THE USE OF CORYNEBACTERIUM XEROSE FOR β-ALANINE ASSAY

by

PHILLIP EDWIN LEVEQUE

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APPROVED:

[redacted]

Professor of Chemistry

In Charge of Major

[redacted]

Head of Department of Chemistry

[redacted]

Chairman of School Graduate Committee

[redacted]

Dean of Graduate School

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TABLE OF CONTENTS

THE USE OF *CORYNEBACTERIUM XEROSE* FOR β-ALANINE ASSAY

INTRODUCTION

β-Alanine was discovered to be a nutrilite for yeast in 1936 (23); later it was found to constitute part of the pantothenic acid molecule (21). Pantothenic acid is required for the growth of many yeasts (9, 11, 22), but may often be replaced by β-alanine. Inasmuch as yeasts can utilize β-alanine for growth in the absence of pantothenic acid, these organisms would appear logical for use in the assay of this vitamin. A satisfactory assay method for β-alanine would be desirable, since it is a constituent of many natural materials, not only in pantothenic acid, but also in others such as carnosine and anserine. Although the pantothenic acid content of natural materials can easily be calculated from determinations thereof, recent studies (7, 8, 10) have revealed that conjugates of pantothenic acid exist which vary in their ability to stimulate growth of the various test organisms employed. Direct determination of pantothenic acid in these conjugates is often not reliable.

In experiments with the effects on yeast growth produced by amino acids and natural extracts, yeast assays for β-alanine have been found to be unreliable due to influence on growth by these materials (17, 18, 19). Lactic acid bacteria, which respond well to pantothenic acid, cannot utilize β-alanine even when the lactone

moiety is also supplied (1, 20); this is also true of rats (4) and chicks (24).

The only other organism which has been reported to utilize β-alanine directly is Corynebacterium diphtheriae (2, 3, 12, 13). This organism has the disadvantage of pathogenicity. In the present work it has been found that a relatively non-pathogenic species of this genus, Corynebacterium xerose, is capable of using β-alanine. In this thesis use has been made of this observation to develop a satisfactory quantitative method for the determination of β-alanine.

EXPERIMENTAL

Culture and Inoculum

Corynebacterium xerose, a facultative anaerobe, grows best near the surface of the medium. All liquid cultures are therefore grown in 50 ml. Erlenmeyer flasks to obtain a larger surface exposure to the atmosphere. C. xerose cultures are maintained at 0° - 5° C. in a refrigerator on veal infusion-agar slants and are transferred monthly allowing a 24 hour growth period at 37° C. after transfer. To prepare an inoculum, a loop from a refrigerated culture is adapted to growth upon the basal medium shown in Table I by means of four successive daily transfers as follows: first day, 8 ml. basal medium plus 2 ml. distilled water plus 10γ pantothenic acid; second day, as above, except that the pantothenic acid is replaced by 100γ of β-alanine; third day and fourth day and for carrying between assays, as in the second except that the aspartic acid is omitted. This routine has been found important to induce growth of the organisms on β-alanine. Although aspartic acid is necessary for good growth, it appears to induce heavy pellicle formation so that a uniform inoculum is diffucult to obtain. For inoculation, the organisms are centrifuged in a sterile test tube. The supernatant is poured off and the organisms are resuspended in basal medium and centrifuged. The process is repeated to the third centrifuge stage. The

TABLE I

BASAL MEDIUM

Group I		
Ethanol (95%)	5	ml.
Lactic Acid (85%)	18	ml.
Casamino Acids (Difco)	17	g.
Cystine	0.6	g.
Glutamic Acid	1.25	g.
Aspartic Acid*	1.25	g.
Pimelic Acid	0.15	mg.
Nicotinic Acid	4.5	mg.
Group II		
KCl	0.8	g.
Na_2HPO_4	3.0	g.
$FeSO_4 \cdot 7\ H_2O$	5.0	mg.
$MnCl_2 \cdot 4\ H_2O$	2.5	mg.
$CuSO_4 \cdot 5\ H_2O$	5.0	mg.
$ZnSO_4 \cdot 7\ H_2O$	9.0	mg.
Group III		
$CaCl_2 \cdot 6\ H_2O$	200	mg.
$MgCl_2$	200	mg.

* Omitted from carrying medium

cells are then suspended in basal medium and 1 drop is used for inoculation.

