NICOTINIC ACID AS A GROWTH ACCESSORY SUBSTANCE FOR THE DIPHTHERIA BACILLUS

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The last papers of this series, dealing with studies of the tissue extract requirements of the diphtheria bacillus for growth, described the separation of the essential substances contained in liver extract into an ether-soluble and ether-insoluble fraction (Mueller and Subbarow, 1937), and the identification of the former as pimelic acid (Mueller, 1937a). It is now possible to throw further light on the nature of the substances not extracted from acid aqueous solution by ether.

Because of the advantages of fractional distillation over methods of precipitation or adsorption, in dealing with the complex mixture which confronts one in a tissue extract, a means was sought of converting as much as possible of the extractive material into a form of sufficient stability to withstand vacuum distillation. Recalling the method suggested some years ago by Cherbuliez, Plattner and Ariel (1929; 1930) for distillation of amino acids after combined esterification and acetylation, it appeared possible that the same procedure might succeed with a somewhat different type of mixture. One would expect to obtain a very complex type of distillate, containing at least (1) simple esters of acids having no group subject to acetylation, (2) acetyl derivatives of amino or hydroxyl compounds, etc., but possessing no carboxyl group, and (3) combinations of the two. In any case, should one or more of the growth-promoting substances appear in such a distillate, careful fractionation might well permit a considerable degree of purification.

The writer was able, in 1932, to confirm the statements of Cherbuliez, Plattner and Ariel in regard to the yield and stability of distillates obtained by their method from protein hydrolysates; he prepared by partial hydrolysis with alkali, several acetylated amino acids in analytically pure form using redistilled fractions of such distillates. Some of these derivatives crystallize in a pure form much more readily than the amino acids themselves, into which they can easily be converted by acid hydrolysis. Since the work was carried out in connection with studies on the amino acids required for bacterial growth, it was not done in such a way as to be particularly suitable for publication, and has never been reported. Although, as far as can be learned, the method has been developed no further, either by its authors or by other workers, it appeared that it had many possibilities in connection with studies of amino acids, and that it might yield equally good results with many extractive materials. The greatest shortcoming of the method as used five years ago was the failure to obtain anything approaching pure compounds by repeated fractional vacuum distillation. Experience gained in carrying out the isolation of pimelic acid indicated the likelihood of much greater success in that part of the method by using a fractionating column such as that of Rittenberg.[1]

A preliminary experiment was carried out using 10 cc. of the alcoholic solution (4b) of the ether insoluble active fraction used in the control solution for the pimelic acid work. This contained about 0.4 gram solid material. The procedure followed was identical with that of Cherbuliez and his co-workers, except that the material was not refluxed either following saturation with HCl or after the addition of acetic anhydride and sodium acetate. The more strictly chemical details of this work will appear elsewhere (Mueller, 1937b).

Distillation of the resulting "acetylated esters," after removal of solvents and excess reagents, was carried out from a very small bulb with a slightly U-shaped side arm as receiver. An oil pump vacuum of about 0.04 mm. Hg was employed, and the temperature

[1] Personal communication.

of the bath carried to 200° before decomposition of the residue began to occur. The distillate weighed 0.233 gram, a very satisfactory proportion of the total material taken. It was a light yellow oil containing some solid indefinitely crystalline material.

The oil was drained from the solid as well as possible, and each was hydrolyzed by autoclaving with N/1 HCl at 10 pounds for 10 minutes. Tests were carried out by adding the material, as indicated, to the following control solution:

Casein hydrolysate (20 per cent)	0.5 cc.
Cystine	1.0 mgm.
Glutamic acid hydrochloride	50.0 mgm.
Lactic acid (as Na salt)	0.1 cc.
Pimelic acid	0.001 mgm.
Salt mixture	
Phenol red	

Quantities given are for 10 cc. medium.

Table 1 shows the effect of the addition of the hydrolyzed distillates to the control solution.

Since the addition of 0.01 cc. of the alcoholic 4b solution to 10 cc. of control medium permits the formation of 3.2 to 3.5 mgm. bacterial N, it is clear that the distillate carries a considerable proportion of the activity. A larger scale preparation was therefor undertaken.

Through the courtesy of Dr. Y. Subbarow of the Department of Biological Chemistry, Harvard University Medical School, and of the Lederle Company, Inc., Pearl River, New York, a concentrate of 300 kilograms of liver was made available. Briefly, this consisted in the 96 per cent alcohol soluble fraction of an aqueous extract, concentrated in vacuo until 1.0 cc. was equivalent to 100 grams liver tissue. Solid material, separating on long chilling, was removed by filtration, and the syrupy filtrate, after acidification, was extracted with isoamyl alcohol. Vacuum distillation of the extract with the occasional addition of water, removed most of the isoamyl alcohol, leaving about 1 liter of an aqueous solution. The extract was supplied in this form by the Lederle Company, to whom the writer wishes to express his appreciation and thanks.

