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ESTIMATION OF THE TIME REQUIRED FOR THE PROCESS OF DIPHTHERIA TOXIN FORMATION

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SUMMARY Employing the isotopic techniques of pulse-labelling with ^{14}C amino acids and their chase, precise kinetic studies were made on toxin production by Park Williams No. 8(Biken) strain of *Corynebacterium diphtheriae* under growth-limiting conditions. The present studies revealed that it required about 3 minutes for the whole process, involving *de novo* synthesis of toxin protein from amino acids and its extracellular release. Evidence was also presented indicating that the extracellular release of toxin takes place within 30 seconds.

INTRODUCTION

The recent establishment by HIRAI *et al.* (1966) of a preferred system for the production of diphtheria toxin, in which non-growing suspensions of iron-depleted diphtheria bacilli continue *de novo* synthesis and release of toxin into the extracellular fluid like growing organisms (PAPPENHEIMER *et al.*, 1962), has provided a very valuable tool for studying the precise kinetics of diphtheria toxin production. Employing this system, together with isotopic techniques, studies were made on the average time required for the whole process involving the synthesis of toxin protein from amino acids and its release into the extracellular environment. We felt that such information is of fundamental importance and would help to clarify the physiology of diphtheria toxin production.

The present paper gives details of this estimation method and results suggesting that the average time required for the synthesis of diphtheria toxin molecule from amino acids is not much less than 3 minutes.

MATERIALS AND METHODS

1. *Organism*

A substrain (Biken) of *Corynebacterium diphtheriae*, Park Williams No. 8 strain, was employed thoughout the study. The organism was subcultured by shaking on a rotary shaker at 160 rpm at 36°C in the CMS medium described below. Cultures with an optical density (OD) at 590 mμ of 2.0–2.4 in a Bausch & Lomb spectrophotometer were usually kept in a frozen state (−20°C) before use as pre-inocula.

2. *Media*

All media described below were deferrated by a slight modification of the calcium phosphate gel method (YONEDA, 1957).

CMS medium for obtaining inoculum culture was prepared according to the method described by HIRAI *et al.*, (1966).

CST-6 *medium* was employed for toxin production under growth-limiting conditions. The composition (per liter) of the semi-synthetic medium was as follows; casamino acid (Difco, certified) 6.0 g, DL-tryptophane 30 mg, L-cystine 80 mg, sodium succinate·$6H_2O$ 3.0 g, NaCl 4.5 g, $CaCl_2 \cdot 2H_2O$ 1.0 g and $MgSO_4 \cdot 7H_2O$ 1.0 g. Materials are dissolved in 1000 ml of 0.05 M Tris-HC1 buffer (pH 7. 4) with the exception of cystine, $MgSO_4$ and $CaCl_2$. After the addition of 1 ml of 50 per cent $CaCl_2$ solution (w/v), the pH is adjusted to 7.4 with NaOH. Then, the mixture is boiled for a few minutes and filtered. This procedure for deferration is repeated twice. Then, 0.8 ml of cystine (10 per cent solution in 10 per cent HC1) is added to the deferrated medium and the pH is readjusted to 7.4 with NaOH. 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 stock medium.

ASP medium, a chemically defined medium, was also employed for toxin production under growth-limiting conditions. The medium was prepared according to the method described by HIRAI *et al.* (1966) with a slight modification. When necessary, L-tyrosine or L-leucine was omitted from the medium.

3. *Culture method*

To obtain the inoculum culture for toxin production, appropriate volumes of a stock culture were inoculated into 100 ml of CMS medium so as to give a final OD of about 0.01. Flasks were then incubated at 35°C on a rotary shaker with shaking at 160 rpm for 17 to 18 hours. Cultures with a final OD of about 2.4 to 2.6 were employed for preparing the cell suspension for toxin production.

4. *Toxin production*

This was done under the growth-limiting conditions described by HIRAI *et al.* (1966).

