MUSCLE EXTRACTIVES IN THE PRODUCTION OF TETANUS TOXIN[1]

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In earlier communications from this laboratory (Mueller and Miller, 1945, 1947) methods have been described for the preparation of highly potent tetanus toxin, methods which have come into rather general use in large-scale toxoid production for human immunization. The procedure involves the use of a variant and somewhat unstable strain of *Clostridium tetani* and a culture medium containing a pancreatic digest of casein with additional cystine and tyrosine, beef heart infusion, glucose, and inorganic salts. A high concentration of iron must be provided. While the use of this method in several laboratories seems to have been reasonably successful, sufficient difficulty has been encountered both by ourselves and by others to warrant careful study of the nutritional factors involved, in the hope that by the recognition and control of the various substances influencing growth and toxin formation a greater degree of uniformity in product may result. Moreover, since the nature of the medium suggests, and since preliminary investigations on the pancreatic digest confirm the belief that hitherto unrecognized nutritional factors are concerned, it is probable that some knowledge of basic and theoretical interest may emerge from such a study.

Since an infusion of beef heart is an essential component of the culture medium, the possibility exists that variations in different batches of this material may account for differences in toxin produced. Earlier work on the growth requirements of *C. tetani* (Feeney *et al.*, 1943) have shown the need for a fairly complex assortment of vitamins and extractives. Certain of these are undoubtedly present in the pancreatic digest of casein, arising either from impurities in the casein or from the pancreatic tissue forming the substance of the enzyme preparation. Here, too, variations from one preparation to another may reflect differences in extractives rather than in amino acid or peptide content. For these reasons, clear definition of the kind and quantity of extractives necessary in toxin formation must be included necessarily in a more comprehensive study. The experiments which are to be presented in this paper provide a certain amount of information on the qualitative and quantitative requirements of a few vitamins and growth accessories and indicate the presence of at least one and perhaps more additional unidentified substances present in beef heart infusion which are essential for maximal toxin formation. The information obtained in these experiments has made possible some modification of the original formula for large-scale toxin manufacture attended by somewhat better and more uniform results and a modest decrease in cost of the medium.

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EXPERIMENTAL METHODS AND RESULTS

Feeney *et al.* (1943) found that maximal *growth* of our strain as of that period occurred when the following vitamins and growth acces-

Substance	*Micrograms per 10 ml medium*
Thiamin	1.0
Riboflavin	1.0
Pyridoxine	10.0
Calcium pantothenate	2.5
Biotin	0.01
Folic acid	0.05
Nicotinic acid	10.0
Adenine	50.0
Uracil	25.0
Oleic acid	25.0

sories were used in supplementing a basic medium composed of pure amino acids, glucose, and inorganic salts.

These experiments were carried out on a 24 hour growth basis and in the presence of trace quantities only of iron since they antedated by two years the experiments on toxin formation which led to the recognition of the effect of high concentrations of iron under special conditions. A reexamination of requirements for growth was carried out therefore as a preliminary to the present investigation.

Methods. A basic medium[3] constituted as follows served this purpose: Pancreatic digest of casein,[4] 150.0 mg; glucose, 75.0 mg; cysteine, 1.25 mg; tyrosine, 2.5 mg; NaCl, 25.0 mg; Na_2HPO_4, 5.0 mg; KH_2PO_4, 1.75 mg; $MgSO_4 \cdot 7H_2O$, 0.5 mg; reduced iron powder, 2.5 to 5.0 mg; H_2O to 10 ml.

The various components of the media were added to 6 by ⅝ inch test tubes, volumes adjusted to 10 ml, and pH brought to 7.2. Following sterilization in flowing steam at 100 C for 25 minutes, the tubes were cooled quickly and inoculated with a loopfull of a 24 hour broth culture of the tetanus strain which was being used currently for toxin production. The seed tubes were prepared daily by inoculating a loopful of the preceding day's culture into 10 ml of ordinary 1 per cent glucose nutrient broth which had been freshly heated for 10 minutes in boiling water and quickly cooled. Incubation was in an anaerobic jar at 35 C for 18 to 24 hours. The experimental media were incubated anaerobically at 35 C in a constant temperature water bath for about two days (usually 41 hours).

The presence of a high concentration of iron and the formation of H_2S during growth resulted in darkening of the cultures to the point that direct turbidimetric readings of growth were not possible. It was found that acidification of the cultures with 0.5 ml concentrated HCl followed by immersion of the tubes in boiling water for exactly 5 minutes dissolved the iron sulfide, leaving degrees of turbidity which could be measured with reasonable accuracy by comparison with standard suspensions of barium sulfate. This procedure clearly is subject to criticism and represents merely a convenient and workable compromise.

The results obtained in an experiment planned to test the effect of each of the above ten growth accessories are shown in table 1.