The procedure followed in converting this material into its

acetylated esters is fully described elsewhere (Mueller, 1937b), and need not be repeated. It differs in no material respect from that recommended by Cherbuliez, Plattner and Ariel.

The first distillation was carried out rapidly from a Claissen flask, and the distillate was divided roughly into (*a*) low, (*b*) medium and (*c*) high boiling fractions by means of a Fischer triangle. The quantities obtained were respectively 14.8, 62.0 and 17.1 grams. Distillation was continued until the bath temperature reached 220°, at which point some decomposition of the residue began to take place.

TABLE 1*

Effect of distillates of acetylated esters of "4b"

TUBE NUMBER	COMPOSITION OF MEDIUM	BACTERIAL N
		mgm.
1	Control + hydrolysate of solid portion, 0.02 mgm.	1.16
2	Control + hydrolysate of solid portion, 0.05 mgm.	1.74
3	Control + hydrolysate of solid portion, 0.1 mgm.	2.20
4	Control + hydrolysate of solid portion, 0.2 mgm.	2.88
5	Control + hydrolysate of liquid portion, 0.03 mgm.	2.66
6	Control + hydrolysate of liquid portion, 0.075 mgm.	3.02
7	Control + hydrolysate of liquid portion, 0.15 mgm.	3.30
8	Control + hydrolysate of liquid portion, 0.3 mgm.	3.54
9	Control	0.58

* In the case of this particular experiment, the National Institute of Health strain of the Park 8 diphtheria bacillus was used instead of the Allen strain. The control was therefor enriched by 1.0 mgm. tryptophane and 0.05 cc. ethyl alcohol.

These fractions, after hydrolysis of small portions with N/1 HCl, when added to control media gave the results indicated in table 2.

Evidently the low boiling fraction alone is either without growth-promoting power, or contains an inhibitory substance. When mixed with the other fractions, the latter effect is indicated.

These distillates were now slowly re-distilled from the Rittenberg apparatus, modified by sealing on a glass bulb to permit dealing with large quantities. In this distillation, the original three fractions were expanded to thirteen. All of these were oils ex-

cept the second, which consisted of 3 to 4 grams of coarse white crystals, which were later identified as acetamide, resulting from the presence of ammonium salts in the extract. This fraction, very troublesome because of the obstruction of the side arm, which necessitated the use of a direct flame to keep the passage clear during distillation, could have been avoided by short alkaline vacuum distillation of the original extract to remove the NH_3.

Hydrolysis of a few milligrams of each of these with HCl permitted the tests of table 3 to be carried out.

In order to get additional information, each fraction was next tested over a wider quantitative range; then combinations of various fractions were tested in twos, attempting so to choose

TABLE 2

Effect of fractions of first distillate (Allen strain)

TUBE NUMBER	COMPOSITION OF MEDIUM	BACTERIAL N
		mgm.
1	Control + fraction (a), 1.0 mgm.	0.21
2	Control + fraction (b), 1.0 mgm.	2.22
3	Control + fraction (c), 1.0 mgm.	2.00
4	Control + fractions (a) + (b), 1.0 mgm. each	1.54
5	Control + fractions (a) + (c), 1.0 mgm. each	1.66
6	Control + fractions (b) + (c), 1.0 mgm. each	3.08
7	Control + fractions (a) + (b) + (c), 1.0 mgm. each	2.69

quantities of each that a mutual or simply an additive effect could be brought out. Then more than two fractions were combined in the same way. It would be quite useless to present these experiments in detail. Of course, no one of these fractions represents a pure compound. It is surprising how far below its boiling point a substance will begin to appear in a distillate, and how far above it, traces will still persist. Had there been any means of knowing the number of compounds in these distillates which either accelerate or inhibit the growth of our organism, and which ones would show an effect in the absence of the others, the analysis of these results would have been simplified. Without such knowledge, it was merely possible to conclude that the lowest and the highest fractions probably contained substances

inhibitory in concentrations of 1.0 mgm. or somewhat less, and that more than one, and possibly several accelerating substances were present.

