GROWTH REQUIREMENTS OF CLOSTRIDIUM TETANI

II. Factors Exhausted by Growth of the Organism

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Preliminary results of an investigation of the growth requirements of a strain of *Clostridium tetani* have been reported from this laboratory by Mueller and Miller (1942). The information obtained in that study has been applied in attempting to devise a peptone-free medium for the production of tetanus toxin intended for conversion to toxoid for human use. A measure of success has attended this effort, but the level of toxin production achieved has been no better than borderline in terms of the potency required by National Institute of Health regulations, (Mueller and Miller, 1943). It has therefor seemed essential to reinvestigate the various factors concerned in growth, and at the same time to check each recognized growth factor against toxin production, in an effort to raise toxin titers three- or four-fold to a concentration comparable with the best yields obtained on peptone-containing media. In this communication, and the one to follow, the results of two separate approaches to the question of growth requirements will be described. The relationship of these findings to toxin production will be presented subsequently.

In the previous work on growth factors, an acid hydrolysate of casein reinforced with tryptophane had been employed as a source of the essential amino acids. This procedure was chosen for two reasons. In the first place, on the basis of much earlier work with various strains of *Corynebacterium diphtheriae* it was shown that the particular amino acids required for prompt and heavy growth varied considerably from one strain to the next. No general conclusions as to the group as a whole could be made from a detailed knowledge of a single strain. It seemed probable that a similar variation would occur with diverse cultures of tetanus. In the second place, as a practical large-scale production method, matters of cost and simplicity made the use of an acid hydrolysate definitely preferable to that of a number of pure amino acids.

There are, however, obvious disadvantages in the use of a relatively crude acid hydrolysate. The amino acids may occur in other than optimal distribution, so that in order to secure an adequate concentration of one, disproportionately large amounts of others will be present, and these may exert an unfavorable influence either through osmotic effects or in other ways. Moreover, when a substance as crude as commercial casein is employed for the preparation of the

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hydrolysate, it is evident that compounds other than amino acids will inevitably be found in the product,—substances which themselves may affect growth and toxin production either favorably or otherwise.

Evidence was at hand which indicated that one or more limiting factors in the hydrolysate medium were responsible for the comparatively low titers of toxin obtained. In the earlier work on diphtheria toxin production it had been shown that toxin production did not cease because the maximum amount compatible with continued growth of the organism had been produced. Rather, growth and toxin production were arrested when some essential component of the medium became used up. By allowing the pellicle of *C. diphtheriae* to form on the surface of a thin layer of fluid medium superimposed on a reservoir of the same medium incorporated in a relatively thick layer of agar, and separated from the fluid phase by a sheet of cellophane, extremely heavy growth and high concentrations of toxin were obtained (Mueller, 1939). The toxin was unable to diffuse away from the fluid whereas any nutrient material which was utilized was promptly replaced from the excess in the agar. Inert ingredients remained equal on both sides of the membrane. A similar experiment was done by Brewer (1941) with *C. tetani*, except that the nature of the growth (absence of pellicle) makes its conduct somewhat simpler. One suspends a loop of cellophane sausage casing in a flask or bottle of the medium, inoculating only the contents of the bag. Here again, and by the same principle, a very considerable concentration of toxin is obtained. This observation has been confirmed in our laboratory.

The identity of the components of the medium which limit growth has therefore been sought, bearing in mind the possibility that there might also be involved the question of a production of inhibitory metabolic products in themselves responsible for the phenomenon. The method has consisted in growing our strain (New York State Laboratories) of *C. tetani* in 4-liter bottles of the hydrolysate medium, prepared as for toxin production, for periods of four to six days, then autoclaving at 15 lbs. to destroy spores and toxin, and to coagulate the greater part of any soluble protein resulting from the metabolism of the organisms. The turbid solution was filtered through paper, and preserved with chloroform in the cold room for use. For experiment, a sufficient amount of such an "exhausted medium" was distributed in 10 ml. amounts in test tubes, suitable additions of test materials were made, the pH adjusted to 7.4–7.6, the mixtures sterilized at 10 lb. for 6–8 minutes, cooled, and inoculated. For this purpose 10 ml. of a 24-hour culture on peptone-infusion broth was centrifuged, the supernatant removed and the sediment suspended in about 1.0 ml. of saline. With a capillary pipette, 0.05–0.1 ml. of the suspension was added to each tube. Incubation was at 34°–36° for 24 hours, and the growth was read in a Gates suspensiometer.

Attention was directed primarily to the casein hydrolysate component of the medium for the reasons outlined above. Preliminary experiments had given evidence of the necessity for histidine, arginine, glutamic acid and tryptophane, as well as for other unidentified amino acids. It was especially in regard to the last named group that it was hoped to obtain information by this method.

As the experiments proceeded it became evident that the most important single

limiting growth factor in the medium as constituted for optimal toxin production was iron. This element, essential for growth of *C. tetani* as for *C. diphtheriae*, prevents in both cases an abundant production of toxin except in extremely low and carefully controlled concentrations. For optimal tetanus toxin, an amount of the order of 0.05 micrograms of Fe per ml. is required. Below this, growth diminishes sharply while with larger quantities growth improves, but toxin diminishes. Exhausted media prepared in this range of Fe could occasionally be reactivated simply by the further addition of Fe salts so as to give a certain amount of growth.

