STUDIES ON CULTURAL REQUIREMENTS OF BACTERIA[1]

VI. THE DIPHTHERIA BACILLUS

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Earlier work (Mueller, Klise, Porter and Grabiel, 1933) has shown that for the growth of a particular strain of diphtheria bacillus which has been investigated—a moderately strong toxin producer, isolated some years ago by Dr. Ho Yu in this laboratory—a combination of meat extract, amino acids and inorganic salts was required for successful growth. The amino acid requirements, in the presence of a crude tissue extract (Liebig's extract of meat) have now been fairly well defined for this strain (Mueller, 1935a). In the preliminary work a certain amount of information was obtained as to chemical methods for the separation of the constituents of the meat extract, using a crude acid hydrolysate of edestin, in place of pure amino acids, as a control. It was stated, in reporting this work, (Mueller, Klise, Porter and Grabill, 1933) that either the greater part of the substance in Liebig's extract, which seemed essential for growth, was lost during the fractionation, or else that additional factors—concerning the nature of which there was no clue—would be found to be involved. Since it is now possible to substitute pure, and to a certain extent, synthetic amino acids for the crude edestin or other protein hydrolysate, we are in a better position to re-

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investigate the tissue extract fraction. Moreover, the quantitative method of estimation of growth now being used (Mueller, 1935b) provides a much more accurate means of following through a series of experiments of this kind. This paper will therefore deal with a further study of the action of the extractives.

METHODS

The same methods used in the experiments to which reference has already been made, are used throughout the present work. The various control substances are added in the amounts shown in table 1 together with stated quantities of the variable material, and a small amount of phenol red. The pH is brought to about 7.6

TABLE 1

AMINO ACID MIXTURE		SALT MIXTURE A	
	mgm.		*mgm.*
Glycine	5.0	NaCl	50.0
dl-Valine	10.0	K_2HPO_4	1.8
dl-Phenylalanine	1.0	$CaCl_2$	0.11
dl-Methionine	2.0	$MgSO_4 \cdot 7H_2O$	0.11
l-Glutamic acid HCl	50.0	$FeCl_3 \cdot 6H_2O$	0.035
l-Cystine	1.0		
l-Tryptophane	1.0		
l-Histidine HCl	1.0		

with sodium hydroxide. The volumes are then adjusted to 10 cc. per tube and the medium sterilized in the autoclave at 10 pounds pressure for ten minutes. Inoculation is made from a twenty-four hour broth culture of the organism and the tubes are incubated in a slanted position at 35° for about sixty hours. Determination of the total nitrogen of the bacterial growth is then carried out by the method earlier described (Mueller, 1935b).

EFFECT OF LIEBIG'S EXTRACT

If, to a control solution of amino acids, ethyl alcohol and salts, a solution of Liebig's extract of meat be added in varying proportions the effect on the growth of this strain of diphtheria bacillus is illustrated in figure 1.

However, as a starting point for the isolation of substances of an unknown nature, Liebig's extract offers certain quite evident drawbacks, notably the fact that the method of its preparation is not under immediate control and is, to a certain extent at least, not generally known. The heating to which the material is subjected during evaporation, quite irrespective of the nature of the substance extracted and the manner of extraction, may well lead to the production of a considerable number of by-products in addition to the numerous normal components of a tissue extract. It therefore seemed wiser to cast about for some other source of

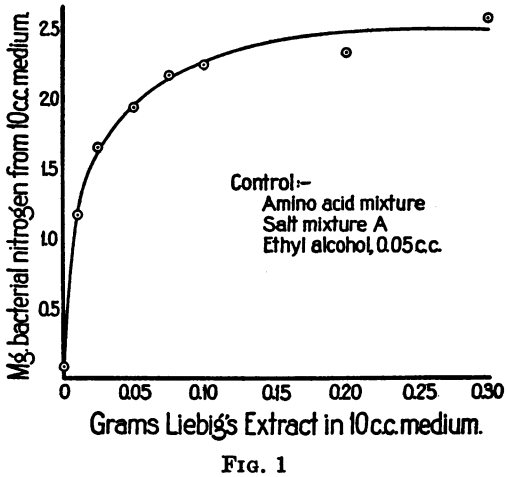

FIG. 1

material equally effective, free from the drawbacks pointed out above and if possible available in considerable quantities at moderate expense. We have considered, and in a superficial way tested the possibility of using plant extractives and although such extracts are efficacious we have discarded this source, for the present at least, as involving still other difficulties.

