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TOXIN PRODUCTION BY *CORYNEBACTERIUM DIPHTHERIAE* UNDER GROWTH-LIMITING CONDITIONS

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SUMMARY *Corynebacterium diphtheriae*, Park Williams No. 8 strain, has been shown to produce toxin without apparent concomitant growth when iron-deficient cells, suspended in phosphate buffer, were incubated in the presence of casamino acid (or 19 amino acids), sodium succinate, magnesium sulfate and sodium chloride. Under these conditions, toxin production occurs almost immediately at the maximal rate and continues for about 2 to 3 hours, whereas the growth of the cells, as measured by the optical density, dry weight and total and viable counts scarcely changes during the incubation. Isotopic studies with L-^{14}C leucine revealed that the rate of cellular protein synthesis by diphtheria bacilli with extremely limited growth was less than 15 per cent of that by growing bacilli, and that the toxin protein released extracellularly under the growth-limiting conditions appeared to be synthesized *de novo* from amino acids.

INTRODUCTION

A precise kinetic study on the relationship between growth and toxin production by *Corynebacterium diphtheriae*, Park Williams No. 8 strain, has recently led us to propose the hypothesis that the rate of toxin production by diphtheria bacilli is expressed primarily as a direct function of the bacterial cells concentration involved and is not dependent on the growth rate (HIRAI, UCHIDA, SHINMEN, FUKUI and YONEDA, 1965 a; HIRAI and YONEDA, 1965). Thus, toxin production may occur irrespective of cellular and population growth and, under suitable conditions, diphtheria bacilli with extremely limited growth or even in the non-growing state can produce toxin just like organisms under normal growing conditions. We thought that, if this were indeed true, it would give strong support for our hypothesis. Moreover, toxin production under such growth-limiting conditions would provide a very good system for studies on the mechanism of formation and extracellular release of diphtheria toxin. Attempts were therefore made to find suitable conditions for this sytem. Preliminary studies (HIRAI, UCHIDA, SHINMEN, FUKUI and YONEDA, 1965 a) showed that replacement of maltose, a main energy source, by sodium succinate in a semi-synthetic medium for *Corynebacterium diphtheriae*, Park Williams No. 8 strain, results in no decrease in the rate

of toxin production but in a great reduction in the growth rate. Thus, essentially based on this finding, it has been possible to prepare a medium in which toxin production occurs without apparent concomitant growth and a method has been devised for kinetic studies on diphtheria toxin production.

The present paper gives details of this method and also results of studies employing this method on the growth and toxin production of diphtheria bacilli.

MATERIALS AND METHODS

1. *Organism*

A substrain (Biken) of *Corynebacterium diphtheriae*, Park Williams No. 8 strain, was employed throughout the study. The organism was subcultured by shaking on a rotary shaker at 34°C in the casamino acid medium described below. Cultures with an optical density (OD) at 590 mμ of 2.0 to 2.5 in a Bausch & Lomb spectrophotometer were usually kept in a frozen state (−20°C) before use as pre-inocula.

2. *Culture medium for inoculum (CMS medium)*

The composition (per liter) of the semi-synthetic medium used for culture was as follows: casamino acid (Difco, certified) 30 g, KH_2PO_4 5.0 g, $CaCl_2 \cdot 2H_2O$(50 per cent solution) 2.0 ml, $FeSO_4 \cdot 7H_2O$ (0.01 per cent solution in 0.1 per cent HCl) 3.0 ml, sodium succinate$\cdot 6H_2O$ 3.0 g, sodium DL-lactate (45 per cent solution, Ishizu reagent), 4.0 ml, L-cystine (10 per cent solution in 10 per cent HCl) 4.0 ml, deferrated maltose (50 per cent solution, YONEDA, 1957) 70 ml, vitamins and metals, solution II (MUELLER and MILLER, 1941) 2.0 ml.

Before addition of iron, cystine, maltose and solution II, the medium was deferrated by the calcium phosphate gel method (YONEDA, 1957). After the addition of solution II and cystine to this deferrated medium, the medium was dispensed in 100 ml volumes in specially designed 500 ml flasks and autoclaved at 115°C for 15 minutes. Before inoculation, the required amounts of iron and deferrated maltose solutions were added aseptically.

3. *Growth-limiting medium for toxin production*

For studying toxin production under growth-limiting conditions, two kinds of media, designated as CST-10 and ASP, found by trial to be optimal for toxin production, were prepared according to the procedures described below.

1) *CST-10 medium*

The composition of this medium is recorded in Table 1. Materials are dissolved in 1000 ml of deionized water with the exception of cystine, $MgSO_4$ and $CaCl_2$. After the addition of 2.5 ml of 50 per cent $CaCl_2$ solution (w/v), the pH is adjusted to 7.6 with KOH. Then, the mixture is boiled for a few minutes and filtered. 1.4 ml of cystine (10 per cent solution in 10 per cent HCl) is then added and the pH is readjusted to 6.9 with HCl. The medium is dispensed in appropriate volumes in bottles, autoclaved at 115°C for 15 minutes and stored. Before use, the requisite amount of magnesium as a 10 per cent solution of $MgSO_4 \cdot 7H_2O$ in deionized water is added to this stocked medium.

