STUDIES ON CULTURAL REQUIREMENTS OF BACTERIA

IX. Tissue Extractives in the Growth of the Diphtheria Bacillus[1]

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Received for publication March 12, 1937

It was shown in the sixth paper of this series (Mueller, 1935c) that the organic substances present in a tissue extract (liver) which are essential for the growth of a strain of diphtheria bacillus, could be very considerably purified. The method consisted in vacuum concentration of a hot aqueous extract, precipitation with alcohol up to 95 per cent concentration, and removal of the alcohol *in vacuo*. The solution was precipitated with neutral lead acetate, the precipitate discarded and excess lead removed from the filtrate with H_2S. The resulting material was adsorbed twice with norit charcoal, and the latter boiled out three times with acid 50-per-cent ethyl alcohol. Evaporation *in vacuo* until the alcohol was removed completed the preparation. The resulting concentrate is a clear, light brown limpid solution, acid in reaction and with an unpleasant bitter taste. One cubic centimeter is equivalent to about 85 grams of liver, and contains 41.0 mgm. solids with 3.9 mgm. nitrogen. (The point to which evaporation may be carried is entirely a matter of convenience.) From 0.1 cc. to 0.2 cc. of this solution, added to 10 cc. of otherwise extractive-free media containing suitable amino acids, inorganic salts and a source of energy, i.e., glycerol or sodium lactate, induces maximum growth with practically all the strains of *Coryne-*

[1] The writers are indebted to the Lederle Laboratories for the preparations of liver extract used in these studies.

bacterium diptheriae which have been tested. The present paper describes various attempts to isolate the one or more compounds which bring about this effect.

CHOICE OF TEST STRAIN

Since the amino-acid requirements for a culture of the Park Williams number 8 strain had been defined (Mueller and Kapnick, 1935) it was thought best to use this organism rather than the strain ("H Y") employed in our earlier work with liver extract, as the Park strain is readily available to all laboratories. It turned out that the choice was an unfortunate one, for reversal of the pH often failed to take place when various fractions of the eluate were tested with the result that little growth was obtained, and it was impossible to be sure whether such fractions were completely inactive or whether more abundant growth would have taken place if the reaction had turned alkaline. Meanwhile it had been found that the advantage of general distribution of this strain was illusory for there is a remarkable and surprising difference in growth requirements for cultures called Park 8 obtained from various sources. A forthcoming paper will deal with a study of these differences.

Attempts were made in two directions to overcome the difficulty due to the reaction. In the first place the media were buffered by the addition of 0.25 per cent $Na_2HPO_4 \cdot 2H_2O$, which resulted in moderate improvement only. Secondly, the replacing of glycerol by sodium acetate or sodium lactate in the usual control formula was attempted with the hope that the base made available in the breaking down of the acid would aid in the reversal of reaction. The latter expedient proved to be extremely satisfactory. In addition, when tested with several strains of the Park 8, and with other more recently isolated cultures, it developed that lactic acid was much more favorable to growth of certain strains than glycerol, even when the question of pH was not involved. The reverse was also true, and the particular Park 8 strain which we had studied in some detail, failed to grow as heavily with sodium lactate as it did with glycerol.

In the course of these experiments on the reversal of reaction,

we encountered a freshly isolated strain of *C. diphtheriae* which grew much more rapidly in the first twenty-four hours than the various Park 8 cultures. With the latter, it was necessary to wait at least 36 to 40 hours before judging grossly of the comparative abundance of growth, and three days to determine the amount for record. With the new strain, on suitable media, abundant growth was present in eighteen hours, and only two days were necessary for complete development. The saving of time made possible by using this culture appears to offset the disadvantage of introducing another non-standard strain. The new culture ("Allen strain"), morphologically exhibits good polar bodies, but shows few club forms. It ferments typically and is virulent for guinea pigs. When freshly isolated, it failed to produce a pellicle on meat infusion-peptone broth even after a considerable number of transplants. Carried during the same period on a medium of the following composition, it formed a well developed pellicle after a few generations.

Casein hydrolysate*	1.0 per cent
Cystine	0.01 per cent
Glutamic acid hydrochloride	0.5 per cent
Salt mixture†	0.25 cc.
Glycerol	0.5 per cent
Liver eluate	2.0 per cent

Transfer from this medium to meat infusion-peptone broth did not result in pellicle formation on the latter. The strain has, therefore, been carried in stock culture by daily transplant on the above medium, and has maintained its virulence and fermentative properties unchanged for 16 months. When it was determined to

* Commercial casein boiled eighteen hours with 10 parts concentrated HCl, evaporated to thick syrup *in vacuo*, taken up in water to make a 20 per cent concentration (calculated on weight of original casein), and stirred cold with sufficient norit charcoal so that the color after filtration is pale yellow.