EFFECT OF FRESH TISSUE EXTRACTS

Animal tissue, of whatever source, appears to supply an active extract. An unexpected difficulty however, was met with, in that the use of considerable quantities of freshly prepared hot-water extract of muscle and other tissue caused an acid reaction

to develop in the cultures which stopped the growth at an early stage through direct inhibition by the pH.

It is probable, but by no means definitely proved, that this effect is due to glucose or other fermentable carbohydrate in the infusion. Thus, it has occasionally been possible to destroy the carbohydrate by fermentation with yeast, thereby avoiding the formation of acid, with consequent heavy growth. Such a procedure naturally introduces the possibility that the yeast itself furnishes an essential element, and is therefor not suitable for the purposes of our work. It was also possible in some experiments, so to alter the infusion by autoclaving with NaOH, which should destroy glucose, that heavy growth resulted. However, controls in which glucose was again added to such treated infusions and in a quantity comparable to that which must have been present in the original infusion have also occasionally permitted heavy growth. We are, therefore, not in a position to state definitely that this peculiarity is due to the presence of glucose, nor to explain why it is not met with in the case of Liebig's extract.

In general, extracts of various animal tissues behave in much the same way as does beef-heart infusion. For example, a boiled and filtered extract of beef liver showed marked growth-stimulating properties in small quantities, but in larger amounts produced an acid reaction which checked the growth, again probably due to the presence of glucose.

LIVER EXTRACT

Liver infusion offers an unusually desirable starting point for an investigation of this type, since it is now being actively studied by a number of workers in connection with its curative effect in pernicious anaemia. We were fortunate in enlisting the interest and assistance of Dr. Yellapragada Subbarow of the Department of Biochemistry at Harvard Medical School in our problem. He was able to supply us with various fractions of a liver extract which had been prepared originally according to his specifications following the method of Cohn, McMeekin and Minot (1933), and could presumably be closely duplicated. We found most of his fractions active in varying degree and were able to obtain

from him one fraction prepared from a ton of beef liver, of no use for his purposes, showing a quite satisfactory degree of stimulation of growth of the diphtheria bacillus. The work to be described below has been carried out with this fraction.

The method of preparation of this fraction as described to us by Dr. Subbarow is roughly as follows: Fresh liver is minced, extracted with water at 70°C. and filtered; the filtrate is concentrated in vacuum to a syrup and is precipitated by the addition of alcohol up to a concentration of 65 per cent and is again filtered. To the 65 per cent alcoholic filtrate more absolute alcohol is added