TABLE 1 *Composition of CST-10 medium**

Casamino acid (Difco, certified)	10.0 g
L-cystine	0.14 〃
L-tryptophane	0.03 〃
Sodium succinate$\cdot 6H_2O$	3.0 〃
$Na_2HPO_4 \cdot 12H_2O$	8.4 〃
KH_2PO_4	0.3 〃
NaCl	4.5 〃
$MgSO_4 \cdot 7H_2O$	0.4 〃
$CaCl_2 \cdot 2H_2O$	1.25 〃

* In 1000 ml of deionized water.

2) *ASP medium*

This medium was designed particularly for isotopic studies and its composition is shown in Table 2.

Materials are dissolved in 1,000 ml of medium with the exception of cystine, $MgSO_4$ and $CaCl_2$. After the addition of 1.0 ml of 50 per cent $CaCl_2$ solution (w/v), the pH is adjusted to 7.6 with KOH and the solution is boiled for a few minutes, filtered and then cystine (10 per cent solution in 10 per cent HCl) is added to this deferrated medium at a final concentration of 0.37 mM. The pH is then readjusted to 6.9 and the medium dispensed in appropriate bottles is autoclaved at 115°C for 15 minutes and stored at room temperature. Before use, the required amount of magnesium is added as a 10 per cent solution of $MgSO_4 \cdot 7H_2O$ in deionized water.

TABLE 2 *Composition of ASP medium**

19 L-amino acids**	—***
Sodium succinate·$6H_2O$	3.0 g
$Na_2HPO_4 \cdot 12H_2O$	8.4 〃
KH_2PO_4	0.3 〃
$MgSO_4 \cdot 7H_2O$	0.4 〃
NaCl	6.8 〃
$CaCl_2 \cdot 2H_2O$	0.5 〃

* In 1,000 ml deionized water

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*** The relative amounts of these amino acids were roughly based on the composition of crystalline diphtheria toxin given by UCHIDA *et al.* (unpublished data, see KATO, 1963.), and the final concentrations (mM) of each were as follows; L-glycine 6.65, L-alanine 5.44, L-valine 4.20, L-leucine 5.00, L-isoleucine 4.22, L-proline 3.43, L-hydroxyproline 3.43, L-phenylalanine 2.60, L-tyrosine 1.84, L-tryptophane 0.63, L-serine 6.54, L-threonine 4.14, L-methionine 0.76, L-arginine 1.90, L-histidine 2.14, L-lysine 5.28, L-aspartic acid 8.40, L-glutamic acid 9.20, L-cystine 0.37.

4. *Iron-free buffered saline for washing cells (PBS-Mg)*

For washing harvested bacterial cells, a iron-free buffered saline containing magnesium sulfate was employed. This medium is prepared as follows: $Na_2HPO_4 \cdot 12H_2O$ 8.4 g, KH_2PO_4 0.3 g and NaCl 6.8 g are dissolved in 1,000 ml of deionized water. After the addition of 1.0 ml of 50 per cent $CaCl_2 \cdot 2H_2O$ solution, the pH is adjusted to 7.6, and the mixture is boiled for a few minutes and filtered. Before use, $MgSO_4 \cdot 7H_2O$ (10 per cent solution) is added to this deferrated buffer at a final concentration of 0.04 per cent.

5. *Method for obtaining toxin production under growth-limiting conditions*

With the exception of the culture and growth-limiting media employed, this method essentially follows that described by YONEDA (1957). Thus, it consists of the following four steps: 1. Cultivation of inoculum in a semi-synthetic medium. 2. Harvesting of the culture and washing it with iron-free buffered saline containing magnesium. 3. Preparation of a suspension of washed cells in growth-limiting medium. 4. Incubation of the cell suspension with shaking.

Step 1. 0.5 to 1.0 ml of the stock pre-inoculum culture described above is usually inoculated into 100 ml volumes of CMS medium and incubated at 34–35°C with shaking at 200 rpm on a rotary shaker.

Step 2. When the OD of the culture at 590 mμ in a Bausch & Lomb spectrophotometer reaches around 2.5 and the toxin content is 6–8 Lf/ml, the culture is harvested and washed once or twice with PBS-Mg with centrifugation at 9,000 × *g* for 8–10 minutes in the cold. The washed packed cells are employed for *Step 3.*

Step 3. The washed packed cells in centrifuge tubes are suspended with the bottom of an acid-clean test tube as evenly as possible in a small amount of growth-limiting medium, CST-10 or ASP, and made up to the required optical density. Ordinarily, a suspension of OD 8 to 10 is prepared for kinetic studies of toxin production.

Step 4. The cell suspension is quickly distributed in 8 to 10 ml volumes in 100 ml Erlenmeyer flasks. The top of the tube is covered tightly with aluminium foil and tubes are incubated at 34°C with shaking at 160 rpm on a rotary shaker in a water bath. When an ordinary rotary shaker in a warm-air incubator is used, the suspension is usually warmed to 34°C just before incubation with shaking.

Except for *step 1*, no strict aseptic manipulation is necessary and this greatly simplifies the whole method. Moreover, when care is taken not to contaminate the medium with iron, the method is very reproducible for toxin production and, as will be seen in later sections, toxin production occurs almost immediately and proceeds linearly during a 2 to 3 hour incubation period. When a bacterial suspension of OD 10 is employed, for instance, the final yield of toxin after 2 hours incubation is usually found to be 22–24 Lf/ml, while the increase in OD of the suspension is almost negligible.