Preparation of Basal Medium

The procedure for preparation of the media is as follows: Group I with the exception of aspartic acid is mixed, diluted to 500 ml., and placed in a cotton plugged flask. In a similar manner the Group II and Group III constituents, as well as aspartic acid (neutralized to pH 7), are diluted to volumes of 200, 50, and 50 ml. respectively, and autoclaved separately. After cooling, all except aspartic acid are mixed and adjusted aseptically to pH 7.5. For carrying medium, 200 - 300 ml. are removed and the aspartic acid solution is then added to the remaining volume which is used as assay medium. This mixing procedure, which is similar to that employed for Corynebacterium diphtheriae (13), was adopted in order to prevent the precipitation of slightly soluble salts which form when the entire medium is heated. Unused portions of either medium are stored in a refrigerator for future use.

Preparation of Samples for Assay

(A) Digestion with alkaline phosphatase.

The following materials are mixed:

(1) Water extract of material to be tested estimated to contain about 100 γ of bound pantothenic acid (equal to 40 γ β-alanine) at pH 7.0-7.5 10 ml.

(2) Alkaline phosphatase (Armour) 50 mg.

(3) $NaHCO_3$, 1% 1 ml.

The above mixture is incubated at 37° C. for 3 to 4 hours. An aliquot may be removed at this point for pantothenic acid assay with Lactobacillus arabinosus 17-5. The remainder of the enzyme digest is then hydrolyzed with NaOH as described below.

(B) Alkaline hydrolysis (the following procedure is sufficient alone for the hydrolysis of free pantothenic acid): A portion of the sample to be tested (0.1 to 1.0 g.) of the enzyme digest from part (A) containing approximately 100 γ of pantothenic acid (now in the free form) is placed in a test tube with 1.0 ml. of 12N NaOH and is autoclaved 3 to 4 hours at 15 pounds pressure to insure complete hydrolysis of pantothenic acid (and presumably other β-alanides). Complete hydrolysis of pantothenic acid is essential since Corynebacterium xerose is about 100 times as sensitive to the complete vitamin as to β-alanine. After cooling and neutralizing to pH 7, the samples are filtered, steamed for 10 minutes, and placed in a refrigerator until assayed.

Assay Procedure

Solutions of materials to be tested at pH 7 are measured into 50 ml. Erlenmeyer flasks, diluted to 2 ml. with sterile distilled water, and 8 ml. of the basal medium are added. One drop of inoculum is added to each

flask except one which is kept for a blank. All operations after plugging and autoclaving of flasks are performed aseptically. After inoculation, the flasks are incubated at 37° C. for 20 hours, at which time the turbidity of the solutions is measured. Turbidity is expressed in terms of optical density (log 100 minus log galvanometer reading) which is obtained on a Pfaltz and Bauer Fluorophotometer.

A standard curve consisting of a duplicate series of flasks containing 0, 0, 1, 3, 5, 8, 13, and 20γ of β-alanine per flask is run concurrently with the substance being tested, and the amount of growth factor present in the samples being tested is determined by comparison to the standards. Samples are tested in duplicate at two or three levels.

Because of the much greater relative sensitivity of this organism to intact pantothenic acid than to β-alanine, all glassware used must be scrupulously clean. Chromic acid has been used routinely for this purpose, since alkaline detergents such as Nacconol are ineffective. After cleaning with chromic acid, assay flasks are thrice rinsed with tap water, thrice rinsed with distilled water, cotton plugged, autoclaved, and stored until used. This routine has been used to minimize possibility of contamination.

RESULTS AND DISCUSSION

The quantitative response of Corynebacterium xerose to 0 - 20γ of β-alanine, as shown in Figure 1, is a smooth curve typical of those obtained with other microorganisms for various growth factors.

C. xerose shows great variability in response to β-alanine; variations such as change in sensitivity and change in the shape of the standard curve necessitate the use of a complete standard curve in every experiment.

Reproducibility of results in regard to β-alanine content calculated from various levels of unknown samples is within ± 15 percent (Table II). The degree of duplication obtained among different experiments also falls within these limits. The yield of β-alanine from pantothenic acid upon alkaline hydrolysis is quantitative, and recoveries of added β-alanine are good (Table III).

Glutamic acid, which has been found to be inhibitory to yeasts (19), does not affect C. xerose in levels up to 50 mg. per 10 ml. of medium. Above this level, inhibition occurs and is most marked at high levels of β-alanine. Similarly, hydrolyzed casein is without effect up to a level of 50 mg. per tube. These levels are higher than are ordinarily encountered among natural extracts which may be present in the assay tubes.

