BETA ALANINE AS A GROWTH ACCESSORY FOR THE DIPHTHERIA BACILLUS

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Received for publication June 7, 1937

In a series of studies carried out in this laboratory (Mueller, 1935, and Mueller and Kapnick, 1935), it has been shown that various strains of diphtheria bacilli grow heavily upon media containing only amino acids in place of peptone, and a considerably purified liver extract in place of meat infusion. Suitable inorganic salts and a source of energy such as glycerol or lactic acid must be added. More recently, it has been possible to identify two of the constituents of the liver extract essential for the growth of our test strain of the diphtheria bacillus, as pimelic acid (Mueller, 1937a) and nicotinic acid (Mueller, 1937b), acting in connection with one or more other unidentified substances. The higher boiling fraction of a vacuum distillate obtained by both esterification and acetylation of the liver concentrate contained the unknown material. The method of preparing this has already been described in the paper on nicotinic acid.

In an attempt to separate the active substance in pure form, the "acetylated esters" containing it were submitted to careful fractionation. The amount available was but 3.0 cc., representing 300 kgm. of fresh liver. The same distilling apparatus (Rittenberg) used in the earlier work was again employed. In a vacuum of about 0.04 mm. Hg, the active material passed over between about 63° and 78°, but it proved impossible to obtain any marked degree of concentration into any one fraction. With the exception of the lowest, measuring 0.15 cc., which contained relatively less activity, the distillates were recombined. Approximately 1.0 mgm. of this oil, after acid hydrolysis, added to a

suitable control gave the maximal effect in producing growth with the test organism.

In an attempt to obtain a crystalline acetyl compound from the oil, a portion was partially hydrolyzed by short boiling with dilute $Ba(OH)_2$ solution. This method was shown by Cherbuliez, Plattner and Ariel (1930) to split the ester linkage of the acetylated esters of amino acids, leaving the acetyl group in place. In this way ,and after removing Ba with H_2SO_4, there was obtained a strongly acid material which showed no tendency to crystallize either as the Ba salt, or as the free acid. The development of the acid reaction, however, indicated that the original, neutral oil probably contained esterified COOH groups.

All of the remaining active distillate, 1.9 grams, was now more thoroughly hydrolyzed by refluxing with about 200 cc. of N/1 H_2SO_4 for six hours. The reagent was accurately removed with $Ba(OH)_2$ and the solution, which was neutral, was evaporated to dryness in vacuo. The residue was partly crystalline and partly syrup. Solution in hot ethyl alcohol resulted in obtaining a small crop of crystals, which, however, were without activity, while the alcoholic solution showed maximal activity with about 0.5 mgm. of dissolved material, which was largely oily, but contained some crystals.

The fact that complete hydrolysis yielded a neutral substance, whereas an acid was formed by splitting off only the ester group, indicated that acetylated NH_2 groups were probably present in amounts equivalent to the COOH groups. In other words, the mixture behaved as though composed of amino acids. Since acid-hydrolyzed casein was a component of the control medium, and tryptophane was not required by the test strain employed, none of the usual amino acids of protein could be concerned, and β-alanine suggested itself as a possible constituent of the mixture, the more so, since the work of Williams and Rohrman (1936) indicates that this substance is a part of the "bios" complex.

A series of tests carried out with synthetic β-alanine quickly showed that it possessed in marked degree the growth-stimulating properties of the distillate fraction; a quantity of approximately 10γ in 10 cc. of medium producing its maximal effect, as did about

0.5 mgm. of the residue left after hydrolysis of the distillate. Assuming the active substance of the latter, therefore, to be actually β-alanine, it would be present to the extent of only 2 per cent in the oily material,—a total amount of perhaps 20 mgm. in the ±1.0 gram of impure material.

Since, as far as we are aware, no compound of β-alanine has been described which would be suitable for its isolation from such a mixture on so small a scale, no further attempt has been made to determine whether or not it actually is the substance present which is essential for growth. That such is probably the case is indicated by the close parallelism of action, by the fact β-alanine is known to occur in tissue extractive, both free and com-

TABLE 1

COMPOSITION OF MEDIUM	BACTERIAL N
	mgm.
Control alone	0.32
Control + β-alanine 0.5	0.30
Control + β-alanine 1.0	0.36
Control + β-alanine 2.5	1.18
Control + β-alanine 5.0	1.58
Control + β-alanine 10.0	2.38
Control + β-alanine 25.0	2.56
Control + β-alanine 50.0	2.58

bined with histidine in the form of carnosine, and that the boiling point of the distillate is within the probable range for that of the acetylated ethyl ester of β-alanine. In any case, this compound is so readily available[1] that even were a more complex substance shown to be equally active, the former would probably still be the more convenient.

