PRODUCTION OF TETANUS TOXIN ON PEPTONE-FREE MEDIA[1]

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An investigation of the growth requirements of *Cl. tetani* was undertaken in 1939, with the hope that through a knowledge of them, it might be possible to produce a uniformly potent toxin on a medium free from peptone, or any other material possessing antigenic effect of its own. Several reports in the literature had described acute anaphylactic-like symptoms following a second or third injection of tetanus toxoid for human prophylaxis, and in a few instances (1 to 3), analysis of the case showed that Witte's peptone, used in the original medium, was responsible.

Considerable progress has been made in the studies on nutritional factors (4). Parallel with these, work was commenced on toxin production, and it soon became evident that with this organism, as with *C. diphtheriae* (5), peptone was not essential and could be replaced by complete acid hydrolysates of protein, and presumably by suitable pure amino acids. Unexpectedly, it developed that the concentration of iron in the medium seemed to play the same crucial part as in diphtheria toxin production (6, 7). With suitable control of this factor, and in media containing the appropriate materials, all of relatively small molecular size and therefore not of themselves antigenic, toxin was obtained with regularity.

Preliminary reports of this work have already been made (8, 9). It is the purpose of the present communication to present, in somewhat greater detail, certain of the experimental aspects of these studies which will serve as a basis for attempts to place tetanus toxin production with simplified media on a practical basis.

PRELIMINARY ASPECTS

The strain of *Cl. tetani* used in the New York State Division of Laboratories was selected by random choice. It is stated to produce toxin of 10,000 to 20,000 guinea pig MLD per cc. We have carried it in stock by the rather unorthodox method of serial daily transplant on ordinary meat infusion peptone broth containing 1.0 per cent glucose, grown in anaerobic jars (10) at 36° C. Probably as a result of this procedure, the ability to form spores seems to have been suppressed and they are not found, even in very old cultures. Toxin production seems to have been unaffected. There is a tradition among producers of tetanus toxin that an inoculum of spores, or at any rate of an old culture yields toxin of higher titer than does a young, actively growing culture. In our experiments, no such difference has been observed in occasional comparisons of our serial transfer strain with older ones.

Experimental media have been handled in two ways—either in 10 cc. amounts in 6 × ⅝ inch test tubes, or in 125 cc. Erlenmeyer flasks. In the former case, the cotton plugged tubes have been autoclaved at 10 pounds pressure for from 7 to 10 minutes, promptly cooled, and inoculated. The centrifuged sediment from a 24-hour infusion broth culture is suspended in 1 or 2 cc. of sterile hydrolysate medium, and an inoculum of 0.05 to 0.1 cc. of the suspension is delivered to the bottom of each tube with a capillary. Incubation at 36° C. is carried out in an anaerobic jar. The flasked media have been autoclaved at 10 pounds for 15 minutes, the containers being about ⅔ full and covered with 30 cc. glass beakers. Immediately upon removal from the autoclave, the flasks to be inoculated are filled to the neck from an extra supply, cooled in water, inoculated with a few drops of a suspension and incubated either anaerobically or in the air, at 36° C. Maximum toxin production is reached after 8 days, but in many experiments, toxin titrations have been done on the fourth or fifth day.

The estimation of toxin has been carried out in mice, using an inbred strain of white Swiss. The toxins to be examined are well mixed with one tenth volume of 5.0 per cent phenol, centrifuged for half an hour, and the supernatants are suitably diluted with 1.0 per cent Witte peptone in saline. The mice are injected subcutaneously along the back, the needle being inserted near the root of the tail, with 0.5 cc. quantities of the desired dilutions. By using 2 or 3 mice for each sample of toxin, a reasonable estimate of comparative potency may be obtained. A number of attempts to estimate the toxin by flocculation have shown no convincing parallelism between the titers obtained and toxicity for animals.