5. *Determination of bacterial protein and its radioactivity*

A 0.5 ml aliquot of bacterial suspension was centrifuged at 9,000 g for 15 minutes and the resulting packed cells were then suspended in 3 ml of 0.05 M Tris-HC1 buffer (pH 7.4) containing 0.14 M NaC1 and 2 mM Mg^{2+}. To this suspension was then added an equal volume of cold 10 per cent trichloroacetic acid (TCA) solution and, after standing the mixture in the cold for about 1 hour, the resulting precipitate was collected, washed with cold and hot 5 per cent TCA solution and finally with an ethanol-ether-chloroform mixture (2 : 2 : 1, v/v/v). The washed precipitate was used for measurement of the protein content by the method of Lowry *et al.* (1951) and of the radioactivity. For the latter measurement, samples were each dissolved in dilute ammonia and dried on stainless steel planchets.

6. *Radioactive amino acids*

L-^{14}C tyrosine and L-^{14}C leucine were obtained from Daiichi Pure Chemicals Co. Ltd. and their specific radioactivities were 322 mc/mM and 214 mc/mM respectively. Radioactivity was measured in a low back ground (background: approx. 1) windowless gas flow counter (Aloka).

7. *Antitoxin*

1) Pepsin-treated horse antitoxin no. 68, containing 1300 limit flocculation unit (Lf)/ml was obtained from the Vaccine Production Plant (The Osaka Microbial Diseases Research Foundation, Kan-onji Institute) of our Institute.

2) Rabbit antitoxin described by YONEDA and MATSUDA (1966) was used for quantitative precipitation of radioactive toxin. It was prepared by immunizing rabbits with a total of 50 Lf (ca. 100 μg protein) of the toxoid from a crystalline diphtheria toxin preparation (3400 Lf/mg. protein N) and it contained 100 antitoxin units per ml.

RESULTS

1. *Effect of duration of pulse-labelling of diphtherial culture with ^{14}C amino acid on the amount of radioactive toxin appearing in the culture filtrate during pulse-labelling and after the chase*

Under the present condition, at any time during toxin production, nascent toxin protein could be considered as being comprised of a heterogeneous population of all varieties of precursor polypeptides incorporating amino acids. Accordingly, on pulse-labelling of the

culture producing toxin for a period corresponding to that required for the completion of the polypeptide, the amount of the radioactivity in the toxin protein completed during the time of pulse-labelling should be equal to that completed after the chase.

Essentially based on this consideration, the following experiment was designed to estimate the average time required for the whole process of toxin formation.

A culture (OD of 2.4) of PW8 Biken strain, producing toxin at the maximal rate (1.2 Lf/OD·hour), was centrifuged at 9.000 *g* for 15 minutes. The cells were collected and suspended in an appropriate volume of CST-6 medium to give a final OD of 9.0. Then 9.4 ml volume of the suspension were distributed into eight 100 ml-Erlenmeyer flasks, which were incubated at 35°C with shaking. After 20 minutes incubation, the culture in each flask was pulse-labelled by addition of 0.6 ml of L-^{14}C tyrosine (5.0 μc/ml) and, during incubation, duplicate flasks were quickly chilled at 0, 2, 3 and 4 minutes after the start of pulse-labelling. Each chilled culture was then centrifuged in the cold to separate cells from culture supernatant and the cells were resuspended at the original concentration in fresh chilled CST-6 medium supplemented with unlabelled L-tyrosine as chaser. A 0.5 ml aliquot was qucikly taken for measuring the radioactivity of cellular protein and then each culture was incubated at 35°C with shaking for a further 20 minutes. After incubation, a 0.5 ml aliquot was again taken for measuring the radioactivity and the remaining culture were centrifuged in the cold to separate cells from culture supernatant. After this chase treatment, practically no increase was seen in the total radioactivity of the whole cellular protein, indicating that the chase was complete.

The radioactivity of toxin was assayed with the specific precipitates obtained by a quantitative precipitation reaction with rabbit diphtheria antitoxin. Thus, the above mentioned culture supernatants were each filtered once through a Millipore filter (HA, 0.45 μ) and then a 7 ml aliquot of each filtrate was dialyzed in the cold against 10 liters of physiological saline for 3 days to remove free ^{14}C tyrosine. After dialysis, was added to each filtrate an appropriate amount of unlabelled diphtheria toxin to make the total Lf about 100, and then an excess (120 units) of rabbit diphtheria antitoxin was added. The mixture was incubated at 35°C for 1 hour and then placed in the cold for 3 days. The resulting precipitates were then each washed twice with chilled CST-6 medium containing 0.14 M NaCl, dissolved in a small volume of dilute ammonia, dried on plachets and their radioactivity was counted in a low background gas flow counter. In each case, the harvested cells were resuspended in CST-6 medium at the original concentration, and washed twice by centrifugation with the medium. A 7 ml aliquot of the washings was treated as described above and the radioactivity of the resulting precipitate was measured. Practically, no measurable radioactivity was found in residues from the 2nd washing.