It was to be expected that even disregarding possible alterations in actual growth requirements of the strain of *C. tetani* over a period of 7 years, the completely different basic medium would lead to findings differing from those of the earlier work. The pancreatic digest of casein surely contained a number of growth accessories or perhaps materials which rendered certain accessories unnecessary. Further, the high concentration of iron probably modified the metabolic processes of the organism in a number of respects.

It appeared that thiamin, calcium pantothenate, nicotinic acid, and uracil were essential under these conditions. Riboflavin, pyridoxine, and adenine produced significant increases over the controls; biotin and folic acid were essentially without effect whereas oleic acid was inhibitory.

The addition of beef heart infusion provides factors which result in growth far above the range obtained from the ten accessory factors under examination. Attempts to obtain comparable growth by inclusion of other known growth accessories have been unsuccessful. These included trace metals, inositol, choline, *p*-aminobenzoic acid, vitamin B_{12}, succinic, fumaric, pyruvic, α-ketobutyric, α-ketoglutaric and pimelic acids, guanine, xanthine, hypoxanthine, thymine, and cytidine. Significant increases, although still far short of the heart infusion controls, were obtained by glutamic acid and glutamine, serine, and histidine.

Various attempts to separate active components from the beef heart infusion indicated that probably at least two substances were concerned, and it appeared unprofitable at this stage of the investigation to undertake the actual isolation and characterization of these compounds.

Toxin formation. Experiments concerning the part played by growth accessories in toxin formation have now extended over a period of four years. With the accumulation of facts from this work it has been possible gradually to

[3] All ingredients except the reduced iron were added from the usual type of concentrated stock solutions so that a final volume of about 4 ml resulted. Any desired additional substances then were added, the volumes were brought to 10 ml, and the pH adjusted to 7.2.

[4] A commercial product, "N-Z-Case," kindly provided by the Sheffield Farms Co., New York, has been used throughout these experiments.

TABLE 1

The effect of various accessories on growth of Clostridium tetani

GROWTH ACCESSORY	AMOUNT ADDED PER 10 ML MEDIUM				
	Nil	1/10 usual amount	Usual amount	10 × usual amount	Usual amount, μg/tube
Thiamin	0	125	140	150	1.0
Riboflavin	90	90	140	110	1.0
Pyridoxine	80	80	140	80	10.0
Ca pantothenate	0	100	140	140	2.5
Biotin	110	110	140	125	0.01
Folic acid	125	130	140	125	0.01
Nicotinic acid	0	150	140	140	10.0
Adenine	80	80	140	140	50.0
Uracil	0	110	140	100	25.0
Oleic acid	185	185	140	25	25.0

Control: basal medium with 2.5 ml beef heart infusion..... 450

Growth is recorded against an arbitrary series of tubes containing $BaSO_4$ suspensions, i.e., the higher the reading the greater the turbidity.

modify the composition of the original toxin medium by decreasing the quantity of beef heart infusion and substituting certain vitamins in pure form. As in the case of the experiments on growth, complete exclusion of the heart infusion has not yet permitted maximal toxin titers with any combination of accessories which has been tried. To summarize the results numerically, it may be stated that titers of 40 to 50 L_f per ml can be attained regularly without infusion in the presence of a few known accessory factors. Occasionally, but irregularly, titers as high as 90 to 100 L_f have occurred, whereas the addition of a small amount of infusion with the same vitamin enrichment leads to titers up to 150 to 160 L_f.

During the course of this work, considerable collateral information on variations in pancreatic digests of casein and on other factors influencing toxin yield has come to light. This has been described elsewhere (Mueller and Miller, 1954), and three successive formulae for complete media have been presented, representing joint outgrowths of those studies and the experiments summarized in the present communication. For the sake of clarity of discussion, these three formulae are reproduced in table 2.

Toxin response to beef heart infusion alone. In table 3 are presented the results obtained by the addition of increasing amounts of heart infusion to a medium having the composition of formula III without added growth accessories; i.e., the pancreatic digest was treated with calcium phosphate and alkali in order to remove certain inhibitory materials, and the glucose, cystine, tyrosine, and minerals given in table 1, column III, were used.

In this experiment toxin increases from "nil" to a maximum of 80 L_f per ml at a concentration of 3 ml of infusion in 20 ml of medium were obtained. With increased amounts of infusion the toxin level falls off. In many other experiments, particularly those carried out with a basic medium having the composition of formula II without added accessories, the decrease has been even more pronounced, and it has become obvious that inhibitory as well as essential factors are present in heart infusion and also in the casein digest. It will be noted that at a level of one ml of heart infusion, the inclusion of the pure accessories of formula III increases the toxin yield from 20 L_f to 150.