Gradually it became evident that the most striking mutual effect was shown by combining suitable quantities of the low-

TABLE 3

Effects of fractions of second distillate

TUBE NUMBER	COMPOSITION OF MEDIUM	BACTERIAL N
		mgm.
1	Control + fraction 1, 1.0 mgm.	0.16
2	Control + fraction 1, 0.1 mgm.	0.14
3	Control + fraction 2, 1.0 mgm.	0.18
4	Control + fraction 2, 0.1 mgm.	1.26
5	Control + fraction 3, 1.0 mgm.	1.38
6	Control + fraction 3, 0.1 mgm.	0.42
7	Control + fraction 4, 1.0 mgm.	2.08
8	Control + fraction 4, 0.1 mgm.	0.52
9	Control + fraction 5, 1.0 mgm.	0.60
10	Control + fraction 5, 0.1 mgm.	0.10
11	Control + fraction 6, 1.0 mgm.	1.42
12	Control + fraction 6, 0.1 mgm.	0.32
13	Control + fraction 7, 1.0 mgm.	0.72
14	Control + fraction 7, 0.1 mgm.	0.28
15	Control + fraction 8, 1.0 mgm.	2.04
16	Control + fraction 8, 0.1 mgm.	0.68
17	Control + fraction 9, 1.0 mgm.	1.38
18	Control + fraction 9, 0.1 mgm.	0.40
19	Control + fraction 10, 1.0 mgm.	0.88
20	Control + fraction 10, 0.1 mgm.	0.24
21	Control + fraction 11, 1.0 mgm.	1.16
22	Control + fraction 11, 0.1 mgm.	0.28
23	Control + fraction 12, 1.0 mgm.	1.50
24	Control + fraction 12, 0.1 mgm.	0.20
25	Control + fraction 13, 1.0 mgm.	0.12
26	Control + fraction 13, 0.1 mgm.	0.32

boiling and of the high-boiling fractions. At this stage, too, the crystals making up the greater part of the second fraction were identified as acetamide, representing simply an inert impurity. The substance was present in solution also in fractions 1, 3 and 4. These fractions were consequently re-distilled using a special

receiver which enabled the side arm of the distilling column more readily to be kept free from solid material. After concentrating the acetamide as completely as possible into a single fraction, it was recrystallized from chloroform, the mother liquors containing the active substance added to the fluid fractions, and again fractionally distilled, this time very slowly in an attempt to obtain the active material in as pure a state as possible.

Acetamide was still present, however, in sufficient quantity to separate on standing in the ice box from the two lowest boiling fractions. These likewise contained the highest concentration of active material.

Meanwhile, all the other middle and high-boiling fractions had again been redistilled, very slowly. The procedure was to start with the lowest boiling, and separate it into perhaps three sub-fractions and an undistilled residue, with the heating of the bath and column carefully controlled by means of rheostats and ammeters, and the vapor temperature accurately noted with a thermocouple. The next higher boiling fraction would then be added, after lowering the bath and column temperatures to those at which the first fraction had begun to distil. By carefully increasing the heat, and noting the temperature of the vapors when distillation commenced, the distillate would be allowed to run into the sub-fraction already obtained which had passed over under similar conditions, commencing a new sub-fraction only when the temperature exceeded that of the highest one already obtained. In this way it was hoped that substances with boiling points differing by only a few degrees from each other would gradually become concentrated into the same fraction.

The redistillation of the entire 13 fractions in this way resulted in a total of but 11 fractions. By far the greatest bulk of the material was contained in fractions 6, 7 and 8.

Tests of the two lowest boiling active fractions, containing acetamide were now carried out, adding fractions 6 and 10 in amounts of 1.0 mgm. and 0.5 mgm. respectively to the control. These two seemed to be the most effective in supplementing the effect of the low-boiling material.

The results are shown in table 4.

This result, repeatedly confirmed and amplified, gave evidence of a substance exerting its effect over a comparatively narrow range of concentration, and either without effect or inhibitory below and above its active zone.

The combined weight of fractions 1 and 2 was 0.68 gram. About half of the material was crystalline (acetamide ?). Hydrolysis of these fractions was effected by refluxing for 1 hour with 25 cc. of about 1.5 N H_2SO_4. The solution was diluted somewhat, and an excess of cold, saturated $Ba(OH)_2$ added, permitting the NH_3 to be distilled out in vacuo. The excess Ba was then accurately removed with dilute H_2SO_4, and the water-clear solution evaporated to dryness in vacuo. The resulting white granular material was recrystallized three times from hot alcohol and in this way were finally obtained about 10 mgm. of white crystals.