Carnosine itself is effective in replacing β-alanine, but a considerably greater concentration is required, indicating perhaps that it must be hydrolyzed by the organisms in order to render its β-alanine available, and that this reaction is not readily brought about by the bacteria.

[1] β-alanine may be purchased from the Department of Organic Chemistry, University of Illinois, Urbana.

Table 1 illustrates the effect of β-alanine when added in varying quantities to the control medium. The test strain is the "Allen," used in much of the earlier work. The control has the following composition per 10 cc. of medium, and the methods of inoculation, incubation and determination of relative growth by means of nitrogen are the same as previously described.

Control

Casein—HCl hydrolysate	0.1 gram
Cystine	0.001 gram
Glutamic acid	0.050 gram
Lactic acid (as Na salt)	0.1 cc.
Salt mixture:	
$NaCl$	0.050 gram
$Na_2HPO_4 \cdot 2H_2O$	0.025 gram
KH_2PO_4	0.0035 gram
$MgCl_2 \cdot 6H_2O$	0.003 gram
Pimelic acid	1.0γ
Nicotinic acid	10.0γ

TABLE 2

COMPOSITION OF MEDIUM	BACTERIAL N
	mgm.
Control + carnosine 5.0	0.29
Control + carnosine 10.0	0.21
Control + carnosine 25.0	0.66
Control + carnosine 50.0	0.90
Control + carnosine 100.0	1.13
Control + carnosine 250.0	2.68

A similar experiment using synthetic l-carnosine, for which the writer is indebted to Professor Vincent du Vigneaud of George Washington University, is presented in table 2.

That the effect of β-alanine, together with nicotinic acid and pimelic acid, in replacing meat or liver extract is not unique on the "Allen" strain, is shown by an experiment with four cultures of the Park-Williams No. 8 diphtheria organism. Earlier work (largely unpublished) with organisms of this "strain" from various sources have brought out differences in nutritional requirements involving both amino acids and energy sources. It is therefore

not remarkable to find differences in respect to these three extractive substances. It is evident that β-alanine and nicotinic acid must be present together to obtain appreciable growth of any strain, whereas pimelic acid increases the growth in some instances but not in others.

The results are summarized in table 3.

TABLE 3

COMPOSITION OF MEDIUM	BACTERIAL N			
	A	B	C	D
	mgm.	*mgm.*	*mgm.*	*mgm.*
Control* alone	0.07	0.06	0.10	0.10
Control + pimelic acid 1γ (1)	lost	0.04	0.14	0.10
Control + nicotinic acid 10γ (2)	0.02	0.04	0.18	0.14
Control + β-alanine 10γ (3)	0.10	0.06	0.10	0.12
Control + (1) + (2)	0.08	0.00	0.17	0.14
Control + (1) + (3)	0.08	0.07	0.11	0.12
Control + (2) + (3)	0.66	1.99	1.92	2.02
Control + (1) + (2) + (3)	2.32	1.86	2.16	1.84

* To the usual Allen control 0.001 gram of l-tryptophane and 0.05 cc. ethyl alcohol are added for the Park 8 strains.

Strain A, National Institute of Health, Washington, D. C.
Strain B, Alabama State Health Department
Strain C, New York State Department of Health, strain 5
Strain D, Toronto, Canada.

DISCUSSION

It is now possible for the first time to obtain heavy growth of certain strains of diphtheria bacilli on a medium which approaches the term "synthetic." It is true that our present control contains hydrolyzed casein but that this may be replaced by known amino acids is indicated by many experiments not presented here. There is still a deficiency of one or more substances, since our present maximum growth with the "Allen" strain of 2.5 to 2.9 mgm. nitrogen can be readily increased to 3.5 to 4.0 mgm. by the addition of whole tissue extract and certain of its fractions. It does not appear to be worth while to complete the substitution of casein hydrolysate with known amino acids until these deficiencies are better understood, and work is being continued along this line.

It is reasonable to hope, however, that even with this amount of growth, which is as good or better than that obtained on the usual peptone-infusion broth, the metabolism of the organism may be nearly enough normal so that toxin of a reasonable degree of potency will be produced. Experiments in this direction are now being made in collaboration with Pappenheimer, whose recent work (1936) on diphtheria toxin places him in an unusually favorable position to carry on this phase of the study.

CONCLUSIONS

1. β-alanine has been found to be a further growth accessory substance for the diphtheria bacillus.

2. This substance, in a concentration of about 1γ per cubic centimeter, together with nicotinic acid in the same concentration and (for some strains) pimelic acid in an even smaller amount, permits the growth of several strains of the organism to the extent of about two-thirds the quantity maximally obtainable with whole tissue extract. It is perhaps not without interest that the cost of sufficient of these substances to prepare 1000 liters of broth would be less than one dollar.

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