FACTORS INFLUENCING THE PRODUCTION OF TETANUS TOXIN: GASEOUS PRODUCTS OF GROWTH

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Tetanus toxin of high potency (100 Lf per ml) is regularly produced in this laboratory (Mueller and Miller, 1947) by a variant strain of *Clostridium tetani.* Maximum yields require the presence of high concentrations of iron and appear to be sharply dependent on the size and type of container in which growth takes place. Titers of 100 Lf are attained in 6-by-$\frac{7}{8}$-inch test tubes containing 20 ml of medium. Flasks or bottles of various shapes and sizes are much less satisfactory, and no attempt at quantity production has resulted in yields of more than about 40 to 50 Lf and frequently less. It has recently been pointed out (Mueller and Miller, 1948) that the temperature of incubation influences the final titer in a relatively critical manner, and may offer a partial but by no means complete explanation for the phenomenon. It now appears that a more important factor is concerned with the free escape by diffusion of the gaseous products of growth and fermentation.

This was first observed in a series of attempts to measure gas evolved during growth and to correlate the measurements thus obtained with the final yields of toxin. It soon became evident that the mere presence of an inverted narrow tube in an ordinary test tube culture of the organism had an entirely unexpected effect on toxin production. Yields were irregular and in general far below those of control tubes. The most obvious difference in conditions was the fact that a certain amount of the gas produced was held in contact with the growing culture, whereas it normally diffused away promptly through the cotton plug. It was a simple matter to test this point, and the following experiment illustrates the effect.

Four tubes of $\frac{7}{8}$-inch diameter each containing 20 ml of the usual medium employed for toxin production were so prepared as to vary the rapidity with which evolved gases could escape during incubation. The first two were 6 inches in length, whereas the second pair were 16 inches long. All four were covered with glass caps and sterilized in flowing steam for half an hour, cooled, and inoculated. Tubes 1 and 3 were then fitted with previously sterilized cotton plugs in the usual way. Tube 2 was closed with a 1-hole rubber stopper through which had been inserted a short length of glass tubing bent to an inverted **U** and drawn to a fine open capillary tip. Tube 4 was fitted with a 2-hole rubber stopper bearing a long glass tube which ended about 2 cm above the surface of the medium, for the entrance of sterile air, and a short exit tube. Incubation

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was for 5 days at 35 C ± 0.1 in a Warburg bath. During this period a very slow stream of sterile air was passed through tube 4. The results follow:

Tube 1—usual cotton stopper (control)	90 Lf
" 2—glass capillary	20 "
" 3—long; diffusion hindered by depth	20 "
" 4—long; ventilated	80 "

It seems reasonably clear that the only variable in this experiment must have been the composition of the gas in contact with the growing cultures. This consists mainly of CO_2 along with some H_2 (Lerner and Pickett, 1945), but contains also appreciable amounts of H_2S or some similar volatile sulfide (odor and lead blackening). Although proof is lacking, it seems probable that the sulfide is the unfavorable component of the mixture, although CO_2 may possibly also exert some influence. It may be noted that the medium invariably blackens (FeS?) in the early stages of growth, and that the black, colloidal material flocculates and settles toward the end of the incubation period. It is thus possible that a part of the role of the iron in the medium is to combine with sulfide. This matter is being investigated further.

Recognition of the injurious effect of the retention of fermentative gases threw light on previously unexplained variations in toxin yields in earlier attempts at production in large containers. Thus, it had been noted on several occasions that less toxin was obtained from cultures in long, narrow-necked flasks (volumetric type) filled well into the neck with broth than in Erlenmeyer flasks three-quarters full. Such differences, of the order of 20 Lf in volumetric flasks as against 40 to 50 Lf in Erlenmeyers, had previously been completely unexpected, since it seemed probable that more favorable conditions for anaerobic growth would be obtained with a minimum exposure of surface to the air.

At present various expedients are being tried in order to provide more adequate disposal of gaseous products of growth in large containers. It is hoped that these will lead to a practicable method adapted to large-scale production which can be reported later.

CONCLUSIONS

Maximal production of tetanus toxin appears possible only if the gases produced during growth can be largely and promptly dissipated from the culture.

This fact offers the most probable explanation yet available for the relatively poor yields of toxin in large containers.

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