Although the observed β-alanine content in beef muscle (Table II) may be reasonable in view of the known

high concentration of carnosine and anserine present, the values of 0.95γ per mg. for rat blood, and 13γ per mg. for yeast extract are much higher than expected. The high values are not due to residual unhydrolyzed pantothenic acid, since assay of these materials with Lactobacillus arabinosus 17-5 (5) after hydrolysis indicated no more than 0.2γ per gram in the blood and less than 1γ per gram in the yeast extract hydrolysate. While the assay values are reproducible and the organism responds quantitatively to β-alanine (either added or produced from alkali hydrolyzed pantothenic acid), these results suggest that (a) other β-alanides present in these materials hydrolyze to produce much greater yields of β-alanine than may be formed from pantothenic acid present or (b) the organism responds to a natural form of pantothenic acid which is stable to alkaline hydrolysis. Such a derivative has been reported which is active for chicks (14). This form, or the related pantothenic acid phosphates (6), are inactive for L. arabinosus (14).

The data in Table V indicate that a substantial part, although not all, of the apparent β-alanine activity in alkali-hydrolyzed yeast is referable to an alkali-stable form of pantothenic acid which is susceptible to hydrolysis by alkaline phosphatase. Thus, the apparent content of 3 - 7γ of β-alanine per mg. of yeast obtained after alkali hydrolysis decreases to 1.0γ per mg. when

the yeast is predigested with alkaline phosphatase. However, even the latter amount is equivalent to 2500γ of pantothenic acid per gram of yeast -- a figure approximately 8 times that of the actual value of 300γ per gram by Lactobacillus arabinosus assay as shown in Table IV. A similar discrepancy is observed for rat blood, and it may be concluded that even under conditions where pantothenic acid is believed to be completely destroyed, the final β-alanine yields do not reflect accurately the pantothenic acid content of the materials assayed. It would appear, therefore, that assays for β-alanine may give a true measure of pantothenic acid only when the materials tested are relatively free from other β-alanides, e. g., in concentrates of Coenzyme A, PAC, or pantothenic acid phosphates. In spite of this limitation, it is believed that the present assay method will be of value in the concentration and isolation of these conjugates where the conjugates themselves are of unknown purity and a useful reference constituent in these molecules (i. e., β-alanine) is needed. Finally, the present method appears to be superior to yeast methods for β-alanine because of its relative insensitivity to α-amino acids and other constituents of natural extracts.

TABLE II

β-Alanine Content of Various Substances

	Beef muscle (fresh)	Yeast extract	Rat blood	Liver extract	Beef extract
	γ/mg.	γ/mg.	γ/mg.	γ/mg.	γ/mg.
	0.45	16	0.83	3.4	11
	0.45	15	0.83	2.6	10
	0.48	11	0.83	1.0*	11.7
	0.44	11	1.17	0.8*	11.7
	0.74*	14	1.0	3.0	12.5
	0.47	11	2.0*	2.9	6*
	0.62	14	1.0	2.7	10.4
	0.57	20*	1.0	3.2	11.8
					10
					9.5
					9.0
					10.0
Average	0.50±0.06	13±2	0.95±0.09	3.0±0.23	10.7±1.0
% Deviation	12	15	9.5	7.6	9.3

* Rejected

TABLE III

β-Alanine Analyses and Recoveries

Material		β-Alanine found		Recovery
	mg.	γ	γ/mg.	%
Beef muscle	6.0	2.7	0.45	
	12.0	5.5	0.46	
3γ β-alanine +	6.0	6.0		110
3γ β-alanine +	12.0	8.7		110
Rat blood	6.0	0.5	0.83	
	12.0	1.0	0.83	
3γ β-alanine +	6.0	3.5		100
3γ β-alanine +	12.0	4.5		110
Liver extract (Wilson's 1:20)	0.4	1.2	3.0	
	0.8	2.2	2.7	
Alkali hydrolyzed pantothenic acid	0.01	4.5		112
	0.04	13.7		86
"Alkali hydrolyzed" β-alanine	0.008	7.7		96
	0.020	18.0		90