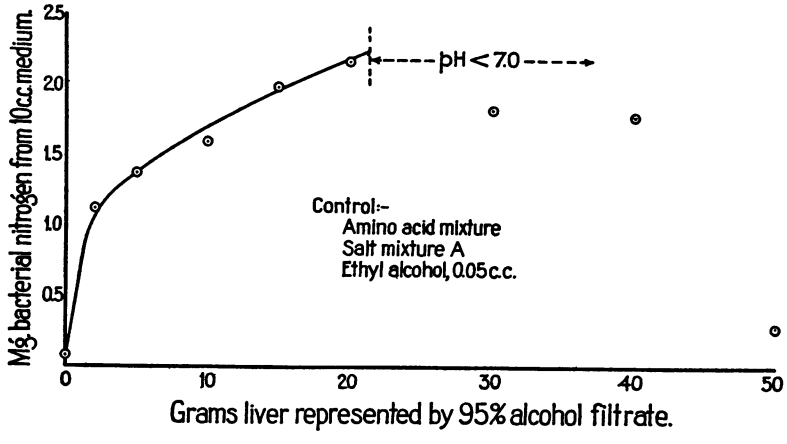

FIG. 2

to a concentration of 95 per cent and the precipitate is again removed. The 95 per cent alcohol filtrate, which has been concentrated in vacuum to a syrup is the material supplied us by Dr. Subbarow. The substances contained in this fraction are almost without effect in pernicious anaemia but show considerable growth stimulation for our strain of diphtheria bacillus. By no means all of the essential material occurring in liver is present in this fraction, but there is a fairly high concentration and it represents a by-product source which is a distinct advantage from the standpoint of expense; and at the same time it is prepared in a manner easily duplicated. In addition, a considerable degree of purification may be assumed to have taken place. Figure 2

shows the effect of the addition of this concentrate to the control solution.

It will be seen that even here one is faced with the problem of removing the acid-forming substances almost as a preliminary step in the investigation. Fortunately this proved to be rather easily accomplished.

The methods which appear to be producing the most promising results at present in the isolation of active substances from such mixtures depend upon adsorption and subsequent elution. Both charcoal or fuller's earth are available as adsorbents and suitable methods of elution for each have now been pretty well worked out. Moreover, Koser and Saunders (1935) have recently shown that adsorption of meat extract by charcoal with subsequent elution by alcohol or acetone yielded solutions which strongly stimulate the growth of certain bacteria including *Corynebacterium diphtheriae*. The writer (Mueller, 1922) in 1921 showed that adsorption of beef-heart infusion with charcoal resulted in the removal of factors necessary for the growth of the hemolytic streptococcus and the pneumococcus. Freedman and Funk subsequently confirmed this observation, and were able to elute most of the active material from the charcoal by means of glacial acetic acid. They believed the material to be similar to "vitamine B."

We have, therefore, adsorbed this liver-extract fraction with norit charcoal and eluted the latter with acid alcohol, very much according to a method outlined to us by Dr. Subbarow and almost identical with that used by Kinnersley, O'Brien, Peters and Reader (1933) in their work on vitamins B_1 and B_4.

Six hundred and fifty cubic centimeters of the syrupy concentrate of the 95 per cent alcohol filtrate of the liver extract, supplied us by Dr. Subbarow, and equivalent to approximately 100 kgm. of beef liver, were diluted to 5,000 cc. and precipitated by the addition of 200 cc. of 25 per cent neutral lead acetate. The precipitate was discarded, the lead removed with H_2S and the latter distilled out *in vacuo*. The filtrate was adsorbed by stirring at room temperature for twenty minutes with 200 gm. norit charcoal which had not been previously treated with acid to remove inorganic material. The charcoal was filtered and washed

with cold water; the filtrate was again adsorbed in the same way with a similar amount of norit. Both adsorptions were carried out without adjusting the pH of the solution which was, of course, fairly acid (4.5–5.0) as a result of the lead acetate precipitation. The filtrate from the second adsorption was concentrated *in vacuo* until crystallization commenced.

The combined washed charcoals were eluted three times as follows: First with 4 liters of 50 per cent alcohol containing 40 cc. of concentrated hydrochloric acid by boiling with constant stirring for fifteen minutes on the steam bath. Second, with 4 liters of 50 per cent alcohol containing 12 cc. of concentrated HCl by ten minutes of boiling and stirring. The third elution was a repetition of the second. The three elutes were mixed after filtration,

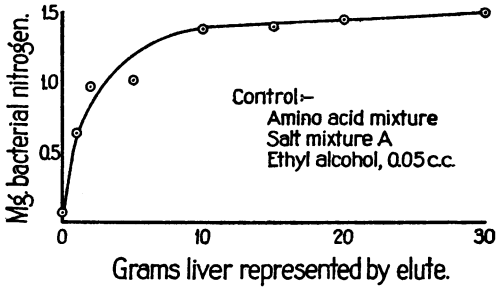

FIG. 3

and concentrated in vacuum to about 1,200 cc. Of this concentrate, 1 cc. is therefore equivalent to about 85 grams of liver.