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COMPONENTS OF MEDIUM

The growth requirements of *Cl. tetani* have proved to be highly complex. The greater part of the early experiments on toxin formation were therefore carried out with relatively uncharacterized mixtures of nutrients. To a certain extent this has continued to be true because of practical difficulties. An acid hydrolysate of casein has been employed to supply amino acids, since it appeared to be the most convenient and least expensive source. Various fractions of liver extract were incorporated to furnish the necessary growth accessories. Eventually, most of the latter could be provided in pure form, but the requirements for the substance which has been named "folic acid," the constitution of which is still unknown, continues to necessitate the use of a concentrate.

There would be no object in recording the numerous experiments carried on over a period of 2 years in the attempt to arrive at uniformly maximum titers. The many chemical factors involved in growth require laborious repetitions of tests in which each individual factor, so far as possible, is varied against a constant of all the others. Only in this way can it be determined which concentrations are critical and must be carefully adjusted. Moreover, the concentration of Fe must be controlled as carefully as in the preparation of diphtheria toxin, and since the permissible level is so low as to be beyond simple and reliable chemical measurement, several tubes of each modification of medium within an experiment, each at a different iron concentration, must usually be made and tested.

It has been possible to obtain toxin of the same, or even a higher, degree of potency as that said to be produced by this strain in the New York State Laboratories (10,000 to 20,000 MLD's for guinea pigs). Efforts to obtain strikingly higher yields have thus far failed, and it is possible that the limit for the particular strain has been reached. An investigation of other cultures will be undertaken in due course. The following experiment describes the production of one of the better lots of toxin.

Experiment March 13, 1941—p. 74.

Casein hydrolysate (15.4 mgm. N per cc.)[2]	240. cc.
Accessories and metals[3]	2.4 cc.
Cystine, 20 per cent in HCl	2.4 cc.
Tryptophane, 1 per cent	12. cc.
Glucose	12. grams
Calcium pantothenate	0.3 mgm.
Liver eluate[4]	9. cc.
Water to make	1200. cc.

After the various ingredients had been mixed and diluted to 1200 cc., the excess iron was removed by adding 6.0 cc. of 10 per cent $CaCl_2.2H_2O$, adjusting the reaction to pH 7.6, bringing to a boil, and filtering. The filtrate was cooled, and further iron removed by adding about 1.5 cc. calcium chloride and repeating the process.

The resulting medium was autoclaved in six 250 cc. Erlenmeyer flasks for 15 minutes, at 10 pounds pressure. Four of the flasks were then filled completely full from the other 2, and cooled promptly. They were inoculated as described earlier, and incubated in anaerobic jars at 36° C. for 5 days.

The resulting toxoid was centrifuged and filtered partly through a Berkefeld "N," the rest through a "V." The "V" filtrate was titrated in both mice and guinea pigs. The results follow:

Mice (Received 0.5 cc. of the stated dilution subcutaneously).

1 : 40,000.....Dead 48 hours
1 : 80,000.....Dead 64 hours
1 : 160,000.....Slight symptoms 96 hours
1 : 320,000.....Slight symptoms 96 hours

MLD>160,000

[2] A low chloride hydrochloric acid hydrolysate, prepared as described by Mueller and Johnson (11), from which excess Cl was removed by PbO. It contains sufficient Na_2HPO_4 and KH_2PO_4 to give a final concentration in the medium of about 0.1 per cent and 0.033 per cent respectively.

[3] Prepared as for diphtheria toxin (12) and contains $MgSO_4$, traces of Cu, Mn, and Zn, together with nicotinic acid, beta alanine, and pimelic acid.

[4] Prepared from the 90 per cent alcohol soluble fraction of liver extract, supplied us through the courtesy of Dr. Defries of the Connought Laboratories, University of Toronto. This was further purified by absorbing with norite charcoal, washing the latter first with water, then with hot 50 per cent alcohol, and then eluting with dilute pyridine-alcohol solution. After removal of the organic solvents *in vacuo*, the aqueous solution was preserved in the ice-box with toluol. One cc. was roughly equivalent to 62 grams of fresh liver.

