[A CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF OREGON STATE COLLEGE]

Pantothenic Acid. IV. Formation of β-Alanine by Cleavage[1]

BY HARRY H. WEINSTOCK, JR., HERSCHEL K. MITCHELL, ERNEST F. PRATT AND ROGER J. WILLIAMS

Since the discovery made in this Laboratory[2] that minute amounts of β-alanine are effective in yeast growth stimulation, we have been led to suspect that there was a structural relationship between β-alanine and pantothenic acid. The observations and experiments which have a bearing on this problem are outlined below.

Production of Pantothenic Acid from β-Alanine by Yeast.—In a previous publication[3] it was noted that G. M. yeast growing in a medium containing a little pantothenic acid does not produce more, and that such a yeast becomes deficient in this essential constituent. In Table I are summarized four experiments bearing on this point. In each case 100 ml. of basal medium[4] was supplemented by the indicated amount of pantothenic acid (in the form of crude rice bran extract), seeded with 0.4 mg. of moist yeast (G. M.) and incubated for forty-seven hours at 30°. The yeast crops were determined turbidimetrically, then centrifuged and autolyzed for two days with toluene at 37°. The autolysates were diluted to 100 times the weight of the original yeast, filtered and assayed for pantothenic acid. In the second two experiments the yeast cultures were agitated occasionally during the growth period. The absolute values of the pantothenic acid found were subject to relatively large errors due to the small amount present. Separate tests were made upon the medium after growth, but any pantothenic acid present was masked by the presence of inhibiting substances.

TABLE I

FAILURE OF YEAST TO PRODUCE PANTOTHENIC ACID

Pantothenic acid in medium, mg. units[a]	Yeast crop, mg.	"Potency" of yeast	Pantothenic acid from yeast crop, mg. units[a]
10	250	0.016	4.0
1	45.5	.021	0.96
10	470	.0028	1.3
1	50	.002	0.1

[a] This is 10^{-3} times the unit previously defined.[5]

When, however, yeast was grown in a medium containing β-alanine, pantothenic acid evidently was formed and the content of the yeast was normal. In a typical experiment 100 ml. of medium, in which the only source of organic nitrogen was 20 γ of β-alanine, was seeded with 0.4 mg. of "G. M." yeast and incubated for forty-four hours at 30° with occasional shaking. The yeast crop was 161 mg., which when autolyzed and assayed for pantothenic acid, as above, yielded 19.3 mg. units and had therefore a "potency" of 0.12, or 8–60 times as high as the deficient yeast (Table I).

That the physiologically active substance in the yeast crop was pantothenic acid, rather than β-alanine itself, was indicated by electrolytic experiments similar to those previously described.[6]

Samples of yeast autolysate, prepared as above, were placed in the cathode end (cell 4) of a small four-cell electrolytic apparatus. The system was maintained at 10,000 v. and 2–6 m. a. current passed for twenty-four hours. The *p*H and pantothenic acid content of each cell were then determined. The results for three such experiments are given in Table II.

In each case, the active principle migrated to the anode compartment, as does a relatively strong acid, and failed to remain in the neutral portion of the system as would have been the case if unchanged β-alanine were present. An electrolysis of β-alanine had indicated that its migration out of cell 4 under similar conditions was negligible.

Moreover, the active principle in the extract of the yeast crop was destroyed by heating with normal hydrochloric acid for three hours in a steam sterilizer. This is characteristic of pantothenic acid, but β-alanine is not destroyed by such treatment.

Results with Other Organisms.—Mueller[7] has observed that β-alanine is an effective "growth substance" for certain strains of diphtheria bacillus. Later work by Mueller and Klotz[8] showed that pantothenic acid is weight for weight more effective in this regard than β-alanine, and

(1) This research is supported by a grant from the Rockefeller Foundation.

(2) Williams and Rohrmann, THIS JOURNAL, **58**, 695 (1936).

(3) Williams, Mosher and Rohrmann, *Biochem. J.*, **30**, 2036 (1936).

(4) The medium and methods of seeding, etc., used in this paper are, unless otherwise indicated, those described in *Biochem. J.*, **28**, 1887 (1934).

(5) Williams, *et al.*, THIS JOURNAL, **60**, 2719 (1938).

(6) Williams, *J. Biol. Chem.*, **110**, 589 (1935).

