The Growth and Toxin Production of Corynebacterium diphtheriae in Submerged Culture

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SUMMARY: The growth curve of Corynebacterium diphtheriae in aerated submerged culture was studied. It showed a logarithmic phase followed by a period in which growth was linear when plotted on an arithmetic scale. There was then a phase of retardation of growth and finally a stationary phase. There was no lag phase. There was no simple correlation between the amount of growth and the amount of toxin produced, although in cultures as growth increased toxin also increased. No relationship was found between toxin production and the formation of catalase or the disappearance of maltose. The relationship between porphyrin and toxin production requires further investigation.

Although surface culture on liquid medium has been the classical means for producing the toxin of Corynebacterium diphtheriae for use in the manufacture of prophylactics, the technique has many serious disadvantages when it is desired to study the growth and metabolism of the organism. The sampling of a single culture over a period was rendered impossible by the fact that without mixing no representative sample could be withdrawn and, since the organism grew as a pellicle, nutrients could only reach the growing mass by diffusion upwards through the medium, thus setting up concentration gradients. Despite these drawbacks much valuable work was done and toxin was produced commercially by this method. With the development of techniques for growing C. diphtheriae in submerged culture (Linggood, Matthews, Pinfield, Pope & Sharland, 1955) it became possible to withdraw successive samples of reasonable size from a single culture without causing any measurable change in the characteristics of the culture and each sample was homogeneous and representative of the whole culture. In the present work it has been possible to investigate accurately the nature of the growth curve for C. diphtheriae and to study the relationship between growth and toxin production. The formation of porphyrin and catalase were also followed since these have been said to be related to toxin production. The disappearance of maltose from the culture was also studied.

METHODS

Organism. A Park-Williams no. 8 strain of Corynebacterium diphtheriae (CN 2000 in the Wellcome Research Laboratories Culture Collection) was used throughout. A 24 hr. growth on a Loeffler slope was used to inoculate 200 ml. medium in a flask which was shaken at 35° for 24 hr. and then used as the tank inoculum.
C. diphtheriae in submerged culture

Submerged culture vessels. Aluminium tanks (80 l.; Linggood et al. 1955) were used, containing 60–65 l. medium and 40 ml. 50% (w/v) antifoam A (Midland Silicone Ltd., 19 Upper Brook Street, London) in liquid paraffin, or, preferably, 40 ml. 25% (w/v) antifoam A dispersed in butyl acetate. The tanks were maintained at 85° and 5 l. air/min. were passed over the surface of the stirred culture. The air was measured and controlled by a flow-meter designed and constructed by T. R. Sharland (to be published). The tank cultures were grown for 48 hr. and samples removed every 6 hr.

Medium. A papain digest of beef muscle like that of Ramon, Pochon & Amoureux (1941) formed the basis of the medium. The digest was yeast treated (Pope & Smith, 1982) and 0.5% (v/v) acetic acid and 2.4% (w/v) maltose were added; the final pH value was ca. 7.8. The medium was sterilized by filtration.

Optical density of cultures. Samples of culture were diluted to a suitable value and the optical density measured by a nephelometer of the type described by Pope & Stevens (1958).

Dry weight of organisms in cultures. A method similar to that of Wade (1952) was used.

Bacterial nitrogen. Organisms were centrifuged down, washed, and their nitrogen content determined by the Hiller, Plazin & Van Slyke (1948) modification of the Kjeldahl method.

Bacterial phosphorus. Organisms were centrifuged down, washed, and total phosphorus determined by the method of Berenblum & Chain (1938).

Viable counts. The method of Miles & Misra (1938) was used, with 20% (v/v) blood agar plates.

Toxin. Toxin was estimated by the flocculation method (Ramon, 1922; Glenny & Okell, 1924). Results are expressed as Lf units/ml.

Porphyrin. Porphyrin was estimated by the HCl extraction method described by Pappenheimer (1947). The colours were read at 401 mμ in a Unicam SP. 600 spectrophotometer using 1 cm. cuvettes. Additional readings were taken at 450, 475 and 500 mμ and these values extrapolated back to give a correction of non-specific absorption at 401 mμ. The results were calculated on the basis of $E^\%_{401}$ for coproporphyrin III being 8100 at 401 mμ (Jope & O'Brien, 1945).

Catalase. The method used was based on that given by Sumner & Somers (1948). The decomposition of hydrogen peroxide in m/150 phosphate buffer (pH 6.8) at 0° was followed by pipetting samples taken at intervals into iodide solution and titrating the iodine so released with standardized thiosulphate. Results are expressed either as the rate constant ($K_1$) or as the rate constant/g. dry weight of organism (Kat. f.).

Maltose. Reducing sugar was estimated by the method of Somogyi (1945). Since chromatography failed to show the presence of any sugar other than maltose the results were calculated accordingly.
RESULTS

In work designed initially to investigate the shape of the growth curve for Corynebacterium diphtheriae four methods of measuring the amount of organism were used. Figure 1 shows curves typical of those obtained throughout this work. It is clear in one case (SC 1101) that the ratio of bacterial phosphorus to bacterial dry weight was not constant nor was the ratio of bacterial nitrogen to optical density (OD) constant in the other (SC 1170). This dependence of the shape of the growth curve on the particular method of measurement used has been found many times in the course of this work, and in a plot of a large number of results of OD and dry-weight determinations there was a very wide scatter among the points which rendered it impossible to use a conversion factor to render OD values in terms of dry weight, though others have apparently managed to convert OD values into bacterial nitrogen when using other strains (see, for example, Yoneda & Pappenheimer, 1957). Part of the discrepancy in the present work appears to arise from changes in the size of the organisms at different times during growth. For example, in one culture (SC 1499) the organisms which were initially about 3.7 \( \mu \) long, were 2.0 \( \mu \) after growth for 12 hr. and then elongated steadily until they were about 5.0 \( \mu \) long at 48 hr.

