	0.225	0.225	0.240
2.0	0.670	0.285	0.265	0.220
0.1 γ pantothenic acid + glutamic acid				
0.5 mg.	0.245	0.120	0.190	0.285
1.0	0.195	0.140	0.165	0.300
2.0	0.150	0.080	0.138	0.280
5.0	0.090	0.030	0.075	0.245

*All amounts of pantothenic acid in this paper are expressed in terms of calcium pantothenate.

**All turbidimetric values in this paper are given in terms of optical density (equals 2 minus log G).

TABLE III

Growth response of yeasts to different levels of β-alanine or pantothenic acid in the presence of 0.5 mg. of glutamic acid per tube

yeast strain / constituents per tube	Turbidimetric readings		
	S. C.	1036	L. M.
pantothenic acid (standard)			
0.0 γ	0.025	0.030	0.020
0.1	0.140	0.150	0.140
2.0	0.580	0.400	0.430
0.5 mg. glutamic acid + pantothenic acid			
0.1 γ	0.025	0.180	0.310
2.0	0.630	0.450	0.550
β-alanine (standard)			
0.2 γ	0.310	0.390	0.090
2.0	0.550	0.320	0.310
0.5 mg. glutamic acid + β-alanine			
0.2 γ	0.030	0.030	0.300
2.0	0.580	0.360	0.560

TABLE IV

Effect of pantoic acid and glutamic acid on yeast growth

yeast strain / constituents per tube	Turbidimetric readings	
	1036	L. M.
β-alanine (standard)		
0.0 γ	0.015	0.000
0.2	0.185	0.040
1.0	0.320	0.345
5.0	0.360	0.460
0.2 γβ-alanine pantoic acid		
0.01 mg.	0.170	0.045
0.10	0.225	0.045
1.00	0.150	0.055
5.00	0.100	0.050
0.2 γβ-alanine 0.5 mg. glutamic acid	0.000	0.290
0.2 γβ-alanine + 0.5 mg. glutamic acid + pantoic acid		
0.01 mg.	0.005	0.300
0.10	0.012	0.310
1.00	0.007	0.275
5.00	0.005	0.075
0.5 mg. glutamic acid + pantoic acid		
0.10 mg.	0.000	0.005
5.00	0.000	0.005
pantoic acid		
0.10 mg.	0.000	0.000
5.00	0.000	0.000

TABLE V

Growth of S. cerevisiae L. M. in the presence of sodium propionate and sodium acetate

test substance / β-alanine	sodium propionate (molar)						sodium acetate (molar)		
	0	0.003	0.002	0.004	0.006	0.072	0.016	0.032	0.072
	turbidimetric readings								
0.0 γ	0.025		0.010	0.005	0.005	0.000	0.025	0.025	0.020
0.2	0.090		0.025	0.025	0.025	0.000	0.080	0.070	0.070
0.5	0.022		0.035	0.030	0.030	0.000	0.210	0.220	0.120
1.0	0.360	0.055	0.150	0.075	0.060	0.000	0.300	0.260	0.140
2.0	0.390	0.150	0.250	0.165	0.110	0.000	0.330	0.330	0.170
5.0	0.430	0.300	0.320	0.270	0.180	0.000	0.370		0.230
20.0	0.430	0.440			0.280				
160	0.450	0.450			0.300				

TABLE VI

Turbidimetric response of growth of S. cerevisiae L. M. to propionic acid esters and propionyl-β-alanine in the presence of β-alanine

test substance / β-alanine	methyl ester			propyl ester		propionyl-β-alanine			
	0	5 mg.	25 mg.	0	25 mg.	0	0.1 γ	10 γ	100 γ
	turbidimetric readings								
0.0 γ	0.020	0.010	0.005	0.040	0.030	0.040	0.040	0.050	0.490
0.2	0.050	0.050	0.020	0.105	0.040	0.115	0.120	0.130	0.500
2.0	0.260	0.280	0.100	0.410	0.040	0.530	0.500	0.510	0.520
5.0	0.360	0.330	0.200	0.450	0.100	0.520	0.510	0.520	0.520

TABLE VII

The effect of propionate on the growth of A. suboxydans

pantoic acid	sodium propionate (molar)				propyl propionate	sodium propionate (0.048 M) + 5γ pantoic acid	
	0	0.012	0.024	0.048	(0.048 M)	β-alanine	turbidimetric readings
	turbidimetric readings						
0.0 γ	0.030	0.030	0.030	0.035	0.030	0.1 mg.	0.230
0.5	0.430	0.340	0.300	0.150	0.290	0.2	0.320
1.0	0.620	0.570	0.540	0.200	0.440	0.5	0.420
5.0	0.630	0.610	0.540	0.240	0.400	1.0	0.400
15.0				0.230			
45.0				0.280			