(7) Mueller, *Proc. Soc. Expll. Biol. Med.*, **36**, 706 (1937).

(8) Mueller and Klotz, THIS JOURNAL, **60**, 3086 (1938).

TABLE II

ELECTROLYTIC STUDY OF "GROWTH SUBSTANCE" FORMED

Yeast autolysate, mg.	"Pantothenic acid" content, mg. units	Cell 1		Cell 2[b]		Cell 3[b]		Cell 4[b]	
		pH	Mg. units	pH	Mg. units	pH	Mg. units	pH	Mg. units
50	16	2.8	14.6	4.8	t	5.3	t	8.5	t
50	7.5	2.6	7.2	4.4	t	5.6	t	9.0	t
50	8.2[a]	2.6	9.8	4.8	t	6.1	t	9.0	t

[a] Based on a single determination. [b] t = slight toxicity was evidenced by a failure of the cultures to grow as well as the blanks.

TABLE III

INCOMPLETE DESTRUCTION BY ACID OR BASIC TREATMENT

Pantothenic acid used, mg. units Potency 6000	Treatment	Medium in which tested	Physiol. activity remaining, mg. units	% residual activity
3600	Concd. H_2SO_4	Asparagin 0.3%	10.5	0.29
3600	100°, 4 hours	Aspartic acid 0.01%	294	8.2
1020	0.1 *N* $Ba(OH)_2$ 100°, 10 min.	Asparagin 0.3%	14.5	1.4
1020	0.1 *N* $Ba(OH)_2$ 100°, 1 hour	Asparagin 0.3%	11.1	1.1
500	0.1 *N* $Ba(OH)_2$	Asparagin 0.3%	Nil	0
500	80°, 30 min.	Aspartic acid 0.01%	109	21.8
500	0.1 *N* $Ba(OH)_2$	Asparagin 0.3%	Nil	0
500	80°, 2 hours	Aspartic acid 0.01%	122	23.8

is stimulatory at a much lower dosage level. Their work, as they indicate, tends to support the supposition that β-alanine is effective for this organism only insofar as it serves as a building stone for the production of pantothenic acid.

In this connection it is interesting also to note that the lactic acid bacteria studied by Snell, Strong and Peterson[9] evidently were *unable* to utilize β-alanine as a building stone for pantothenic acid production, since pantothenic acid stimulated growth whereas β-alanine is inactive.

Acid and Basic Cleavage.—Previous investigation in this Laboratory[10] indicated that pantothenic acid has a peptide structure since it contains nitrogen which is very slightly basic in character and yet contains no pyridine or similar aromatic nucleus. In keeping with this supposition is the fact which was reported several years ago[11] that pantothenic acid is destroyed by heating in either acid or alkaline medium.

A highly important observation escaped us at that time, namely, that the physiological potency of pantothenic acid is not *completely* destroyed either by acid or basic treatment. The percentage of the original activity which remains varies with the treatment, the basal medium used, the physiological state of the yeast and the amount of toxic material produced by the treatment. If the medium contains, as in the experiments cited below, 3.0 g. of asparagin per liter, the residual activity is low. If the basal medium contains little or no organic nitrogen (in the form of asparagin or aspartic acid) the residual activity may be 20% or more. This is illustrated in Table III.

The fact that β-alanine is much less effective when tested in a medium of high asparagin content has been alluded to.[2] Experiments showing this are illustrated in Table IV. In these experiments β-alanine was tested for physiological activity using a pantothenic acid preparation as a standard.

TABLE IV

EFFICACY OF β-ALANINE IN DIFFERENT MEDIA

Amt. β-alanine tested	Medium used	Physiol. activity found, "mg. units"	"Potency" of β-alanine
5. γ	Asparagin 0.3%	1.15	230
1. γ	Asparagin 0.3%	0.20	200
0.1 γ	Aspartic acid 0.005%	.21	2100
2.9 γ	Asparagin	.84	286
1.95 γ	0.15%	.63	322
0.16 γ	Aspartic acid	.40	2500
.08 γ	0.01%	.26	3100

(9) Snell, Strong and Peterson, THIS JOURNAL, **60**, 2825 (1938).
(10) Williams, *et al.*, *ibid.*, **61**, 454 (1939).
(11) Williams, *et al.*, *ibid.*, **55**, 2912 (1933).