Even at this point, however, it became evident that tryptophane in the amount employed was a second limiting factor. This amino acid had been reduced, because of its expense, to a relatively low concentration, and evidently dangerously near the level of deficiency. When both tryptophane and iron were increased in the initial medium, or when the medium originally prepared as described above was exhausted a second time after the addition of supplements of Fe and tryptophane, a more complex set of deficiencies was uncovered.

In the first place, a definite requirement for biotin appeared. The necessity for this substance had been suggested in earlier work by the inhibition of growth by avidin, and it was assumed to be present in the casein hydrolysate or other components of the medium as an impurity. Such is evidently the case, and under suitable conditions the small amount available from these sources becomes exhausted. Either free biotin or the methyl ester, (both prepared from naturally occurring sources), will supplement such media, the amount required being of the order of 0.001 microgram per ml. or less.

A new and unanticipated growth factor appeared in the form of oleic acid. This substance, shown by Cohen, Snyder and Mueller (1941) to be essential for the growth of *C. diphtheriae* from minute inocula, obviously occurs in casein hydrolysate due to the presence in commercial casein of small amounts of butter fat. Its function as a growth factor for *C. tetani* was demonstrated through the finding that the exhausted medium, reinforced with Fe, tryptophane and biotin was still deficient in a substance present in the "proline" fraction obtained by Dakin's butyl alcohol extraction of casein hydrolysate. The active material could be extracted from this fraction with ether (evidently as the butyl ester), and also it could be extracted from whole commercial casein by ether or alcohol. The knowledge that oleic acid had been found essential under certain circumstances for the diphtheria bacillus quickly led to its successful substitution for the casein factor. Since oleic acid is not available in synthetic form, the possibility remains that another substance present as an impurity, rather than oleic acid itself may be concerned. This possibility had previously been considered in connection with the work on *C. diphtheriae* mentioned above, and had been excluded so far as possible by chemical purification of commercial oleic acid. This was accomplished by converting the material to its methyl ester, which was fractionally distilled. The appropriate fraction of distillate was converted by bromination to dibrom-oleic-acid-ester, which was again purified by fractional distillation, and finally reduced by means of zinc dust and hydrolyzed. The resulting oleic acid retained fully its effect on growth of *C.*

diphtheriae. A specimen of the same preparation was quite as effective as commercial oleic acid in the present experiments with *C. tetani*.

Folic acid also may become a limiting factor under suitable conditions—(either two exhaustions or adequate Fe and tryptophane in the initial formula). The addition to such media of 0.005 micrograms per ml. of a concentrate of folic acid kindly supplied by Prof. R. J. Williams renders the medium again suitable for use, in the presence of the other factors previously mentioned.

Of the amino acids other than tryptophane, only glutamic acid and histidine appear to be used to the point where their concentrations may become limiting under the experimental conditions here employed. There has appeared to be nothing to be gained through carrying the exhaustion experiments further, for obviously such results could have no bearing on the immediate problem of limitation of toxin production.

It does not seem worth while presenting detailed protocols of experiments by which the above points have been established. In the nature of the case, no two lots of the exhausted base are completely identical, and each batch has had to be treated somewhat as an individual problem,—its particular deficiencies determined and eventually replaced by known compounds and more or less well defined concentrates. It may be said, however, that in the case of each of the substances discussed above, it has been possible to carry through one or more experiments in which little or no growth occurred in the absence of the compound in question, while its addition in suitable quantity resulted in growth approximating that obtained on a peptone-infusion broth.

SUMMARY

Growth of *Clostridium tetani* on a casein hydrolysate medium reinforced with various growth accessories and certain inorganic ions eventually ceases because of the depletion of the medium in respect to one or more nutrilites. Under conditions of maximal toxin production, the iron seems to be the first one to disappear, being presumably linked in organic combination in the cells. In the presence of somewhat more iron, the following substances disappear more or less in the order named: Tryptophane, biotin, oleic acid, folic acid, histidine and glutamic acid.

It appears that iron is the first substance which sharply limits growth as well as toxin and that little is therefore to be expected through the addition of larger quantities of other growth factors to the medium. The actual application of these results to toxin formation will be described later.

REFERENCES

BREWER, JOHN H. Personal communication.

COHEN, S., SYNDER, J. C., AND MUELLER, J. H. 1941 Factors concerned in the growth of *Cornyebacterium diphtheriae* from minute inocula. J. Bact., **41**, 581–591.

MUELLER, J. H. 1939 Factors concerned in the formation of diphtheria toxin. Third Intern. Congress Microbiol. Report of Proceedings, pp. 502.

MUELLER, J. H., AND MILLER, P. A. 1943 Large scale production of tetanal toxin on a peptone-free medium. J. Immunol., **47**, 15–22.

MUELLER, J. H., AND MILLER, P. A. 1942 Growth requirements of *Clostridium tetani*. J. Bact., **43**, 763–772.