Guinea Pigs (Received 1.0 cc. subcutaneously).

1 : 20,000 ..... Dead 60 hours
1 : 30,000 ..... Dead 62 hours
1 : 40,000 ..... Dead 93 hours
1 : 50,000 ..... Dead 93 hours
1 : 60,000 ..... Dead 98 hours

MLD 50,000

The following experiment illustrates the marked inhibition of toxin formation by traces of iron salts.

Experiment May 2, 1941—p. 105.

Casein hydrolysate (17.0 mgm. N per cc.)	18 cc.
Accessories and metals	0.2 cc.
Cystine, 20 per cent	0.2 cc.
Tryptophane, 1 per cent	1.0 cc.
Glucose	1.0 gram
Calcium pantothenate	0.025 mgm.
Liver eluate	0.75 cc.
Water to make	100. cc.

The iron was removed twice by precipitating together with calcium phosphate. Five tubes, of 15 cc. each, were prepared and the following quantities of $FeSO_4.7\ H_2O$ were added: 0, 4, 8, 16, and 32 micrograms. The tubes were autoclaved, cooled, inoculated, and incubated anaerobically for 5 days. One and a half cc. of 5 per cent phenol was then mixed with the contents of each tube. After centrifuging for half an hour, the supernatants were diluted with 1 per cent Witte peptone in saline, and 0.5 cc. subcutaneous injections given a series of mice.

Toxin dilution	$FeSO_4.7H_2O$/15 cc. medium				
	0	4 gamma	8 gamma	16 gamma	32 gamma
1 : 20,000	Dead 70 hours	D 90 hrs.	SS	S	0
1 : 40,000	Dead 70 hours	SSSS	SS	0	0
1 : 80,000	Dead 90 hours	SS	S	0	0
1 : 160,000	SSSS	S	0	0	0

Increased degrees of paralysis at 96 hours are indicated as S, SS, etc.

EFFECT OF OTHER INDIVIDUAL COMPONENTS

Variation, within reasonable limits, of the concentrations of the other ingredients of the medium appears to be without marked effect on toxin formation. Increase in the amount of liver extract concentrate, seems, on the whole, to be unfavorable to toxin, whereas its reduction results in failure to obtain growth. The casein hydrolysate can be somewhat diminished without loss of toxin, while its increase retards toxin and growth, probably through osmotic effects and perhaps through excessive concentrations of certain amino acids.

Omission of traces of Cu, Mn, and Zn tends to lower the amount of toxin somewhat, but it has been difficult to obtain clear cut results. Nicotinic acid, beta-alanine, and pimelic acid are without effect, so far as it is possible to observe, and, if required, are evidently supplied by the liver concentrate in adequate quantity.

Cystine and glucose probably contribute to the production of anaerobic conditions in the freshly heated medium. Either one may be omitted with no impairment of growth or toxin. In the absence of both, growth fails.

Tryptophane also is required for multiplication of the organisms, as are the inorganic salts of K, Mg, and PO_4. The concentrations are not particularly critical.

It is clear that while using such complex mixtures as casein hydrolysate and the liver extract concentrate, the appraisal of optimal concentrations of many individual factors is impossible. When it becomes possible to substitute pure amino acids and growth accessories for these materials, progress in this direction may be expected, and, conceivably, higher yields of toxin may be obtained.

SUMMARY

The production of tetanus toxin on a medium free from peptone has been accomplished with one strain of *Cl. tetani*. As good, or somewhat better, toxin titers have been obtained as this strain (New York State Department of Laboratories) repeatedly yields on peptone-infusion media. The composition of the medium is very similar to that developed in this laboratory for diphtheria toxin, but is relatively more complex because of the greater number of growth factors demanded by the tetanus bacillus. As with diphtheria toxin, a very low and accurately controlled concentration of Fe is essential for optimal yields. Attempts to effect further simplifications and improvements in the medium are being continued.

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