6. *Determination of growth*

Growth was followed by measuring the optical density of samples diluted with physiological saline at 590 mμ, using a Bausch & Lomb spectrophotometer, by dry weight determination and, by total and viable counts.

1) Dry weight was determined by filtration of the bacterial culture on weighed Millipore filters (HA, 0.45 μ). The bacteria were washed thoroughly on the filter with distilled water and then dried at 100°C to a constant weight. In this study, an OD of 1.0 was found to correspond to 0.75±0.05 mg dry weight of bacteria per ml.

2) Preparation of a homogenized bacterial suspension for total and viable counts was done as follows: an 0.5 ml aliquot of bacterial culture was first added to 9.5 ml of suspending medium containing 0.5 per cent Tween 80, 2 mM Mg^{2+} and 0.14 M NaCl. After thorough mixing, an aliquot of about 2 ml of the diluted culture was introduced into the top of a sterilized 2 ml syringe fitted with a tuberculin test needle, and then quickly forced out by the piston into a sterilized test tube. This procedure was repeated three times with each sample under aseptic conditions and the resulting well homogenized suspension was employed for both total and viable counts.

3) Total counts were performed in a Petrof-Hauser counting chamber under a phase contrast microscope using the homogenized suspension described above containing a drop of 1 per cent Merthiolate.

Viable counts were made as follows: a series of appropriate dilutions of material was prepared in phosphate buffer (pH 7.0, M/50) containing 0.14 M NaCl and 4 mM Mg^{2+}. Then, 0.1 ml of each diluted sample was evenly spread on a chocolate-blood agar plate and the plates were incubated at 37°C for three to four days. After incubation, the number of colonies formed on the plate was counted and the viable cell number/ml of the original sample was calculated.

Tripricate samples were always employed for both total and viable counts.

4) Determination of bacterial protein and nucleic acid

An 8 ml aliquot of bacterial suspension was centrifuged at 9,000 ×*g* for 15 minutes and the resulting packed cells were suspended in phosphate buffer (pH 7.0, M/50) containing 0.14 M NaCl and 2 mM Mg^{2+}. After washing the cells twice by centrifugation at 9,000 ×*g* for 15 minutes, they were resuspended in 3 ml of the same buffer. To this suspension was then added an equal volume of 10 per cent cold TCA solution and, after standing the mixture in the cold for about one hour, the resulting precipitate was collected and washed three times with cold 5 per cent TCA solution with centrifugation at 5,000 ×*g* for each time 20 minutes. The wased precipitate was finally used for measurement of the protein and nucleic acid contents.

Nucleic acid was extracted by a slight modification of TANNHAUSER-SCHNEIDER (1946). RNA was determined by the method of MEJBAUM (1939) and DNA by that of BURTON (1956). Protein was determined by the method of LOWRY *et al.* (1951) on the residual material obtained after extraction of nucleic acids. The intensity of the color developed in each reaction was measured in a Hitachi-Perkin Elmer spectrophotometer Model 139. As standard, commercial preparations of yeast RNA, sperm DNA and bovine serum albumin respectively were used.

7. *L-* ^{14}C *leucine*

This was obtained from Daiichi Pure Chemicals Co. Ltd. and its specific activity was 160 mc/mM. Radioactivity was measured in a windowless gas flow counter (Nuclear Chicago).

8. *Antitoxin*

1) Pepsin-treated horse antitoxin no. 68, containing 1300 flocculating units/ml was obtained from the Vaccine Production Plant (Kanonji Institute) of our Institute.

2) Rabbit antitoxin was prepared by immunizing rabbit with a total of 50 Lf of toxoid from a crystalline diphtheria toxin preparation (3400 Lf/mg protein N) and it contained 100 antitoxin units per ml. The toxoid was prepared by a slight modification of Neumueller (1958).

3) Standard antitoxin containing 10 antitoxin units/ml was kindly provided by the National Institute of Health of Japan.

9. *Crystalline diphtheria toxin*

Crystalline diphtheria toxin was prepared by a combination of ammonium sulfate fractionation, zone-electrophoresis and DEAE cellulose column chromatography (HIRAI and YONEDA, unpublished; ref., KATSURA et al., 1957; RELYVELD and RAYNAUD, 1964). Its specific activity was 3,400 Lf/mg protein N and it gave a single precipitation line against crude horse antitoxin both in an Oakley's tube and on an Ouchterlony's plate.

10. *Measurement of toxicity*

Toxicity of the crude diphtheria toxin produced under growth-limiting conditions was measured by a quantitative rabbit skin reaction test using diphtheria toxin no. 108 from the Vaccine Production Plant of our Institute as a standard.

11. *Double diffusion precipitation in agar gel*

Precipitation reactions in agar gel were carried out by the double diffusion technique of Ouchterlony (1948) using 0.7 per cent Special Agar Noble (Difco) dissolved in physiological saline containing 0.01 per cent Merthiolate. The reaction was read after 4 days incubation at 34°C.

RESULTS

1. *Effect of various energy sources on growth and toxin production in a semi-synthetic medium*

Since a preliminary experiment showed that, when a heavy inoculum of Park Williams No. 8 strain was employed, removal of maltose from the CMS medium resulted in a great reduction in the growth rate but in almost no decrease in the rate of toxin production, the effects of various energy sources on growth and toxin production were studied.