The results are summarized in Table 1.

TABLE 1 *Distribution of radioactivity in toxin protein in culture fluid during pulse-labelling with L^{14}-C tyrosine and its chase with an excess of L^{12}-C tyrosine*

Labelling time (minutes) t	Total radioactivity (*cpm*)* ** per toxinantitoxin precipitate from 7 ml culture ffltrate	
	Pulse(P)	Chase(C)***
2	430	780
3	945	928
4	1364	941

* Corrected for self-absorption and zero-time counts.

** 460 *cpm* = 1 Lf

*** As mentioned in text, fresh medium was employed at the time of chase.

In this table, the figures are given as the sum of radioactivities (counts per minute; *count/min*) of the specific precipitates obtained from

the culture filtrate and the 1st washing of cells respectively. This table shows that the amount of radioactive toxin appearing in chased culture fluid is so great that it is comparable with that in the pulse-labelled sample and, after 2 min pulse-labelling, it is nearly twice the latter. The presence of radioactive toxin in chased culture fluid could not be seen when similar suspensions of pulse-labelled cells were incubated at 3–4°C with shaking. But radioactive toxin began to appear in the culture immediately after raising the temperature to 35°C. Moreover, no matter how long the duration of pulse-labelling, only negligible amounts of radioactive toxin could be detected in the soluble fraction obtained from disrupted labelled cells by centrifugation at 105,000 *g* for 90 minutes. These findings suggest that the radioactive toxin, found in chased culture fluid, represents radioactivity of the nascent toxin protein which had been being synthesized on the site associated with 105,000 *g*-sedimentable cellular structure. Incidentally, our recent studies have provided evidence suggesting the close association of membrane with the site of diphtheria toxin synthesis (UCHIDA & YONEDA, 1967).

In the table, it may be seen that the amount (945 *count/min*) of radioactive toxin in 3 min pulse-labelled culture filtrate nearly coincides with that (928 *count/min*) in its chased sample. Moreover, the increase in the amounts of radioactive toxin in chased culture filtrates almost ceases after 3 min pulse-labelling, while the radioactive toxin in pulse-labelled filtrates increases from 430 to 1364 *count/min* with increase in the duration of pulse-labelling. Similar results were obtained in an experiment with L-^{14}C leucine. Therefore, these data indicate that the average time required for the whole process of toxin formation which includes the intracellular synthesis of the toxim molecule from external amino acids and its extracellular release is about 3 minutes.

Assuming that the number of sites of toxin protein synthesis does not increase during the production and that the incorporation of external ^{14}C amino acids into nascent toxin protein commences immediately after the start of the pulse-labelling, the actual time (T) required for toxin production can be calculated by the following equations*;

$$T = (P+C)t/2P \qquad (P \leqq C) \qquad (1)$$

$$T = 2Ct/(P+C) \qquad (P \geqq C) \qquad (2)$$

where P represents the amount (*count/min*) of radioactive toxin in pulse-labelled culture filtrates, C, that (*count/min*) in their chased ones and t, the time (minutes) of pulse-labelling. Using the actual values given in Table 1, the time (T) for toxin production was calculated from these equations as about 3 minutes.

2. *Continued production of radioactive toxin in culture fluid of ^{14}C amino acid pulse-labelled cultures after chase with an excess of unlabelled amino acid*

This experiment was designed to confirm that the average time required for the formation of toxin protein molecules is about 3 minutes. If this is so, the liberation of radioactive toxin into culture filtrates should continue for about 3 minutes after the chase.