TABLE 4

Effect of low-boiling material on controls supplemented by middle and high fractions

TUBE NUMBER	COMPOSITION OF MEDIUM	BACTERIAL N
		mgm.
1	Control with fractions 6 and 10 + fraction 1, 1.0 mgm.	1.10
2	Control with fractions 6 and 10 + fraction 1, 0.1 mgm.	1.84
3	Control with fractions 6 and 10 + fraction 1, 0.01 mgm.	2.29
4	Control with fractions 6 and 10 + fraction 2, 1.0 mgm.	1.22
5	Control with fractions 6 and 10 + fraction 2, 0.1 mgm.	2.40
6	Control with fractions 6 and 10 + fraction 2, 0.01 mgm.	2.04

Controls without fraction 1 or 2, but containing 6 and 10 produced regularly about 1.0 mgm. bacterial N.

Even assuming this material to be approximately pure, too little was available for analysis with the available facilities. Fortunately it was possible to identify it another way almost as soon as obtained.

Knight (personal communication) had recently shown that his "staphylococcus vitamine" was a mixture of nicotinic acid and vitamin B_1. Both these substances were at once tried in various combinations with our distillate fraction, and on the same day that the above crystals were obtained it became evident that nicotinic acid, when substituted for the low-boiling material, in

TABLE 5

Comparison of the effects of the crystals from liver and nicotinic acid

TUBE NUMBER	COMPOSITION OF MEDIUM	BACTERIAL N
		mgm.
1	Control with fractions 6 and 10 + crystals, 1.0 mgm.	1.10
2	Control with fractions 6 and 10 + crystals, 0.1 mgm.	1.16
3	Control with fractions 6 and 10 + crystals, 0.01 mgm.	2.12
4	Control with fractions 6 and 10 + crystals, 0.001 mgm.	1.80
5	Control with fractions 6 and 10 + crystals, 0.0001 mgm.	1.10
6	Control with fractions 6 and 10 + nicotinic acid, 1.0 mgm.	1.12
7	Control with fractions 6 and 10 + nicotinic acid, 0.1 mgm.	1.32
8	Control with fractions 6 and 10 + nicotinic acid, 0.01 mgm.	2.92
9	Control with fractions 6 and 10 + nicotinic acid, 0.001 mgm.	1.54
10	Control with fractions 6 and 10 + nicotinic acid, 0.0001 mgm.	1.10
11	Control with fractions 6 and 10	0.92

TABLE 6

Further comparison of effects produced by crystals from liver, nicotinic acid and its amide

(Each tube contains 1.0 mgm. fraction 10 in the control)

SUBSTANCE	N		
	Crystals	Nicotinic acid	Nicotinamide
Control			0.34
mgm.	*mgm.*	*mgm.*	*mgm.*
0.0001	0.66	0.66	
0.00025	0.86	0.86	
0.0005	1.07	1.11	
0.001	1.51	1.51	
0.002	1.54	1.79	
0.003	1.89	1.84	
0.004	2.15	2.02	
0.005	2.20	2.18	
0.01	2.20	2.29	0.84
0.02	2.04	1.96	
0.025	1.62	1.68	(0.03 mgm.) 1.34
0.05	1.54	1.45	1.63
0.1	1.37	1.38	2.24
0.5			1.88
1.0			1.56

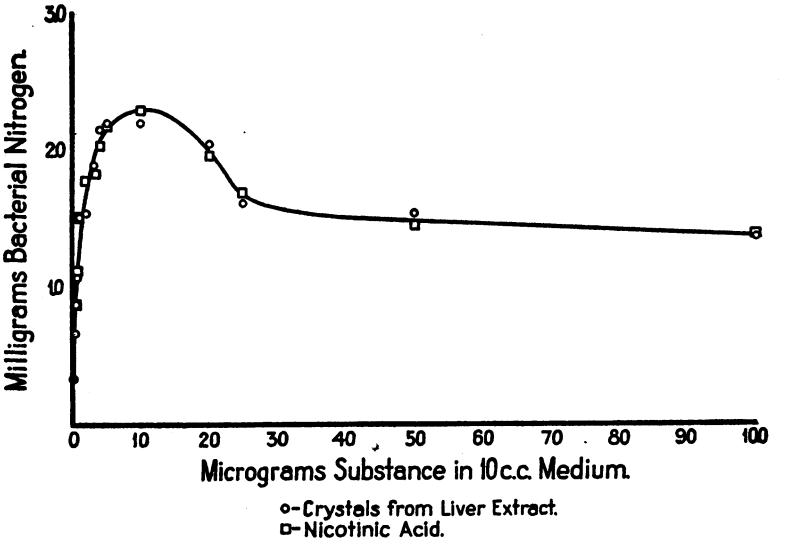

FIG. 1

TABLE 7

The effect of crystals from liver, nicotinic acid and its amide in the absence of fractions 6 and 10