Figure 3 demonstrates the effect of the addition of the adsorbed and eluted material to the control solution.

It is evident that one or more substances taking part in the growth-stimulating effect of the liver extract have been removed by charcoal and can again be obtained from it by the method of elution above described. The effect of this elute, however, is much less marked than the effect of the whole crude fraction of the liver extract. Tests carried out with the filtrate from the charcoal showed almost no growth-stimulating properties to be present in this material but, when it was mixed with the elute and again tested, a marked increase in growth over that produced

by the elute alone resulted, as shown in figure 3. However, the charcoal filtrate still contained the substances responsible for the development of an acid reaction and smooth curves could not be obtained. The actual figures for such experiments may therefore perhaps be omitted, but it was clear that there were at least two types of substance having an effect on the growth of our organism present in the liver-extract fraction. One class is readily adsorbed on charcoal and eluted from it by acid alcohol, the other class is not adsorbed by charcoal and is present in the filtrate.

It seemed desirable to investigate, first, the nature of the supplementing material present in the charcoal filtrate since it is clear that substances not readily adsorbed would be expected to withstand the usual chemical manipulations more readily than those which, being easily adsorbed, would probably suffer heavy losses on inert precipitates, etc.

The charcoal filtrate when concentrated in vacuum evidently contained considerable quantities of organic material and a large crop of crystals grossly resembling impure leucine separated. Since it is quite certain that various amino acids occur in tissue extracts, in small amounts at least, and since, because of the method of fractionation employed in the preparation of this material, they would almost surely be present in this fraction, it was assumed, to begin with, that there was still perhaps a deficiency of one or more amino acids from the control solution. This was a possibility which had been kept in mind in our earlier work on the amino acid requirements, since there was every reason to believe that Liebig's extract contained certain, at any rate, of these compounds. A total acid hydrolysate of casein prepared in the ordinary way was therefore substituted for the charcoal filtrate and it was found that quite as heavy growth resulted as in the case of the liver-extract fraction. For the sake of brevity this protocol, as well as some others which served only to point the direction, may be omitted.

The attempt was made in the usual manner to determine into which main group of the amino acids the new essential factor fell and results were obtained which were not clear cut, but which, after a time, suggested that we were not dealing with an amino

acid but rather with some quite different type of material, possibly only an inorganic salt.

Throughout the work, a mixture of inorganic salts containing the usual elements and groups had been supplied (salt mixture A). The proportions, however, were perhaps carelessly fixed upon, for it was felt that as long as tissue extractives in crude form were being supplied, the inorganic elements would, to a certain extent, be sufficiently well taken care of. Now, however, upon increasing tenfold the previously used concentrations of KCl, $CaCl_2$, and $MgSO_4$, growth with the usual amino acid control and the charcoal elute was quite satisfactorily heavy.

The optimal concentrations of the various inorganic constituents in the presence of the liver elute and the amino acid control have been determined as accurately as possible by means of curves which, for the sake of brevity, may be omitted. Should it become possible to substitute pure compounds for the liver elute which almost surely contains some inorganic material, it may be worth while to reinvestigate the salt requirements which could then be done with some accuracy. At present, as a result of a considerable number of experiments, a new salt mixture has been determined upon as giving excellent results. Probably the SO_4 and Fe may be omitted, but their presence makes little or no difference. There is some indication that the Fe is even slightly inhibitory, but the effect is so small as to be in doubt with the present set up. The effect of the sodium ion is also in doubt, its omission necessitating the use of KOH to adjust the reaction of the medium in rather considerable amounts because of the HCl present in the liver elute and in the hydrochlorides of glutamic acid and histidine. Growth in such media is poor, but whether on account of the lack of Na or the great excess of K cannot now be stated.