There would be little point in presenting large numbers of protocols from which the composition of formula III was derived. In table 4 is given an experiment showing the effect of the omission of each in turn of the accessories used.

These results are entirely typical of those obtained in many similar experiments. Pantothenic acid and uracil seem to be the two substances having the most striking effect. The other accessories, or materials replacing them, obviously are supplied at almost the optimal levels by the small amount of heart infusion and by the pancreatic digest of casein. Adenine or

TABLE 2

Comparison of various formulae for toxin production

COMPONENTS PER 1,000 ML	I, 1947	II, 1951	III, CURRENT
Pancreatic digest of casein*	15 g	15 g	22.5 g
Beef heart infusion†	250 ml	37.5 ml	50 ml
Glucose	7.5 g	8.75 g	11 g
NaCl	5.0 g	2.5 g	2.5 g
Na_2HPO_4	0.5 g	1.0 g	2.0 g
KH_2PO_4	0.17 g	0.1 g	0.15 g
$MgSO_4 \cdot 7H_2O$	0.05 g	0.1 g	0.15 g
Cystine	0.125 g	0.125 g	0.25 g
Tyrosine	0.125 g	0.5 g	0.5 g
Ca pantothenate‡	—	1.75 mg	1.0 mg
Uracil	—	0.875 mg	2.5 mg
Nicotinic acid	—	0.25 mg	—
Thiamin	—	0.25 mg	0.25 mg
Riboflavin	—	0.25 mg	0.25 mg
Pyridoxine	—	0.25 mg	0.25 mg
Biotin	—	2.5 μg	2.5 μg
Vitamin B_{12}	—	0.5 μg	—
Reduced iron§	0.3–0.4 g	0.5 g	0.5 g
Average yield of toxin (L_f/ml)	60–80	80–100	130–150

* Prepared by method of Gladstone and Fildes (1940) or a commercial product such as "N-Z-Case", Sheffield Farms Co., Norwich, New York. In formula I the digest was used without preliminary treatment. In II, a 10 per cent solution of the digest was stirred for 20 minutes with 15 g norit charcoal per 1,000 ml solution and filtered. In III, a 20 per cent solution of the material was heated to boiling, 3.75 g anhydrous $CaCl_2$ were added, followed by 7.5 g K_2HPO_4. The solution was adjusted to about pH 9.3 with strong NaOH and filtered. It was neutralized to pH 7.2 to 7.6 with concentrated HCl. (Procedure of Hendry and Wheeler, 1954.)

† Minced beef heart muscle (2 lb) suspended in 1,000 ml tap water, brought rapidly to active boiling, then strained through cheese-cloth and filtered through paper.

‡ In the 1951 formula, two vitamin solutions were prepared in bulk and stored in the cold room for use as needed. These had the following composition: I. Thiamin, 5.0 mg; riboflavin, 5.0 mg; pyridoxine, 5.0 mg; Ca pantothenate, 20.0 mg; biotin, 50.0 μg; vitamin B_{12}, 1.0 μg; in 100 ml 25 per cent ethanol. II. Nicotinic acid, 5.0 mg; uracil, 25.0 mg; in 100 ml 1 per cent HCl. Currently, all vitamin stock solutions are prepared and stored separately.

§ This should be added to the otherwise complete medium, allowing it to settle to the bottom, and *not stirred or mixed with the medium.*

other purine is without effect under these conditions although clearly essential for growth under somewhat different ones.

If now, one reexamines the influence of the heart infusion in the complete formula III, results such as those shown in table 5 are obtained.

DISCUSSION

In the complete absence of infusion, toxin of 50 L_f per ml was obtained in the experiment of table 5. As stated above, this is entirely typical and even higher levels may occur sporadically. This fact renders attempts at concentration and characterization of the additional substances supplied by the infusion a troublesome matter. When the "negative control" quite regularly gives a result of 30 per cent, and sometimes 50 to 60 per cent of the maximum, it is clearly difficult to appraise results of chemical fractionation. To what extent the additional factor or factors occur in the casein digest is not yet clear, and perhaps as it becomes more nearly possible to replace this material with known compounds, the problem may become simpler. By various methods of fractionation of the heart infusion, preliminary evidence for the participation of at least two substances not yet identified with

TABLE 3

Effect of beef heart infusion in formula III without accessories

HEART INFUSION ML PER 20 ML	TOXIN TITER (Lf/ML)
0	nil
0.25	nil
0.5	20
1.0	20
1.5	20
2.0	20
2.5	65
3.0	80
3.5	65
4.0	65
4.5	65
5.0	70
6.0	60
7.5	65
10.0	65
Control containing 1.0 ml heart infusion and accessories of formula III	150

TABLE 4

Influence of individual growth factors on toxin production

SUBSTANCE OMITTED	TOXIN TITER (Lf/ML)
None	145
Thiamin	105
Riboflavin	120
Pyridoxine	115
Ca pantothenate	40
Biotin	110
Uracil	nil

any of the known growth promoting factors has been obtained. This is still too unsatisfactory to warrant giving detailed experiments, and for the present this aspect of the problem is being held in abeyance.