Because of the parallel in the physiological activity of β-alanine and the residual activity after acid or alkaline treatment of pantothenic acid, we were led to conclude that probably β-alanine itself was produced by cleavage of a peptide linkage in pantothenic acid.

The value of this hypothesis was enhanced by the fact that when pantothenic acid was destroyed by heating in basic solution, the acid and base produced were in equivalent amounts. (This agrees with the behavior of a peptide.) The production of an amino acid by the hydrolysis was indicated by quantitative formol titrations (Table VI).

That the physiologically active cleavage product of pantothenic acid was β-alanine rather than some closely related substance was indicated by the fact that the effect of β-alanine on yeast growth seems to be highly specific. All of the available α-amino acids, including threonine, have been tested and found to give negative results. β-Amino acids, including β-aminobutyric, β-phenyl-β-alanine, isoserine, anthranilic acid, α,β-diaminopropionic acid, and nicotinic acid failed to give responses in any way comparable to that of β-alanine. γ-Aminobutyric acid and nicotinamide were likewise inactive.

Quantitative Tests for the Cleavage Product.—By using basal media containing a small amount of *l*-aspartic acid (0.1 g. per liter) instead of the larger amount of asparagin, a quantitative test for the physiologically active cleavage product was devised, based upon its effect on yeast growth.

Assuming tentatively that the cleavage product was β-alanine, we were able to compare its effects with those of authentic β-alanine and thus determine the amount of "β-alanine" recovered from a given pantothenic acid preparation.

Numerous preliminary experiments showed that approximately the same amount of "β-alanine" was obtained from one unit of alkali-treated pantothenic acid, regardless of the purity of the sample used. This was tested further in experiments recorded in Table V.

In a typical experiment, 0.8 mg. of calcium pantothenate was dissolved in a known amount of water. A portion of the solution was used to determine quantitatively the amount of pantothenic acid present. Six growth tests compared with three sets of standards, in asparagin media, indicated that there were present 678 mg. units, showing that the material being used had a potency of 825. Another portion of the solution was made 0.07 *N* with barium hydroxide solution and heated at 80° for forty-five minutes. It had been found previously that such conditions rapidly destroyed pantothenic acid, and that the amount of active cleavage product remained constant even though the treatment was at 90° for two hours. After neutralization with sulfuric acid, six growth tests compared with two sets of β-alanine standards, in aspartic acid medium (0.1 g. per liter), indicated that 22.7 γ of "β-alanine" had been produced, or 33.4 γ per gm. unit of pantothenic acid. Similar experiments with calcium pantothenate of potencies 5300 and 11,100 are summarized in Table V. When 0.250 mg. of authentic β-alanine was treated with alkali, in exactly the same manner, the recovery, by test, was 0.251 mg.

TABLE V

QUANTITATIVE YIELDS OF "β ALANINE"

Sample, mg.	Gm. units of pantothenic acid	Potency	Gammas of β-alanine after hydrolysis	β-Alanine per gm. unit pantothenic acid
0.8	0.678	825	22.7	33.4
.9	4.75	5300	158	33.4
.467	5.20	11,100	169	32.5

These results demonstrate conclusively that the active cleavage product is derived from the physiologically active pantothenic acid and is not a contaminating "growth substance" carried along during the process of purification. Results in substantial agreement with these were obtained in experiments where pantothenic acid was split by acid treatment.

Assuming that the active cleavage product was β-alanine and that its physiological effect was not altered by other cleavage products, we found by this method that 0.467 mg. of calcium pantothenate (potency 11,100) yielded 0.169 mg. of β-alanine. Theoretically, if the formula $C_8H_{14}O_5NCa/_2$, which we have derived is correct, 1 mg. of calcium pantothenate should yield 397γ of β-alanine. Our preparation is calculated to yield 362 γ per milligram. On the above assumptions the material which we analyzed was 92% pure.

Formol Titrations.—For the purpose of quantitative chemical determinations of the amino acid produced by the hydrolysis of pantothenic acid, the standard formol titration method was reduced to a submicro scale using the apparatus of Kirk.[12]

(12) Kirk, *Mikrochemie*, **14**, 1 (1933).

Results on formol analysis of β-alanine and hydrolyzed pantothenic acid solution are compared to results obtained by yeast tests, in Table VI. The pantothenic acid was hydrolyzed with 0.2 *N* sodium hydroxide at 100° for thirty minutes and the β-alanine was treated in a like manner.