The results in Fig. 1 seemed to suggest that there was no lag phase in the growth of this organism. This was further investigated and viable counts were done on SC 1101 and SC 1162 which are shown in Fig. 2. In both cases
division appears to have begun immediately after inoculation and both cultures have grown logarithmically for 18 hr. There appears to have been no lag phase. The counts diminished after 24 hr. growth in one case and after 30 hr. growth in the other, though the other measures of growth continued to increase after this time.

Figure 2 also shows that in tanks giving similar viable counts, the toxin titres produced may none the less vary a great deal. The toxin production curves differ mainly in the rate at which toxin is produced after the 18th hr. of growth, and it is not simply a question of toxin production starting later as it would be if ferrous iron were responsible for the effect (Edwards & Seamer 1960). These curves for toxin production start at 12 hr. or slightly earlier, but it is not claimed that toxin synthesis is initiated at this point and undoubtedly

animal tests, with their great sensitivity, would detect the presence of small amounts of toxin before that time. Any toxin that is formed however must represent less than 0.5% of the final total in each case. In terms of dry weight, one culture (SC 1162) had by 12 hr. produced almost 7% of its final dry weight while in SC 1101 almost 12% had been formed. Thus a considerable discrepancy existed between toxin and growth which suggests that very little toxin is produced per organism in the early stages of the culture and that toxin production is therefore not parallel to growth.
Figure 3 shows typical curves for maltose metabolism and for porphyrin, catalase and toxin production. The maltose curve is one of disappearance and exhibits none of the irregularities found in pyruvic acid production (Edwards, unpublished observations). The constancy of the Kat. f. values ($K_f$ per unit weight of organism) shows that the catalase curve is merely another expression of the growth curve. The porphyrin and toxin production curves are steeper than the catalase curve after 30 hr. and could be said to be almost parallel for this culture. Analysis of three cultures after 48 hr. growth gave toxin titres of 118, 142 and 168 Lf units/ml and porphyrin concentrations of 3.55, 5.80 and 4.98 µg./ml respectively. Thus in two cases the ratio toxin/porphyrin is constant, while in the third it is 27% less. Further work is required before firm conclusions can be drawn on this point.

Analysis of four cultures after 48 hr. growth revealed no correlation between toxin titre and either maltose metabolism or catalase ($K_f$) production. The results for Kat. f. tended to increase with increasing toxin titres for values for Kat. f. of 16.1, 14.3, 29.3 and 33.3 corresponded to toxin titres of 118, 142, 168 and 235 Lf units/ml respectively. The relationship however is by no means linear.

**DISCUSSION**

The possibility of correlating toxin production with some aspect of the growth of the organism has attracted much attention but does not appear to have been successful. Mitsuhashi, Kurokawa & Kojima (1949) stated that toxin formation was approximately parallel to bacterial growth and ceased when growth ceased, but Pappenheimer (1955) replotted their results and concluded that no toxin was produced until after the end of the exponential phase, at which time iron was assumed to limit the growth rate. Raynaud, Turpin, Mangalo and Bizzini (1954) concluded that there was a constant relationship between the increase in toxin and that of bacterial nitrogen during growth. They also considered that toxin production as measured by minimum reacting dose started immediately their culture began to grow. Nishida (1954) considered that toxin production was parallel to growth, and further that the growth curve was an accumulation of dead cells. The present results show that under the conditions studied, toxin is not synthesized in significant amounts until at least the 12th hr. of growth and further that by this time the growth has reached as much as 7–12% of its final value. Thus it would appear that toxin synthesis per unit weight of organism must have accelerated and that it is not a linear function of growth. Logarithmic growth also ceases around 18 hr. and in this respect the present results agree with the conclusions reached by Pappenheimer (1955). It appears also that toxin production is not linked to any loss of viability in the cultures as seems to be implied by Nishida (1954). Indeed it is much more likely that it is linked to the viability of the culture, for as the number of viable organisms diminishes so also does the rate of toxin production tend to diminish, but tank cultures with very similar viable counts may none the less produce very different toxin titres, and it is possible to conclude as do Raynaud, Alouf & Mangalo (1959) that no simple relation-
ship can be expressed between the amount of toxin produced and the number of organisms present.

The shape of the growth curve of *Corynebacterium diphtheriae* growing in submerged culture has been commented on before in the literature, especially by the Japanese workers. Mitsuhashi & Takeuchi (1951) showed that the growth rate did not stay exponential for very long and that it was followed by a long period of relatively slow growth. This is borne out by the results presented in the present paper and by the results of Raynaud *et al.* (1959). Pappenheimer (1955) replotted results given by Mitsuhashi *et al.* (1949) and concluded that the deviation from logarithmic growth was due to the iron supply becoming limiting. In the following paper (Edwards & Seamer, 1960) it will be shown that excess iron did not greatly affect the growth rate of the organism; it appears more likely that the factor which limited the growth rate was the availability of oxygen.

Pappenheimer & Hendee (1947) showed that with an increase in the iron content of the culture the catalase activity (Kat.f.) increased while toxin synthesis decreased. In the present study there appears if anything to be a direct relationship between the two which could perhaps be explained if the iron concentrations of the various media were too low and toxin production was being restricted for this reason (Pappenheimer & Johnson, 1936). The relationship, however, is by no means a linear one and in addition the viable counts for SC 1101 and SC 1162, tanks which produced Lf/ml. of 285 and 168 respectively, have already been shown to be very similar, and presumably any serious deficiency in iron would be expected to have an adverse effect on growth.

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REFERENCES


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