A culture (OD of 2.4) of PW8 Biken strain, producing toxin at the maximal rate (1.2 Lf/OD·hour), was centrifuged at 9,000 *g* for 15 minutes and the harvested cells were suspended in an appropriate volume of ASP medium, from which all amino acids were omitted, at a final OD of 5.0. The bacterial suspension was then incubated at 35°C with shaking for 20 minutes. After incubation, the cells were collected by centrifugation and resuspended in 90 ml of ASP medium from which L-leucine was omitted. The suspension of OD 9.0 was then distributed in volumes of 9.75 ml in eight 100 ml-Erlenmeyer flasks, which were incubated at 35°C with shaking. After 10

* These equation and those with modifications may be generally applicable for estimating the average time for synthesis of a protein molecule from amino acids. The basis will be described in some detail elsewhere.

minutes incubation, the culture in each flask was pulse-labelled by addition of 0.25 ml of L-^{14}C leucine (5 μc/ml). At 0 and 1.5 minutes after the start of pulse-labelling, one flask each was quickly chilled and unlabelled L-leucine was added to a final concentration of 0.32 μM. At 3 minutes after the start, the same amount of unlabelled L-leucine was added to the other falsks and flasks were taken out after 0, 1.5, 3.0, 6.0, 12.0 and 24.0 minutes. From each flask, duplicate 0.5 ml aliquots were taken for measuring the bacterial protein and its radioactivity by the methods described in the section of Materials and Methods. The remaining suspension was then centrifuged at 9,000 g for 15 minutes and the resulting supernatants were each filtered through a Millipore filter (HA, 0.45 μ). A 2.5 ml aliquot of the filtrate was employed for the approximate determination of its toxin content by flocculation with horse diphtheria antitoxin. The following procedures were used for determining the radioactivity of the toxin in the culture filtrates. A 5.0 ml aliquot of each filtrate was first dialyzed in the cold against Tris-buffered physiological saline for 4 days to remove free ^{14}C leucine. Then, it was transferred to a flask quantitatively, washing with ASP medium, and the resulting 15 ml of dialyzed filtrate was mixed with an appropriate amount of unlabelled diphtheria toxin to make the total Lf about 100. Then an excess (120 units) of rabbit diphtheria antitoxin was added. The mixture was incubated at 35°C for 1 hour and then placed in the cold for 3 days. The resulting precipitate was then washed three times with chilled Tris-buffered physiological saline containing unlabelled L-leucine (0.32 μM), dissolved in dilute ammonia, plated and counted as described previously.

The result is shown in Figure 1. As is seen, ^{14}C leucine-labelled toxin is liberated into the culture fluid either during 3 min pulse-labelling or during a further 3 minutes after the application of a chase with excess ^{12}C leucine. The amount of the radioactive toxin thus produced

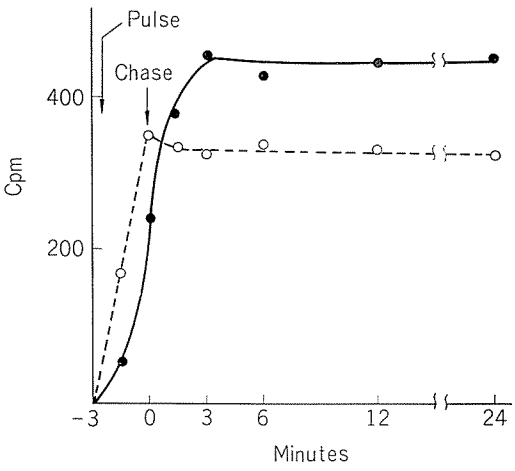

FIGURE 1 Cessation of L-^{14}C leucine incorporation into cellular and toxin proteins after the application of a chase of excess L-^{12}C leucine.
*cpm of specific precipitate per 5 ml of culture (—●—)
*cpm of cellular protein per 0.25 ml of culture (—○—)
*Corrected for self-absorption and zero-time counts.

increases sigmoidally with time and the increase stops 3 minutes after the chase. Careful inspection also shows that the zero-time point is almost in the middle of the curve, indicating that the amounts of radioactive toxin appearing in the 3 minutes before and after chase treatment are nearly the same. This is in sharp contrast to the case of cellular protein, where the radioactivity increases almost linearly with time and the increase stops almost instantly at the time of application of a chase with ^{12}C leucine. Incidentally, an almost linear increase of toxin was observed when the toxin content was measured by flocculation.