TABLE VI

β-ALANINE ANALYSES

Compound	Sample (γ)	β-Alanine found by formol titration (γ)	β-Alanine found by yeast test (γ)
β-Alanine	83	83.5 84.3 83.8	85
Hydrolyzed pantothenic acid (potency 8300)	206	54.4[a] 53.8[a]	57

[a] A blank determination on unhydrolyzed pantothenic acid indicated these results might be as much as 10% high.

Diffusion Experiments.—In order to check further the formation of β-alanine from pantothenic acid and in order to rule out the possibility that acid or alkali treatment might cause the opening of a ring and not the cleavage of the molecule, we carried out diffusion experiments on authentic β-alanine and on the cleavage product from pantothenic acid. The proportion diffusing in each case was determined by yeast growth tests, and the rate of diffusion served as a measure of molecular size.

The diffusions were carried out in double rotating cells[13] in the presence of 0.01 *N* potassium chloride and 50 mg. per liter of gallic acid. The amount of β-alanine present in the original solution was 2 mg. per liter.

The diffusion constants of authentic β-alanine and of the cleavage product were found to be 0.811 and 0.807 cm.^{2} per day at 25°, respectively.

Amino Group in the Cleavage Product.—The presence of an amino group in the cleavage product was indicated not only by the results of formol titration already discussed, but also by the fact that its physiological activity was destroyed to the extent of 77% by nitrous acid in fifteen minutes at room temperature. Pantothenic acid itself is not destroyed by such treatment.

Two other lines of evidence indicating that the cleavage product could not be an α-amino acid, were the failure of the material to give the ninhydrin test and lack of destruction of activity by oxidation with silver oxide.[14]

Presence of a Carboxyl Group.—The presence of a carboxyl group in the cleavage product is indicated by the fact that the physiological activity disappeared when it was subjected to conditions for esterifying β-alanine.[15] The activity could be recovered by alkaline treatment.

The physiologically active fragment is practically a neutral substance since it does not migrate to either end of an electrolytic system. This means that it has an acid group which neutralizes the amino group known to be present.

Distillation Experiments.—Three milligrams of pantothenic acid (potency 8000) was treated for three hours at 95° with normal hydrochloric acid. Distillation was carried out in a 3–4 mm. capillary with a 10-mm. bulb blown on the end. A fraction of about 0.2 mg. was collected up to 140° (mostly below 100°) at 0.05–0.07 mm. pressure and discarded. A 0.205 mg. fraction of colorless liquid was obtained from 140–160° at the same pressure. (Authentic β-alanine hydrochloride distils as a colorless liquid under these conditions.) A quantitative physiological test showed the equivalent of 0.195 mg. of β-alanine hydrochloride in the sample. In view of the highly specific nature of our physiological test for β-alanine this finding also pointed to its presence in hydrolyzed pantothenic acid.

β-Naphthalenesulfo-β-alanine.—This derivative is prepared relatively easily. To 50 mg. of β-alanine in one equivalent of *N* alkali was added 250 mg. of β-naphthalene sulfochloride in 3 ml. of ether. The mixture was shaken mechanically and one equivalent of *N* alkali was added in three portions at one and one-half hour intervals. The ether layer was removed and the aqueous solution acidified. The weight of the crude product, which immediately crystallized, was 140 mg., m. p. 131–132.5°. The material crystallized from water as colorless platelets, and after three recrystallizations melted at 135.5–136.5°.

Anal. Calcd. for $C_{13}H_{13}O_4NS$: N, 5.02; neut. equiv., 279. Found: N, 4.98; neut. equiv.,[16] 278.

The compound was moderately soluble in hot water, 1.0 mg./ml. remained in solution at room temperature. The solubility in ether was 3.8 mg./ml. at room temperature.

Fifty milligrams of calcium pantothenate of potency 6500 (which should yield about 10 mg. of β-alanine) was treated with oxalic acid to remove the calcium ion. After heating at 100° with three equivalents of 0.425 N alkali for one-half hour, one and a half equivalents of acid were added and the solution treated essentially as above. There was obtained 19.7 mg. of crystalline material. An additional treatment gave 10.5 mg. The total yield was 88% of the amount which theoretically could be obtained from the β-alanine determined to be present by physiological test. After two recrystallizations the melting point was 135–136°. A mixed melting point with an authentic sample of β-naphthalenesulfo-β-alanine was 134.5–135.5°.