As a basal medium, the growth-limiting medium CST-10, from which sodium succinate had been omitted was employed. The cultural and incubation procedures were as described in the section on Materials and Methods. In the growth experiment, 9 ml volumes of a bacterial suspension of OD 0.5 were dispensed in 100 ml Erlenmeyer flasks and, before incubation at 34°C with shaking at 200 rpm, 1 ml of one of the 10 test matereials listed in Table 3 was added to each flask at a ffnal concentration of 20 mM. Their effects on growth are shown in Table 3.

It will be seen from the table that, of the 10 materials tested, only three, namely maltose, glucose and sodium lactate appear to support growth under these conditions. No appreciable increase of OD of the culture was seen on addition of the other test materials, and sodium succinate, fumarate and citrate especially had no effect on 4 hours incubation.

TABLE 3 *Effects on various carbon sources on growth of a strain (PW8) of Corynebacterium diphtheriae in a casamino-acid medium**

Source	mM	Bacterial growth (OD)		
		0 hour	2 hour	4 hour
Maltose		0.50	0.70	1.18
Glucose	20	0.50	0.83	1.50
Na lactate		0.50	0.63	1.20
Glycerine		0.50	0.52	0.65
Na pyruvate		0.50	0.55	0.58
Na citrate		0.50	0.50	0.56
Na α-keto glutarate	20	0.50	0.54	0.71
Na succinate		0.50	0.50	0.56
Na fumarate		0.50	0.53	0.56
Na malate		0.50	0.50	0.55
No addition		0.50	0.50	0.55

* Basal medium: CST-10 minus succinate, plus vitamins and metals.

Next, the effect of addition of the four test materials which did not support growth on toxin production of the organism was studied. The same procedure was used except that the OD of the bacterial suspension was 10. It was found that, except for sodium citrate, all these materials supported maximal toxin production during a 2 hours incubation period. Thus, the yield of toxin after incubation was about 20 Lf/ml in each case and the rate of toxin production (Lf/OD. hour) was found to be 1.0 to 1.1. This is similar to the toxin production of the organism under normal growth conditions.

These results clearly indicate that sodium succinate does not support the growth of the organism but has a favorable action on toxin production, justifying the use of CST-10 as a medium for studying toxin production under growth-limiting conditions. The results also suggest that succinate may be replaced by sodium fumarate or sodium malate.

2. *Growth and toxin production of Park Williams No. 8 strain in CST-10*

Employing the method described previously, the toxin production of Park Williams No. 8 strain and the changes in optical density and dry weight of the cells during incubation in CST-10 medium were studied kinetically. About 70 ml of a bacterial suspension of OD 10 was first distributed in 10 ml volumes in six 100 ml Erlenmeyer flasks, which were then immediately incubated at 34°C with shaking at 160 rpm. During incubation, duplicate flasks were used at one hour intervals for measuring the OD and dry weight of the cells and the toxin content (Lf/ml) of the culture supernatant. The remaining suspension was placed in the cold and used as the zero-time sample. In each case, 2 ml aliquots from each were taken in tripricate for determining the dry weight. The result is illustrated in Fig. 1.

From the figure, no detectable increase of the cell mass, as measured by either optical density or dry weight, can be seen during the entire period of incubation. This is a sharp contrast to obvious toxin production which occurs. The organism produces toxin from almost the beginning of the incubation period and the toxin contents of the culture filtrate continue to increase linearly until the end of the 3 hours incubation period. The final yield of toxin was 32 Lf/ml on the average, giving a rate of toxin production (Lf/OD·hour) of about 1.0.

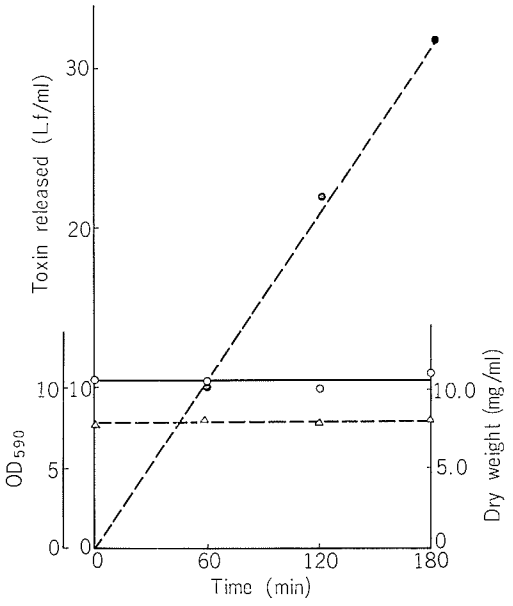


FIGURE 1 Toxin production by *Corynebacterium diphtheriae* strain Park Williams No. 8 under growth-limiting conditions.
●------● Toxin (Lf/ml)
○———○ OD at 590 mμ
△------△ Dry weight (mg/ml)

3. *Changes in total and viable counts of toxin producing cultures during incubation under growth-limiting conditions*

The result of the preceding experiment stimulated us to see if there was any change in the cellular population of a bacterial suspension incubated under growth-limiting conditions. Thus, the total and viable cell numbers of a suspension in CST-10 medium during a 3 hour incubation period were measured.