3. *Rate of incorporation of ^{14}C amino acid into cellular and toxin proteins*

This experiment was made to find the time required for extracellular release of toxin protein and whether the incorporation of external ^{14}C amino acids into intracellular nascent proteins starts immediately after the onset of pulse-labelling.

The bacterial suspension (OD of 9.0) in CST-6 medium of PW8 Biken strain was first distributed in 9.6 ml volumes in five 100 ml-Erlenmeyer flasks and then these were incubated at 35°C with shaking. After 20 minutes incubation, the cultures in the flasks producing toxin at the maximal rate (1.2 Lf/OD·hour) under growth-limiting conditions, were each pulse-labelled by the addition of 0.40 ml of L-^{14}C tyrosine (5.0 μc/ml) for 3 minutes. At 0, 0.5, 1.0, 2.0 and 3.0 minutes during the pulse-labelling, one culture was quickly chilled and, from each, duplicate 0.5 ml aliquots were taken for measuring the cultural protein and its radioactivity by the methods described in the section of Materials and Methods. For determining the radioactivity of toxin, the chilled culture was centrifuged to separate cells from culture supernatant, and a 6 ml aliquot of the resulting supernatant was treated as mentioned in a previous section (Results, 1).

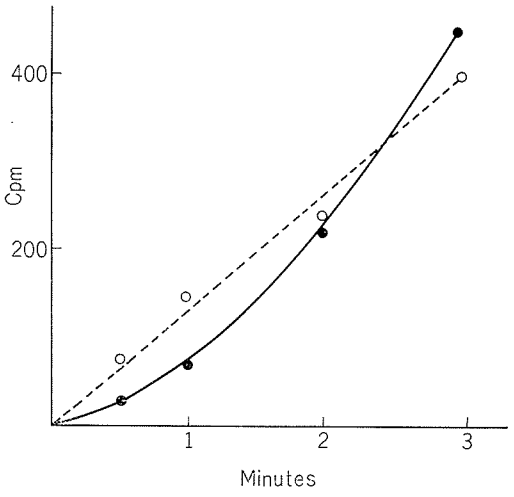

FIGURE 2 Rate of incorporation of L-^{14}C tyrosine into cellular and toxin proteins.
*cpm of specific precipitates per 6 ml culture (—●—)
*cpm of cellular protein per 0.17 ml of culture (—○—)
*Corrected for self-absorption and zero-time counts.

The result is illustrated in Figure 2. As may be seen, radioactive toxin appears in the culture fluid within 30 seconds after the start of pulse-labelling, and increases in amount with time as if the rate of ^{14}C tyrosine incorporation into toxin protein were accelerating. In a similar experiment, attempts were made to measure the amount of radioactive toxin in culture fluid at 10 and 20 seconds after the addition of ^{14}C tyrosine. However, since the differences between the actual radioactive counts and the zero-time counts were very small, it was impossible to obtain reliable values. At any rate, the result at least indicates that the release of toxin protein into the extracellular environment takes place within 30 seconds. A similar rapid extracellular release of diphtheria toxin was suggested by Matsuda and Barksdale (1967) in their system for phage-directed toxin production.

The incorporation of ^{14}C tyrosine into whole cellular protein occurs immediately and quantitatively after the addition of labelled amino acid. As may be seen in the figure, the radioactivity of the protein increases as a linear function of time and the line can be virtually extraporated to zero-time. This indicates that there is no measurable lag period before the start of the incorporation.

Experiments were repeated several times using either ^{14}C tyrosine or ^{14}C leucine and each time gave similar results.

DISCUSSION

The equations (1) and (2), described previously, are based on the assumptions that the number of sites of toxin protein synthesis does not increase during the production and that the incorporation of external amino acids into nascent toxin protein starts immediately after the onset of pulse-labelling.

As reported by HIRARI *et al.* (1966), under similar growth-limiting conditions to those employed here, the curve of accumulation of toxin in culture fluid becomes virtually linear

when the toxin content is plotted against time in terms of flocculation units. Since no growth of either the bacterial population or of the cells themselves takes place under such conditions, the linear increase strongly suggests that the number of the sites of toxin synthesis may be constant during the production, provided that the rate of toxin synthesis is constant at each site.