† Salt mixture:

NaCl	20.0 grams
$Na_2HPO_4 \cdot 2H_2O$	10.0 grams
KH_2PO_4	1.4 grams
$MgCl_2 \cdot 6H_2O$	1.2 grams
HCl	5 cc.
Water to 100 cc.	

adopt this organism as the test strain, a number of ampules of a young culture were frozen and dried on the Flosdorf-Mudd apparatus. Recently it has been necessary to go back to this material since the passage culture suddenly became quite different in certain of its growth requirements although still virulent and typical in other respects. The dried culture, eleven months old, was living and perfectly characteristic.

METHODS

The procedure is described in the earlier papers of this series to which reference is made (Mueller 1935a, b and c, and Mueller and Kapnick 1935). The relative quantities of growth are indicated by the milligrams of nitrogen in the centrifuged and washed bacteria after either two or three days incubation.

EXPERIMENTAL

I. Selective adsorption. A large number of experiments were carried out in which it was attempted to effect further purification of the eluate by adsorbing with fuller's earth, charcoal or lead sulfide. In general, the results were unsatisfactory, although it was possible to remove considerable inert material, amounting perhaps to a third or more or the total, including practically all the color, by a single adsorption with a small amount of fuller's earth followed by a similar treatment with a small quantity of norit. The activity was removed, however, by further adsorption with either reagent and could not be quantitatively eluted. It appears from these results that some essential component is either destroyed or becomes so firmly attached to the adsorbing material when exposed in a moderately purified form, that it cannot be removed.

On the whole, the results with selective adsorption were so difficult to duplicate that it seemed wise to abandon them and search for a better method.

II. Various precipitants. The reagents used were $HgCl_2$ in both aqueous and alcoholic solution, $HgSO_4$, Ag_2SO_4 with acid, neutral and alkaline reaction, and $Ca(OH)_2$ and alcohol-ether.

The results with the Hg and the Ag salts were not promising.

In general, the active material was not precipitated, although silver in alkaline solution brought down part of it. Moreover, there was usually considerable loss, due perhaps to adsorption on the sulfide precipitates formed in removing the metals.

Of all of the precipitation methods tried, that in which calcium salts were thrown down with alcohol and ether seems to give the most encouraging results. The procedure consisted in adding $Ca(OH)_2$ suspension to the eluate until alkaline, then adding 10 volumes each of ethyl alcohol and ether. The precipitate was removed by filtration, and the alcohol and ether removed from the filtrate by vacuum distillation. Although some activity was lost in the precipitate, the greater part of the material passed into the filtrate. A moderate amount of purification could be effected in this way with considerable regularity and the method was utilized in some of the further procedures to be described. On the whole, however, it is doubtful whether enough is to be gained to be of any real value and no detailed protocols of these experiments need be given.

III. Extraction with immiscible solvents. The excellent results obtained with butyl alcohol in the separation of amino acids suggested its use for our present purpose. A number of experiments were carried out, of which the following is an example:

March 6, 1936. The material used was a calcium-alcohol ether filtrate prepared as described above from a liver eluate made rather differently from the one which has been employed in most of the experiments. In this instance, a liver concentrate (Lederle Solution Liver Extract Parenteral Refined and Concentrated, NNR), and containing considerably more total material than the 95-per-cent alcohol filtrate which we had used, was absorbed on charcoal and eluted with acid alcohol in the same way as the other preparation. The method is described by Subbarow, Jacobson and Fiske (1936).

Ten cubic centimeters of this preparation, of which 1 cc. represented 100 grams liver, were made strongly alkaline by the addition of 1 cc. of 10 N NaOH. The solution was then extracted five times by shaking in a test tube with 10 cc. butyl alcohol, centrifuged in order to hasten the separation, and the butyl alcohol extracts pipetted off. The aqueous residue was then acidified with 2.5 cc. concentrated HCl and the acid solution again extracted five times as before. Each fraction, mixed

with about two volumes of water was neutralized to litmus, the butyl alcohol distilled out *in vacuo*, adding more water as required, and the solutions each brought to a final volume of 40 cc.

Media were prepared as follows and inoculated with the Allen strain. Nitrogen determinations were made on the washed bacteria after about 40 hours growth.

1. Control solution* 9.6 cc. + NaOH extract 0.4 cc..... 0.74 mgm. N
2. Control solution 9.6 cc. + HCl extract 0.4 cc..... 3.36 mgm. N
3. Control solution 9.6 cc. + Residue extract 0.4 cc..... 0.20 mgm. N

Nitrogen determinations on the original material and the three fractions gave the following results:

Ca-alcohol-ether filtrate	6.62 mgm./100 grams liver
NaOH extract	1.56 mgm./100 grams liver
HCl extract	2.23 mgm./100 grams liver
Residue	2.24 mgm./100 grams liver
	6.03 Loss about 10 per cent

Experiments of this type invariably gave results of the same sort, and it was evident that nearly two-thirds of the total nitrogenous material of the calcium-alcohol filtrate could be removed with very little loss of activity. The moderate growth with the alkaline butyl fraction and the fact that not infrequently somewhat better growth was obtained by mixing the three fractions together in the media, than by the use of the acid extract alone, may be significant. For the present, the relatively feeble effect of the alkaline extract and the residue have been disregarded.