Anal. Calcd. for $C_{13}H_{13}O_4NS$: N, 5.02; neut. equiv., 279. Found: N, 4.90; neut. equiv.,[16] 276.

The preparation of this derivative leaves no doubt but

(13) Mouquin and Cathcart, THIS JOURNAL, **57**, 1791 (1935).

(14) Herbst and Clarke, *J. Biol. Chem.*, **104**, 769 (1934).

(15) Lengfeld and Stieglitz, *Am. Chem. J.*, **15**, 510 (1893).

(16) Potentiometrically determined using the glass electrode.

that β-alanine is actually a cleavage product of pantothenic acid.

Discussion.—The fact that β-alanine, as a cleavage product of pantothenic acid, may show considerable biological activity in certain media and not in others, serves to explain some of the contradictions in the literature with regard to the stability of "bios" in alkaline and acid condition. It seems likely from our findings that β-alanine is of widespread importance in biochemistry and that its importance for various organisms is connected with the fact that it makes up a part of the pantothenic acid molecule. This question is being investigated more fully in this Laboratory.

Summary

1. Pantothenic acid appears to be synthesized by yeast only when β-alanine is furnished in the culture medium.

2. Several lines of evidence indicate β-alanine to be a cleavage product of pantothenic acid. It finally was isolated from this source in the form of β-naphthalenesulfo-β-alanine.

3. The yield of β-alanine from the pantothenic acid preparation of which an analysis has been reported, indicates that it was not more than 90% pure.

4. The significance of these findings for "bios" studies has been discussed briefly.

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Electrical Properties of Multimolecular Films

BY H. H. RACE AND S. I. REYNOLDS

A. Introduction

In 1935 we made some preliminary attempts to measure the dielectric constant and dielectric loss of 101 layer barium stearate films deposited on a polished chromium surface. Measurements of such films should be extremely interesting because of the known arrangement of organic molecules which normally can be measured only in conditions of random orientation. We immediately found three experimental limitations. First our existing bridge equipment had insufficient sensitivity and accuracy. Second, we needed more accurate measurement of the area of contact of a small drop of mercury used as one electrode. Third, the metal and water surfaces must be kept dust free while the film is being prepared. Occasional dust particles have little effect on optical measurements but cause conducting holes or cracks in the film which prevent electrical measurements. Nevertheless, the preliminary results were encouraging enough so that last year we refined the necessary bridge equipment,[1] developed a means for accurate measurement of a few square millimeters of mercury drop contact area and built a box in which films could be deposited with a much lower concentration of dust particles.

The results of recent studies on films made from a number of metal soaps, using this improved equipment, are described in this paper.

(1) S. I. Reynolds and H. H. Race, *G. E. Rev.*, **41**, 529 (1938).

B. Preparation of Specimens.—Multilayer films were built by depositing monolayers on a clean polished chromium slide as described by Blodgett[2] except that we took special precautions to prevent the deposition of dust particles in or on the film.

It was found necessary to control very closely the acidity of the water-bath from which the various types of films were made in order to obtain good reproducibility. This was done by measuring the *p*H of the water with a glass electrode.[3]

C. Apparatus and Technique of Measurements

1. Electrodes.—The metal slide on which the multimolecular films were laid down constituted one electrode. A number of types of contacts were tried on the surface of the film for the other electrode. Using very thin pieces of metal foil, we were unable to get intimate contact without mechanically damaging the film, and the accurate determination of area was very difficult. Using drops of aqueous electrolytes, we were unable to find any of sufficiently low resistivity not to affect the dielectric loss measurements particularly at high frequencies. A mercury drop is the only satisfactory contact which we have found.

The electrode assembly is shown in Fig. 1. The chromium plated slide on which the film is built is held in a microscope stage, which is insulated from the base plate by mica strips. The mercury drop upper electrode is suspended from an amalgamated copper rod which can be lowered by a micrometer screw to make contact with the film surface. The upper electrode assembly is insulated from the base plate by long Pyrex rods. The whole assembly is mounted on a heavy cast iron base to prevent vibration and changes in area of the mercury drop contact during measurements.

(2) K. B. Blodgett, THIS JOURNAL, **57**, 1007 (1935).

(3) D. A. MacInnes and M. Dole, *ibid.*, **52**, 29 (1930).