It may not be out of place to point out that here, as in many other types of bacterial growth phenomena, there are certain interrelationships involving proportions of certain constituents, the reasons for which may not be apparent immediately. These may become evident only by a laborious series of cross-variations or may emerge accidentally. One such case in point is a somewhat critical proportionality between cystine and uracil. When it became evident that a higher concentration of the calcium treated pancreatic digest of casein could be used than was possible with the charcoal adsorbed product of formula II, it was necessary to reexamine optimal concentrations of all other components of the medium. It then developed that both cystine and uracil had to be increased simultaneously in order to achieve regularity of result. Too little uracil, or conversely, too much cystine resulted in early autolysis of the cultures and poor toxin production. It does not seem necessary to present detailed protocols to illustrate this point which is mentioned merely as an instance of one of the many complexities of such biological systems.

TABLE 5

Effect of beef heart infusion in formula III with accessories

HEART INFUSION ML PER 20 ML	TOXIN TITER (Lf/ML)
0	50
0.1	95
0.25	115
0.5	125
0.75	135
1.0	140
1.5	145
2.5	135
5.0	115
7.5	105
10.0	55

A further point worthy of comment concerns the stock solutions of the accessory factors. In preparing media according to formula II, stock solutions were employed as indicated in table 2. Often these stock solutions, kept in the refrigerator, were used repeatedly over considerable periods of time. Periodically throughout the entire duration of this work "positive controls" have failed to come up to expectation, and many experiments consequently have failed. Eventually a part, at any rate, of such difficulties has seemed to correlate with the age of the stock vitamin solutions, and substitution of freshly prepared mixtures has apparently put matters right.

Unfortunately, it has not been possible to obtain clear-cut evidence for the phenomenon, and consequently attempts to define the substance at fault have failed. One might assume that riboflavin, well known to be unstable to

light, might have been altered gradually by short daily exposure when removed from the refrigerator for use. However, complete omission of riboflavin usually permits better toxin than is obtained during these periods of "depression". A further possibility exists that riboflavin in the presence of light exerts a photodynamic effect resulting in destruction of one of the other components of the mixture, or even perhaps in the formation of a slightly modified form of one of the others which may be directly inhibitory. Again, attempts to establish such a situation experimentally have failed. There appears on the whole to have been less trouble when the individual accessory solutions are maintained separately and renewed at reasonable intervals, and at present it is probable that this procedure should be followed in using formula II or III in large-scale toxin production.

It will be noted that nicotinic acid and vitamin B_{12}, present in formula II, have been omitted from the third medium. Evidence for the participation of vitamin B_{12} was never convincing, either on growth or on toxin formation, and in many recent experiments its omission has been entirely without effect. Nicotinic acid, on the contrary, is essential to growth, but its omission in formula III is without effect. The substance itself, or something replacing it, must be present therefore in adequate quantities in the small amount of beef heart infusion or in the tryptic digest of casein, or in both.

One may speculate that the charcoal treatment of the pancreatic digest employed in formula II may remove significant amounts of certain accessory factors present as impurities, and that these remain following the calcium precipitation of formula III. This may well be true of nicotinic acid and is almost surely the case with pantothenic acid. Omission of the latter from formula II almost invariably resulted in little or no toxin, whereas, as indicated in the experiment of table 4 and in others of similar nature, readily measurable toxin is formed without the added vitamin.

Possibly many of the complexities of the nutritional requirements of *C. tetani* could be eliminated by adaptation experiments designed to simplify the needs of the organism while perhaps retaining its toxigenicity. This has been accomplished for the diphtheria bacillus by Yoneda (1951) with a measure of success. Stone (1953) reports partial success in the adaptation of our strain of *C. tetani* to formula II without meat infusion. It is not clear, however, from his report exactly what has been accomplished since his adapted strain has yielded L_f values ranging from 10 to 40 in 27 lots in large-scale preparation while our own results, as stated above, range from 40 or 50 to as much as 90 L_f in test tube experiments. The writers confess, however, a certain predilection for taking the organism as it occurs in nature and attempting to untangle its nutritional complexities at the level of maximum toxigenicity in the hope that practical application will follow and that new facts in connection with bacterial nutrition may emerge.

SUMMARY

The requirements of a highly toxigenic strain of *Clostridium tetani* have been investigated under the conditions necessary for good toxin production. A formula has been devised in which beef heart infusion is reduced to a fairly low level and replaced by a few known vitamins and accessories. It has not yet been possible to achieve maximal levels of toxin production in the complete absence of infusion.

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