About 40 ml of a bacterial suspension of OD 5.0 was distributed in 10 ml volumes in three 100 ml Erlenmeyer flasks which were immediately incubated at 34°C with shaking at 160 rpm. During incubation, flasks were taken at hourly intervals to measure the total and viable cell number/ml of suspension and the toxin contents (Lf/ml) of the supernatants obtained by centrifugation. The result is presented in Fig. 2.

As may be seen in this figure, no increase or decrease in either the total or viable cell number/ml was observable and the ratio of viable to total cell number was almost constant, with an average value of about 0.96 during the whole period of incubation. In this experiment, the average total cell number/OD of the bacterial suspension was found to 1.69×10^9. This clearly indicates that, under the growth-limiting conditions described above, all cells in the bacterial suspension seem to be viable and yet no measurable multiplication occurs. On the other hand, the toxin content of the supernatant increased in a similar manner to that observed in the preceding experiment. The rate of toxin production (Lf/OD·hour) was also

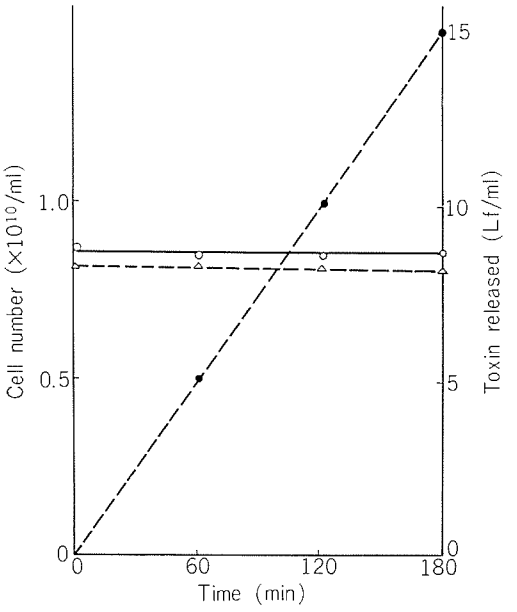


FIGURE 2 Total and viable counts of toxin producing cells during incubation under growth-limiting conditions.
●------● Toxin (Lf/ml)
○——○ Total counts
△------△ Viable counts

found to be about 1.0.

4. *Changes in protein, RNA and DNA contents of bacterial cells during incubation under growth-limiting conditions*

Since the preceding experiments demonstrated that toxin could be produced by apparently non-growing cells, it seemed that unbalanced synthesis of intracellular materials occurred in cells incubated under the growth-limiting conditions described above. An attempt was therefore made to follow the changes in the cellular protein, RNA and DNA contents under these conditions and to compare them with those of growing cells.

Two kinds of media, CST-10 and CMS, were employed in this experiment. The former was used for obtaining cells with limited growth and the latter, for obtaining growing cells. These media were first distributed in 8 ml volumes in eight 100 ml Erlenmeyer flasks. Then 2 ml aliquots of bacterial suspension of OD 2.5 were introduced into each flask and six of the flasks were incubated at 34°C with shaking at 200 rpm, while the other two flasks were immediately cooled in an ice-water bath for use as zero-time samples. During incubation, two flasks were taken at one hour intervals and immediately cooled. The contents were mixed and used for measurement of the protein, RNA and DNA contents of the cells.

The results are shown in Fig. 3. As may be seen, there was no appreciable increase in the protein, RNA or DNA contents of cells incubated in CST-10 medium, while those of cells in CMS medium increased nearly exponentially during incubation. The figure

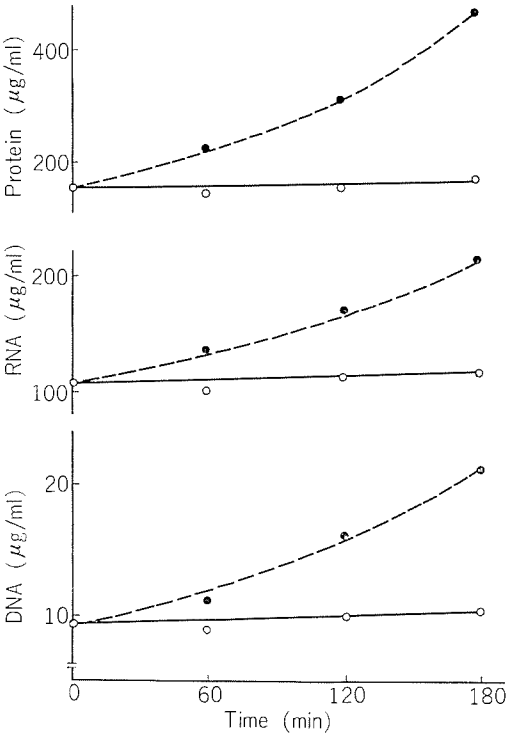


FIGURE 3 Changes in protein, RNA and DNA contents of bacterial cells during incubation under growth-limiting conditions.
○——○ Growth-limiting
●------● Growing

also shows that, in each case, the contents of protein, RNA and DNA appear proportional during the entire period of incubation. Incidentally, the change in optical density of the culture was also found to be proportional to those of these cellular materials. It thus appears that no unbalanced synthesis of cellular materials occurs in cells during incubation under the growth-limiting conditions employed. However, it could be that a change occurs which is so small that it is not detectable by the method employed. Further precise isotopic studies are necessary to decide this.