The presence of the Ca ion is apparently unnecessary, at any rate in excess of that which may be present in the liver elute. Since the charcoal was not acid-washed it is altogether probable that some calcium phosphate is derived from that source and would be present in the medium. Additional amounts of $CaCl_2$ however, lead to the formation of precipitates which apparently contain phosphates of both Ca and Mg and the growth is less

good than when Ca is entirely omitted. The precipitate forms, usually, during the autoclaving of the medium and may be for the most part avoided by the addition of sterile $CaCl_2$ by means of a sterile pipette after the medium has been autoclaved. Even with this technique, however, the growth is not so good as with the omission of the element.

TABLE 2

SALT MIXTURE B

	mgm.
NaCl	50.0
KH_2PO_4	3.5
$MgCl_2 \cdot 6H_2O$	3.0
H_2SO_4	1.0
$FeCl_3 \cdot 6H_2O$	0.1

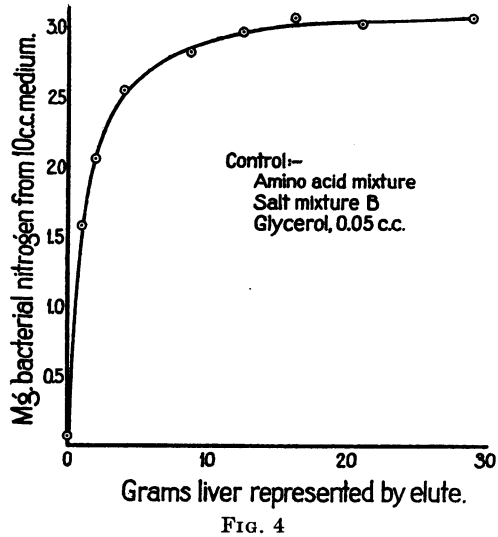

FIG. 4

The composition of the salt mixture as employed at present is as shown in table 2.

Figure 4 shows the effect of the addition of the liver elute to a control solution containing salt mixture B in place of salt mixture A and may therefore be compared directly with figure 3 as

illustrating the difference in effect produced by the increased concentration of the inorganic elements. It will be noticed that glycerol has been substituted for ethyl alcohol, which, however, should lead to no material difference in the growth. For various reasons, in the latter part of the work glycerol has been used routinely and its effect is apparently quite similar to that of the alcohol.

The striking effect of the liver elute produced by a relatively minute amount of material suggests, of course, something of the action of a vitamin-like substance. Certain components of the B Vitamin complex are almost surely present and obviously also, many other substances of still unknown composition are to be found in such a preparation. It is quite impossible at this time to state whether one substance or several will be found to be involved. It has been possible thus far to attempt to substitute crystalline vitamin B_1 obtained from Merck & Company, the *Staphylococcus* vitamin of Knight, a small specimen of which was kindly placed at our disposal by Dr. Knight, and a preparation of pantothenic acid, for which we are indebted to Dr. Roger J. Williams; none of these three substances by itself showed any appreciable growth-stimulating effect when substituted for the liver elute. This, of course, does not mean that one or all may not be involved along with additional compounds. Work on this question is now under way and it is hoped to be able to obtain more definite information to be reported later.

CONCLUSIONS

1. Animal tissue extracts, apparently of whatever source, contain substances essential to the growth of our strain of diphtheria bacillus.

2. There is also present in most freshly prepared tissue infusions a substance, perhaps glucose, which inhibits growth through the formation of acid.

3. An extract of beef liver has been found to be highly effective in stimulating growth of the organism and a considerable proportion of the effective material is present in a 95 per cent alcohol filtrate of an aqueous liver extract, which is available in con-

siderable quantities as a by-product in the preparation of the pernicious anaemia curative fraction.

4. One or more substances essential to growth may be removed from such a solution by means of adsorption on wood charcoal and may be recovered from the latter by elution with acid alcohol. The nature of the compounds here involved is still unknown but is being investigated.

5. The filtrate from such charcoal adsorptions also contains growth-stimulating materials which have been fairly well defined as inorganic in nature, probably for the most part K and Mg.

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