The next step was to attempt the further purification of the HCl-butyl alcohol extract by shaking out with ether. Surprisingly, this resulted in a separation of the active material into two fractions, neither one alone producing maximal growth, but when mixed, being fully active. The following is a typical experiment:

March 19, 1936. A portion of the same calcium alcohol-ether filtrate used in the experiment of March 6 was diluted with three parts of water.

* Control solution has same composition as formula for stock media, page 155 without the liver eluate, and with 0.1 cc. lactic acid, as sodium lactate, replacing the glycerol. It is so prepared for stock purposes that the quantities required for 10 cc. media are contained in 2.5 cc. of solution. This is diluted as required for use.

One hundred cubic centimeters of this solution were adsorbed by stirring for five minutes with 3.2 grams fuller's earth, filtered, and the filtrate treated in the same way with 1.25 grams norit charcoal. These quantities had previously been shown to remove a considerable amount of inert material and to cause little change in growth stimulating properties. The filtrate was concentrated *in vacuo* to 25 cc.

To 10 cc. of this material was added 1.0 cc. of 10 N NaOH, and the alkaline solution was extracted five times with 10 cc. portions of butyl alcohol. The aqueous portion was then neutralized with concentrated HCl, and 1 cc. additional acid added. Ten extractions with 10 cc. ether were then carried out, followed by five more with 10 cc. butyl alcohol. The acid butyl and the ether extracts were freed from solvent by vacuum distillation, after neutralizing, and made up to 50 cc. each with water. The following experiment was set up in duplicate:

	Mgm. bacterial N A	B
The loss, of course, is purely mechanical.		
1. Control solution 9.5 cc. + ether extract 0.5 cc.....	1.37	1.60
2. Control solution 9.5 cc. + acid butyl extract 0.5 cc...	1.39	1.39
3. Control solution 9.5 cc. + both extracts 0.5 cc.....	3.26	2.86

The control solution alone gives a negligible amount of growth. Doubling, or increasing still more the quantity of either fraction, does not give an increase of growth comparable to that produced by the mixture.

Several further experiments indicated that the order of carrying through these procedures was unimportant. The ether extraction could be done on the original eluate or calcium-alcohol-ether filtrate, or the butyl alcohol extraction could be done first, and later, after removing butyl alcohol, the material shaken out with ether. In all cases the separation took place only from an acid solution.

DISCUSSION

The comparative ease with which the activity of these preparations is lost by adsorption on various substances leaves any type of purification involving the production of precipitates open to considerable risk. In the course of an investigation of this type there is no means of knowing how many separate substances are

involved in the effect being studied. It is clear that if two or more compounds are required the loss or partial destruction of any one will be followed by greatly diminished growth. In all experiments, therefore, the possibility of a separation into two fractions, each necessary, was kept in mind, but it was only with the employment of extraction with butyl alcohol and ether that facts were observed which indicated that this was really the case.

The observation that a second adsorption could not always be followed by satisfactory recovery of the material, renders such methods of purification on a large scale hazardous. The same applies to precipitation methods. Were it not for this fact, and perhaps even in spite of this drawback, a considerable degree of purification might be accomplished by removal of inert materials by suitable combinations of these methods. Extraction with organic solvents, however, should avoid any such danger and from a number of such small-scale experiments there is every indication that their behavior is uniform and that a satisfactory separation of the activity into two fractions can be made by butyl alcohol and ether with a minimum of loss. Neither of these fractions alone is entirely without activity in promoting a certain amount of growth of the organism, but quantitative experiments in which varying amounts of both fractions are used have indicated clearly that the combined action of the two is essential for optimal growth. The further studies of these two fractions will be the subject of the next paper of this series.

CONCLUSIONS

1. The liver eluate previously described can be freed from a small proportion of inactive material by adsorption with either fuller's earth or charcoal.

2. The use of larger quantities of adsorbants results in a partial loss of activity either by destruction or failure of elution.

3. Purification by means of precipitating with heavy metal salts has not proved useful.

4. A certain amount of inert material is removed by precipitation with lime and alcohol and ether.

5. The active materials are extracted from strongly acidified aqueous solution by butyl alcohol but not from an alkaline one.

6. The materials so extracted can be separated into two fractions by repeated extractions with ether; also only from acid solution. Neither of these fractions alone shows the full effect of the combination of the two in stimulating growth of the diphtheria bacillus. These fractions are being further studied.

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