5. *^{14}C leucine incorporation into bacterial cell protein under growing and growth-limiting conditions*

The bacterial suspension was incubated in ASP medium to give limiting-growth conditions and in the same medium supplemented with iron (3 μg $FeSO_4 \cdot 7H_2O$/ml), maltose (2 per cent) and vitamins and metals (Mueller's II solution minus Mg^{2+}) to give growing conditions. The latter was designated as ASP-G medium for convenience.

ASP and ASP-G media were first distributed in 8 ml volumes in eight and six 100 ml Erlenmeyer flasks respectively, and 2 ml aliquots of a bacterial suspension of OD 2.5 were put into each flask. After thorough mixing, flasks were placed in an ice water bath (0–2°C) and then 0.2 ml of a solution of 1 μc of ^{14}C leucine/ml was added. Then, all flasks except for two of the flasks containing cells suspended in ASP medium were immediately incubated at 34°C in a water bath with shaking at 200 rpm on a rotary shaker. During incubation, two flasks were taken from each series at one hour intervals and quickly cooled. Two flasks were not incubated and were used as zero-time samples. After determining the OD, 8 ml aliquots of each suspension were washed three times with PBS-Mg containing 5 mM unlabeled L-leucine by centrifugation at 9,000$\times g$ for 20 minutes. The protein fraction of the resulting packed cells was prepared by a slight modification of the method of SIEKEVITZ (1952). After dissolving the protein fraction from each 8 ml aliquot in 1 ml of 0.2 N NaOH solution, a 0.5 ml sample was placed on a planchet and dried. The radioactivity of the dried samples were counted in a windowless gasflow counter (Nuclear Chicago). The counts per minute per ml (cpm/ml) were calculated making correction for self absorption and the value of the zero-time sample.

Fig. 4 shows the results obtained. It may be seen that the incorporation of ^{14}C leucine into the cellular protein takes place in both series, but the rate of incorporation by cells incubated in the growth-limiting medium, ASP, is far less than that by those in medium ASP-G which supports growth. Thus, even after 3 hours incubation, only 102 cpm/ml were incorporated into the protein of the cells with limited growth, while 682 cpm/ml were incorporated into growing cells. It was found

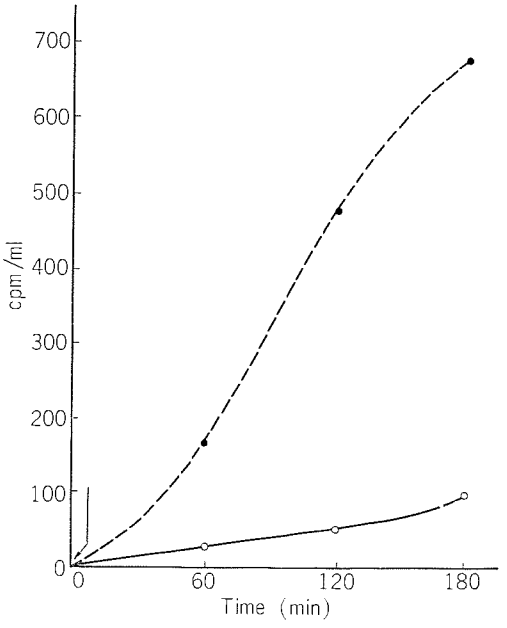


FIGURE 4 ^{14}C-leucine incorporation into cellular protein under growing and growth-limiting conditions.
○———○ Growth-limiting
●------● Growing
Arrow indicates the addition of ^{14}C-leucine.

TABLE 4 *^{14}C leucine incorporation into toxin protein released during incubation under growth-limiting conditions*

Time (min)	OD of the culture at 590 mμ	Total toxin specifically precipitable ^{14}C (Lf/ml)	(counts/min/ml)	Specific radioactivity Observed value (counts/min/Lf)	Corrected value* (counts/min/Lf)
30	10	5	22	4.4	6.6
60	10	12	58	4.84	7.3
120	10.5	22	107	4.86	7.3

* Corrected for self-absorption.

that the percentage incorporations into growth-limited cells to those into growing ones were 15, 10.4 and 14.7 after 1, 2 and 3 hour incubation, respectively. In triplicate experiments, the values varied from 3 to 15, de pending on the actual counts of the zerotime sample. These results clearly show that limited synthesis of cellular protein occurs in diphtheria bacilli incubated under growth-limiting conditions.

6. *^{14}C leucine incorporation into toxin protein released extracellularly by diphtheria bacilli under growth-limiting conditions*

Diphtheria toxin protein was previously shown to be synthesized *de novo* from amino acids by growing organisms (PAPPENHEIMER, MILLER and YONEDA, 1962). The present experiment was to see if this was also the case with toxin protein produced by organisms under the extremely growth-limiting conditions described above.

Volumes of 7.8 ml of ASP medium were dispensed in ten 100 ml Erlenmeyer flasks and 0.2 ml of ^{14}C leucine solution (1 μc/ml) was added to each flask. Then 2 ml of bacterial suspension (OD 50) in PBS-Mg was added and the flasks were incubated at 34°C with shaking at 160 rpm. Five flasks were cooled after 30 minutes incubation, 3 after 60 minutes and 2 after 120 minutes. The contents in the flasks were mixed and centrifuged at 9,000 × *g* for 20 minutes. The resulting supernatants were filtered through a Millipore filter (HA, 0.45 μ) and the filtrates were employed as test materials.