SUBSTANCE	BACTERIAL NITROGEN		
	Crystals	Nicotinic acid	Nicotinamide
Control			0.10
mgm.	*mgm.*	*mgm.*	*mgm.*
0.0001	0.28	0.27	
0.00025	0.40	0.40	0.13
0.0005	0.64	0.56	0.23
0.001	0.91	0.98	0.48
0.0025	0.89	0.86	0.45
0.005	0.30	0.08	0.80
0.01	0.17	0.08	1.14
0.025	0.22	0.06	1.33
0.05			0.87
0.1			0.23
0.25			0.28

the presence of fractions 6 and 10, showed the same property of growth-acceleration when the amount used was neither too great nor too little. A comparison of the solubilities and crystal form showed close similarity, and a mixed melting point made the identity practically certain. The crystals from the liver, a mixture of these with nicotinic acid, and the latter alone, melted at 234.0, 234.4 and 235.0 (uncorrected).

Growth tests set up with the crystals and nicotinic acid in parallel, shown in table 5 give equally convincing biological proof of their identity.

In table 6 and figure 1 are shown the results of a further comparison of the crystals from liver and nicotinic acid through the zone of maximum effect. Results obtained with nicotinamide, the form in which the substance may well occur in the original tissue extract, are also included in the table.

The part played by fractions 6 and 10, which have been included in the control media of the last three tables, is shown in table 7.

DISCUSSION

There can be no reasonable doubt that the 10 mgm. of crystalline material isolated from liver extract by the procedure outlined is nicotinic acid, and that this substance accounts for a part of the growth-promoting activity of tissue extracts for the diphtheria bacillus. That additional substances play a part of equal or greater importance, is also clear.

The effect of the nicotinic acid is peculiar in that there is evidently a well defined optimal concentration, below which, of course, no effect is exerted, and above which the effect is greatly diminished. The concentration necessary to produce maximal growth is evidently a function of the concentration of one or more other substances. For example, in the absence of "fraction 10," the heaviest growth takes place with a concentration of about 0.1 γ per cubic centimeter of medium, while in the presence of this fraction, about ten times that concentration must be supplied. Curiously, too, the amide of nicotinic acid appears to be only about one-tenth as effective, weight for weight,

as the acid, itself, although the general type of growth curve produced is the same.

The discovery by Knight that nicotinic acid is required by the staphylococcus, taken together with the findings here presented for the diphtheria bacillus, increase the probability that a general group of substances will eventually become recognized as being the components of meat or tissue extracts so essential to the growth of many of the bacteria pathogenic for man and animals. Certain of the more fastidious organisms may require several, others only one or two. Knight finds that vitamin B_1 is required by the staphylococcus in addition to nicotinic acid, and other workers have found it essential for quite different types or organisms. So far, it has not been possible to demonstrate an effect of this compound on the diphtheria bacillus.

That several different extractive substances should be required by the diphtheria bacillus is not surprising. The work on amino-acid requirements reported in the earlier papers of this series suggests that the organism is definitely limited in its synthetic capabilities. There is no reason to assume that this limitation applies only to amino acids; as our general knowledge of various enzyme systems increases, to select but a single example, it becomes more and more evident that many of these involve a complex interaction of several different substances. The fact that nicotinamide takes part in at least two such systems has recently become known, due to the work of Warburg et al. (1935) and of Euler et al. (1935) and their collaborators. It is altogether probable that its requirement by the diphtheria bacillus is in connection with some such enzyme-co-enzyme mechanism, and that the organism, being unable to synthesize the pyridine nucleus, must obtain it, preformed, from its substrate.

That the greater part of the remaining growth-promoting effect of tissue extract on the diphtheria bacillus will be found due to comparatively simple substances is definitely indicated by the fact that they can be distilled without decomposition after esterification and acetylation, and will with stand acid hydrolysis. The writer hopes to be able to report later on the results of work now being carried on with these remaining factors.

CONCLUSIONS

1. The method of Cherbuliez, Plattner and Ariel for preparation and distillation of the acetylated esters of amino acids has been applied to a study of tissue extraction required by the diphtheria bacillus for growth.

2. Distillates so prepared from a liver-extract concentrate contain the greater part of the growth-promoting activity of the extract.

3. One of the active substances has been isolated in a quantity of about 10 mgm. from 300 kgm. of liver and identified as nicotinic acid. Its maximum effect appears to be exerted at a concentration of about 1 microgram per cubic centimeter of medium but varying somewhat with the composition of the control medium. Nicotinamide is effected in approximately ten times the concentration of the free acid.

4. One or more additional active substances are also present in the distillate and are now being investigated.

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