With regard to the second assumption, the results described in a preceding section clearly indicate that this may be so. Thus, the incorporation of ^{14}C tyrosine or ^{14}C leucine into whole cellular protein took place immediately after the addition of labelled amino acids to the external medium and the radioactivity of the protein increased linearly with time during pluse-labelling. Therefore, as far as the ^{14}C amino acids employed in the experiment are concerned, the concentration of the amino acids in the intracellular pool can be considered to reach equilibrium with the amino acids in the external medium almost immediately.

During 3 min pulse-labelling, radioactive toxin in the culture fluid did not increase linearly but it appeared as if the rate of ^{14}C amino acid incorporation were accerelating (Figs. 1 and 2). This could be predicted on the assumption that the formation of toxin protein molecules from amino acids takes about 3 minutes. As mentioned previously, at any time in toxin production, the neascnt toxin protein can be considered as being comprised of a heterogeneous population of all varieties of precursor polypeptides incorporating amino acids, so that the increase in amount of ^{14}C amino acid incorporated into completed toxin molecules would take place, as a whole, as a linear function of time. Since the extracellular increase of radioactive toxin is expressed as intergral of the linear increase in number of ^{14}C amino acids incorporated, the curve of accumulation of radioactive toxin in the culture fluid should become quadratic by the end of 3 min pulse-labelling. For a similar reason, a similar reciprocal curve would be obtained for the increase in radioactive toxin which occurs for 3 minutes after the application of chase treatment. The result in Figure 1 clearly shows that this is so, thus giving a sigmoidal curve for the increase in radioactive toxin during the 6 minute period. From the above, it seems that, at least under the growth-limiting conditions employed, the average time required for the whole process of toxin production including its synthesis and extracellular release must be about 3 minutes. The time required for toxin production under growing conditions has not yet been measured. However, since the maximal rate (1.0–1.2 Lf/OD·hour) of toxin production was found to be almost constant with the organism employed regardless of its stage of growth (HIRAI *et al.*, 1965; 1966), the time could well be the same as that estimated under growth-limiting conditions.

From the above and the data shown in Figure 2, which indicate that extracellular release of toxin protein takes place within 30 seconds, it also seems that the average time required for the *de novo* synthesis of a complete toxin molecule from amino acids, is not much less than 3 minutes. If this 3 minutes actually means the average time for assembling and releasing the polypeptide chain at the site of toxin synthesis, it is surprisingly long if we compare it with the length of time (about 15 seconds) reported by MCQUILLEN *et al.* (1959) for synthesis of cellular protein molecules in growing *E. coli*. This difference may be due to a difference between the mechanisms for regulation of synthesis in these oranisms or perhaps between the natures of the proteins themselves. In a preliminary experiment, we have recently obtained results suggesting that the average time required for the synthesis of intracellular soluble protein in the PW8 strain of *C. diphtheriae* is 15–30 seconds. It is thus possible that this long period required for synthesis of a protein molecule may represent a unique feature of diphtheria toxin protein or generally of the extracellular proteins such as this toxin. However, this problem requires further precise studies. Incidentally, our re-

cent finding (UCHIDA *et al.*, 1966) indicating the stability of messenger RNA relevant to toxin formation (its half-life was estimated to be about 9–10 minutes at 35°C in the presence of Actinomycin S) may be consistent with this long synthesizing period.

With regard to the occurrence of radioactive toxin in chased culture filtrates, it is of interest to refer to the data obtained by PAPPENHEIMER *et al.* (1962). In kinetic experiments on diphtheria toxin production, they found that a small amount (corresponding to 0.45 Lf/ml) of radioactive toxin was released by washed, ^{35}S methionine-labelled cells when they were resuspended in fresh, unlabelled medium and then incubated under growing conditions. They suggested that the radioactive toxin might have been carried over with the inoculum or been formed from a ^{35}S methionine pool carried over in the inoculum. However, as amply shown by the present study, this radioactive toxin could be formed from nascent toxin protein, which had already been partially labelled with ^{35}S mthionine, using unlabelled amino acids in the fresh medium.

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