TABLE IV

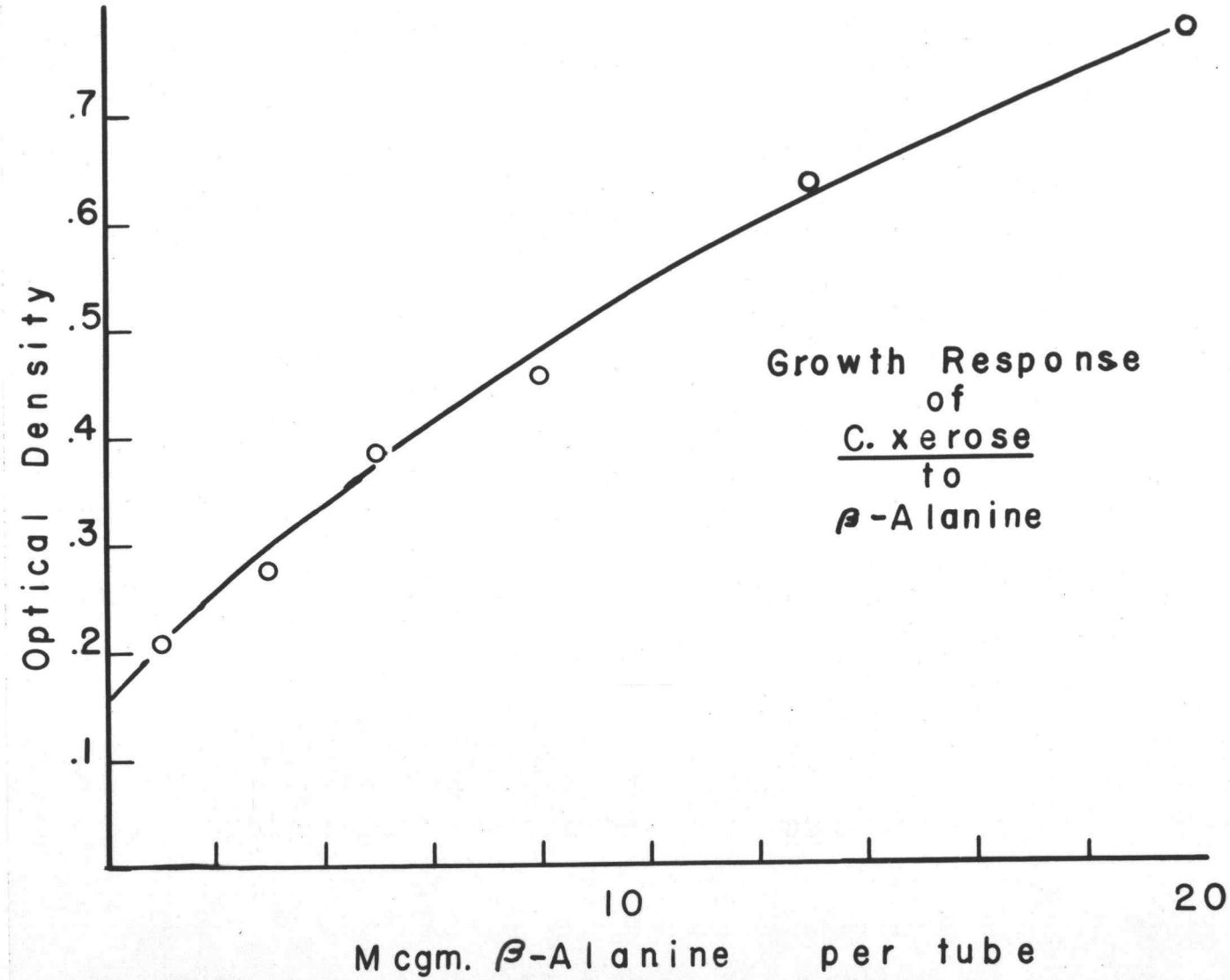
P. A. Content of Natural Materials
Assay Organism: L. arabinosus

Material tested	Pantothenic acid content	
	Before alkali hydrolysis	After alkali hydrolysis
	γ/g.	γ/g.
Rat Blood		0.2
Yeast Extract	300	< 1
Beef muscle, fresh		0.3
Beef extract, Difco		< 1

TABLE V

Comparison of Enzymatic and Alkaline Hydrolyses of Yeast

Material	Treatment	Apparent β-alanine found (C. xerose)
		γ/mg.
Yeast	Hydrolysis with 1N NaOH	3.4 - 7.0
Yeast	Alkaline phosphatase + hydrolysis with 1N NaOH	1.0


Optical Density
.7
.6
.5
.4
.3
.2
.1
10
20
Mcgm. β-Alanine per tube
Growth Response
of
C. xerose
to
β-Alanine

BIBLIOGRAPHY

1. Cheldelin, Vernon H., Edward H. Hoag, and Herbert P. Sarett. The pantothenic acid requirements of lactic acid bacteria. Journal of bacteriology 49: 41-45. 1945.