These filtrates were designated as F1 (30 minutes), F2 (60 minutes) and F3 (120 minutes). In the flocculation test, values of 5 Lf/ml for F1, 12 Lf/ml for F2 and 22 Lf/ml for F3 were obtained. The toxin in each filtrate was precipitated quantitatively and specifically with the rabbit antitoxin (100 units/ml) described in the section on Materials and Methods as follows: Duplicate aliquots of 20 ml of F1, 9 ml of F2 and 4.5 ml of F3 were introduced into conical centriguge tubes, made up to a final volume in 20 ml with physiological saline and 1.2 ml of rabbit antitoxin was added to each. As controls, duplicate tubes were set up with 5 ml of unlabeled crude toxin (prepared in ASP medium, 20 Lf/ml), 0.4 ml of ^{14}C leucine solution (1 μc/ml), 1.2 ml of rabbit antitoxin and 14.6 ml of physiological saline. These mixtures were incubated in a water bath at 40°C for 3 hours and then kept at 4°C for 4 days. They were centrifuged and washed three times with chilled saline containing 5 mM unlabeled L-leucine. The washed floccules were then completely dissolved by addition of 1 N NH_4OH solution, and the resulting solutions were carefully transfered to planchets, dried and counted. The results are shown in Table 4 and Fig. 5.

From Table 4, it may be seen that the specific radioactivity (cpm/Lf) of the toxin formed under the present conditions was constant, irrespective of incubation time, and thus, the cpm/Lf (average value from duplicate samples, corrected for self absorption and the zero-time value) was 6.6 after 30 minutes, 7.3

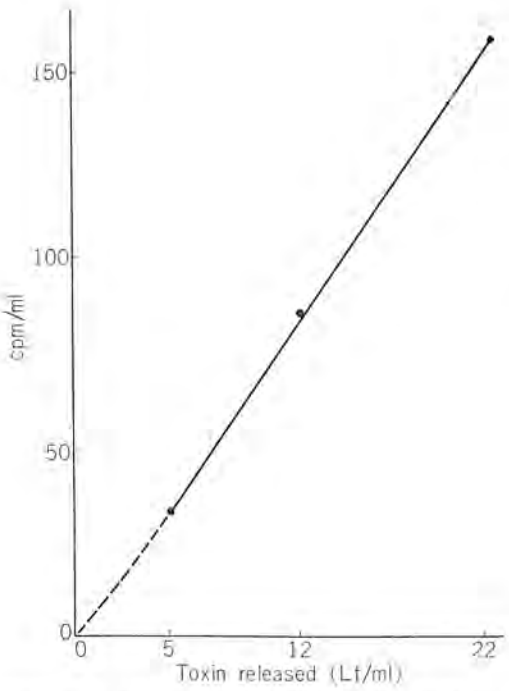


FIGURE 5 ^{14}C-leucine incorporation into toxin protein released extracellularly during incubation under growth-limiting conditions.

after 60 minutes and 7.3 after 120 minutes. Accordingly, when the specifically precipitable radioactivity per ml of filtrate is plotted against the toxin concentration (Lf/ml) at each incubation time, a linear relationship should be obtained and this was indeed obtained as shown in Fig. 5.

From the value for the specific radioactivity of 7.3 cpm/Lf obtained from this experiment, it was calculated that there were about 33 moles of L-leucine per mole of toxin protein assuming the molecular weight of diphtheria toxin to be 70,000, and that there is 1.89 μg protein per Lf (3300 Lf/mg protein N) and 8200 cpm/μM leucine in the medium. The value of 33 indicates that there is about 6.2 per cent (w/w) leucine in toxin protein and this agrees fairly well with the analytical value (6.82 per cent) obtained by UCHIDA *et al.* (unpublished data, see KATO, 1963). These results suggest that exogenous leucine is directly utilized by diphtheria bacilli for synthesizing toxin protein even under growth-limiting conditions.

7. *Characterization of the toxin produced under growth-limiting conditions*

The toxicity and antigenic specificity of the toxin produced under the growth-limiting condition described above were examined by the rabbit skin test reaction and by Ouchterlony's immunodiffusion technique, and compared with those of crystalline diphtheria toxin. The test toxin employed was an aseptic filtrate of the supernatant of a bacterial suspension incubated at 34°C in ASP medium with shaking for 2 hours and its toxin content was 20 Lf/ml. A quantitative rabbit skin test revealed that the toxicity of test toxin was about 10^5 mrd/Lf and it was completely neutralized by ordinary antitoxin. The result of Ouchterlony's immunodiffusion test is presented in Fig. 6.

The single line formed between the test toxin and crude horse antitoxin completely fuses with the single precipitation line with crystalline diphtheria toxin suggesting the identity of these two toxins in terms of antigenic specificity.

Although further precise studies are needed before reaching any conclusion, these results

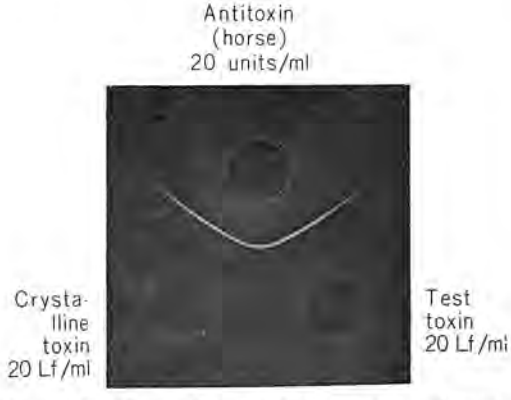


FIGURE 6 Agar gel precipitation pattern demonstrating the antigenic indentity of test toxin with crystalline diphtheria toxin.

suggest that there is at least no great difference between the toxin proteins produced under growing and growth-limiting conditions.