2. Evans, W. C., W. R. C. Handley, and F. C. Hapfold. The nutrition of Corynebacterium diphtheriae. Pantothenic acid as an essential growth factor for certain strains of D. diphtheriae gravis. The synthesis of some physiologically active compounds by C. diphtheriae cultures in synthetic media. British journal of experimental pathology 20:396-408. 1939.

3. Evans, W. C., F. C. Happold, and W. R. C. Handley. The nutrition of Corynebacterium diphtheriae (types mitis, gravis, and intermediate). Ibid 20:41-48. 1939.

4. Gyorgy, Paul, C. E. Poling, and Y. Subbarow. Experiments on the antidermatitis component of the filtrate factor in rats. Proceedings of the society of experimental biology and medicine 42: 738-740. 1939.

5. Hoag, Edward H., Herbert P. Sarett, and Vernon H. Cheldelin. Use of Lactobacillus arabinosus 17-5 for microassay of pantothenic acid. Industrial and engineering chemistry, analytical edition 17:60-62. 1945.

6. King, Tsoo E., Personal communication.

7. King, Tsoo E., I. Gordon Fels, and Vernon H. Cheldelin. Pantothenic acid studies. VI. A biologically active conjugate of pantothenic acid. The journal of the American chemical society 71:131-135. 1949.

8. King, Tsoo E., Leanor M. Locher, and Vernon H. Cheldelin. Pantothenic acid studies. III. A pantothenic acid conjugate active for Acetobacter suboxydans. Archives of biochemistry 17:483-485. 1948.

9. Leonian, Leon H., and Virgil G. Lilly. The effects of vitamins in ten strains of Saccharomyces cerevisiae. American journal of botany 45:459-464. 1942.

10. Lipmann, Fritz, et al. Coenzyme for acetylation, a pantothenic acid derivative. The journal of biological chemistry 167:869-870. 1947.

11. Lochhead, A. G., and G. B. Landerkin. Nutrilite requirements of osmophilic yeasts. Journal of bacteriology 44:343-351. 1942.

12. Mueller, J. Howard. Substitution of β-alanine, nicotinic acid, and pimelic acid for meat extract in growth of diphtheria bacillus. Proceedings of the society of experimental biology and medicine 36:706. 1937.

13. Mueller, J. Howard, and Sidney Cohen. β-Alanine as a growth accessory for diphtheria bacillus. Journal of bacteriology 34:381-386. 1937.

14. Neal, A. L., and F. M. Strong. Existence of an alkali-stable derivative of pantothenic acid in biological materials. The journal of the American chemical society 65:1659-1660. 1943.

15. Neilands, J. B., and F. M. Strong. The enzymatic liberation of pantothenic acid. Archives of biochemistry 19:287. 1948.

16. Nishi, Hiroshi, Tsoo E. King, and Vernon H. Cheldelin. Pantothenic acid studies. IX. The influence of dietary pantothenic acid upon a pantothenic acid conjugate (PAC) in rat tissues. Journal of nutrition 41:279-292. 1950.

17. Nielsen, N. Ergangende Untersuchungen uber die Wuchsstoffwirkung der Aminosauren auf Hefe. Biochemische Zeitschrift 307:187-193. 1941.

18. Nielsen, N., and G. Johansen. Uber die Wirkung verschiedener β-alanine Derivative als Wuchsstoff oder Antiwuchsstoff auf Hefe. Naturwissenschaften 31:235. 1943.

19. Sarett, Herbert P., and Vernon H. Cheldelin. The utilization of β-alanine and pantothenic acid by yeasts. Journal of bacteriology 49:31-39. 1945.

20. Snell, Esmond E., F. M. Strong, and W. H. Peterson. Growth factors for bacteria. VIII. Pantothenic acid and nicotinic acid as essential growth factors for lactic and propionic bacteria. Journal of bacteriology 38:293-308. 1939.

21. Weinstock, Harry H., Jr., et al. Pantothenic acid. IV. Formation of β-alanine by cleavage. The journal of the American chemical society 61: 1421-1425. 1939.

22. Williams, Roger J., Robert E. Eakin, and Esmond E. Snell. The relationship of inositol, thiamine, biotin, pantothenic acid, and vitamin B_6 to the growth of yeasts. The journal of the American chemical society 62:1204-1207. 1940.

23. Williams, Roger J., and Ewald Rohrman. β-Alanine and "bios". The journal of the American chemical society 58:695. 1936.

24. Woolley, D. W., H. A. Waisman and C. A. Elvehjem. Nature and partial synthesis of the chick antidermatitis factor. The journal of the American chemical society 61:977-978. 1939.