DISCUSSION

The results presented in this paper show for the first time that diphtheria bacilli in an almost non-growing state can produce toxin at the same rate as growing organisms. Thus, under the conditions described here, toxin production by the Park Williams No. 8 strain occurs with almost no apparent concomitant net increase in cell mass. Since synthesis of toxin protein normally occurs irrespective of synthesis of bulk intracellular protein, it now appears that the mechanisms regulating the former synthesis may differ from those acting in the latter. Although the question of whether or not there is any co-ordination between these mechanisms is yet unanswered, these different systems must be present because of the converse observation that diphtheria toxin protein synthesis by the organism is completely stopped by addition of iron to the medium without affecting the growth rate (HIRAI, UCHIDA, SHINMEN, FUKUI and YONEDA, 1965 b; HIRAI and YONEDA, 1965).

The main factor in our proposed method, to which success in obtaining toxin production under growth-limiting conditions is mainly attributed, seems to be the employment of an incubation mixture in which maltose, an ordinary major energy source used for the strain employed, is replaced by sodium succinate. As seen in Table 3, addition of maltose to the basal medium employed completely restores the growth rate of the organism, while addition of sodium succinate does not. Conversely, the removal of maltose from the growth medium greatly reduces the growth rate irrespective of the presence of sodium succinate, while the rate of toxin production is unchanged. Thus, our method somewhat resembles the so-called "shift-down" procedure by which one may observe unbalanced synthesis of cellular macromolecules in an exaggerated form (KJELDGAARD *et al.*, 1958). In the present experiments, however, no unbalanced synthesis of cellular macromolecules such as protein, RNA or DNA appeared to take place, their synthesis being suppressed under the present extremely growth-limiting conditions. In this connection, it is significant to note that, under these conditions, no change was seen in either the total or viable cell numbers of the bacterial suspension during the entire incubation period. This clearly indicates an almost complete suppression of cellular multiplication, strongly suggesting that toxin production takes place irrespective of cell division of the organism.

The actual role of sodium succinate in the synthesis of toxin protein is not yet known. Since available data (unpublished) show that succinate is metabolized by the organism and that the removal of succinate from CST-10 or ASP medium results in a considerable reduction in the yield of toxin, it may be that this organic acid is somehow related to the synthesis of toxin protein. However, whether it acts as an energy source for the synthesis of toxin protein or indirectly by stabilizing the cellular structure with which the site of toxin synthesis is associated, must await further investigation.

Isotopic studies showed that the net incorporation of ^{14}C leucine into proteins in cells incubated in the growth-limiting conditions described above is only 10 to 15 per cent of that in growing cells. Hence, it is not surprising that we could not detect any increase in the protein contents of cells incubated under these conditions, because the increment was so small that it could probably not be measured by ordinary method for protein determination, such as that of Lowry *et al.* The slight but definite net increase in cellular protein, shown by ^{14}C leucine incorporation, however, raises the interesting questions of which cellular protein is responsible for the increase and of whether or not the cellular protein which increases is somehow related to the synthesis of toxin protein. These pro-

blems are now being studied.

From the results of studies on ^{14}C leucine incorporation into toxin protein, the bulk of the leucine molecules in newly synthesized toxin protein appear to be derived from exogenous leucine added to the incubation mixture. This strongly suggests that the toxin protein, produced under growth-limiting conditions, must be synthesized *de novo* from amino acids, like the toxin protein produced by growing diphtheria bacilli (PAPPENHEIMER, MILLER and YONEDA, 1962). Incidentally, it should be mentioned here that the value (6.2 per cent) for the leucine content of toxin protein calculated from the present *in vivo* experiments is based on the several assumptions, particularly on the value of 8,200 cpm/μM leucine, as described in a previous section. This value was calculated from the amount (4 μM/ml) of unlabeled leucine added to the medium, without considering the amount of leucine possibly already present in the cells in the amino acid pool. Since we used a heavy bacterial suspension with OD 10 (corresponds to about 7.5 mg dry weight/ml) for toxin production, the amount of leucine in the pool may be considerable, and if this is the case, the true value may be much more than 6.2 per cent. In a recent publication, RAYNAUD *et al.* reported (1965) the amino acid composition of a crystalline toxin preparation in which leucine comprises about 7.7 per cent of the total. Even if this is so in the present case, more than 80 per cent of the leucine in toxin protein would have been derived from leucine added to the medium. At any event, these results suggest that exogenous leucine is directly utilized by diphtheria bacilli for synthesizing toxin protein even under growth-limiting conditions.

Finally, it may be significant to mention that toxin production under the growth-limiting conditions described above may provide a very good and simple system for studying the mechanism involved in the synthesis and extracellular release of diphtheria toxin. Thus, using this system together with both cellular fractionation and isotope techniques, studies on the site of synthesis of toxin in the cell and on the inhibitory action of iron on the synthesis are now in progress and results will be published elsewhere.

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