Kinetics of Diphtheria Toxin Formation

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SUMMARY

Studies on the kinetics of diphtheria toxin formation in iron-free culture media by variants of the PW no. 8 strain of *Corynebacterium diphtheriae* labelled with ^{14}C -phenylalanine or ^{35}S -methionine, showed that the toxin protein was synthesized *de novo* from amino acids by growing organisms. Release of toxin into the extracellular medium occurred without lysis of more than a minor proportion of the bacterial population.

INTRODUCTION

Diphtheria toxin is only synthesized by strains of *Corynebacterium diphtheriae* which are lysogenic for a particular bacteriophage or one of its mutants (Freeman, 1951; Groman, 1953; Barksdale, 1955) and which are growing under conditions of decreasing bacterial iron content (Pappenheimer, 1955). The toxin is released into the external culture medium as it is formed during the terminal stages of growth, and at any given time, only traces can be extracted from the bacteria themselves (Raynaud, Turpin, Mangalo & Bizzini, 1954). While there seems to be general agreement that the genetic information which controls toxin synthesis is carried by the prophage, the exact relationship between phage and toxin formation has not been established. Barksdale and his co-workers (Barksdale, 1959; Barksdale, Garmise & Horibata, 1960; Barksdale, Garmise & Rivera, 1961) presented evidence which suggests that under certain conditions at least, induction of prophage to the vegetative state by ultraviolet (u.v.) radiation may accelerate the release of toxin and enhance its yield several fold. These authors suggested that under the usual conditions for producing toxin, its formation may be a consequence of 'autoinduction' and phage multiplication resulting from a decreased bacterial iron content. Barksdale *et al.* (1961) observed a steady decline in viable count during toxin production in cultures of a variant of the PW no. 8 strain of *C. diphtheriae*. Unfortunately, this strain has been reported to carry a 'defective' prophage which does not give rise to plaque-forming particles following induction (Barksdale, 1959) so that it was not possible for them to establish a direct relationship between the decrease in viable count and phage multiplication.

From the earlier work by Barksdale & Pappenheimer (1954), Hatano (1956), Yoneda & Pappenheimer (1957), Yoneda (1957*a*), Edwards (1960) and others, it seems most unlikely that the liberation of toxin is associated with the lysis of any significant fraction of the bacterial population. Nevertheless, because of the recent suggestion that phage multiplication may be directly involved in toxin production,

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it seemed to us worthwhile to re-investigate the kinetics of toxin formation as a function of bacterial growth and lysis. In the present work we have followed toxin production by organisms of *C. diphtheriae* PW no. 8 strains labelled with ^{35}S -methionine and ^{14}C -phenylalanine growing in an iron-free unlabelled medium. The results have shown conclusively that toxin is synthesized *de novo* from amino acids and that its liberation into the extracellular medium is not associated to any significant degree with bacterial lysis.

METHODS

Organisms. Variants of the classic Park-Williams strain of *Corynebacterium diphtheriae* were used including the SM-1 strain of Yoneda (1957*b*) and the rough and smooth strains, PW no. 8_r (Pd) and PW no. 8_s (Pd), kindly sent us by Dr W. L. Barksdale.

Culture media and toxin production. The PW no. 8 (Pd) strains were grown on the casein hydrolysate medium of Mueller & Miller (1941). SM-1 was grown on the AMC medium of Yoneda (1957*b*). For toxin production the organisms were grown overnight on media supplemented with $0.1\text{ }\mu\text{g./ml. FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 2% (w/v) maltose to an optical density (OD) at (590 m μ) of 2–2.5. The culture was centrifuged and the bacteria washed with iron-free medium and resuspended to OD = 5–6 in iron-free medium containing 4% (w/v) iron-free maltose as described by Yoneda (1957*b*). In all experiments the organisms were grown on a rotating shaking machine at 33–35°.

Growth was followed by measuring optical density at 590m μ , of samples diluted in distilled water, in a Bausch and Lomb Spectronic 20 and by dry-weight determinations. Dry weight was determined by filtration of suitable samples (5 or 10 ml.) on weighed Millipore filters. The bacteria were washed thoroughly on the filter with distilled water and then dried to constant weight. An OD of 1.0 was found to correspond to 0.47 ± 0.02 mg. dry weight bacteria/ml.

Amino acids. ^{35}S -L-methionine was obtained from Abbot Laboratories; ^{14}C -L-phenylalanine from Oak Ridge Laboratories. Radioactivity was measured in a low background (2.7 counts/min.) windowless gas counter (Nuclear Chicago).

Antitoxin. The rapidly flocculating pepsin-treated horse antitoxin no. 5353A was used. This antitoxin precipitates toxin completely over a broad zone (Pappenheimer & Yoneda, 1957) and has been shown to give only one band of precipitate on immuno-electrophoresis (Raynaud & Relyveld, 1959).

Fumarase was determined spectrophotometrically at 24° by following the rate of fumarate formation from malate at 240 m μ according to the method of Racker (1950).

Coproporphyrin III was determined by adsorption on alumina at pH 5 followed by elution with *N*-HCl as described by Yoneda & Pappenheimer (1957). Porphyrin concentration was calculated from the extinction at 403 m μ , assuming a molar extinction coefficient of 5.3×10^5 (Jope & O'Brien, 1945).

RESULTS

Toxin production and growth

A 500 ml. culture of *Corynebacterium diphtheriae* PW no. 8_r (Pd) was grown overnight on casein hydrolysate medium to OD = 2.5. It was centrifuged, washed twice with iron-free medium and resuspended in 200 ml. iron-free medium containing 4% (w/v) maltose. After a homogeneous suspension had been obtained by shaking at 35° for 45 min., 100 ml. portions were placed in two 1 l. flasks. Samples were removed immediately from each flask and at intervals thereafter for determination of optical density, pH value and dry weight of organisms. Porphyrin and toxin were determined in the bacteria free filtrate. The final yields of toxin in the two flasks after 30 hr. shaking at 35° were 116 and 108 Lf/ml., respectively. Table 1 shows that optical density and bacterial mass, as measured by dry weight, were strictly proportional over the entire 30 hr. period. Coproporphyrin release at a linear rate began almost immediately after the bacteria were suspended in the iron-free medium. Toxin appeared in the filtrate soon afterward and increased at a maximum rate over a 15 hr. period during which the bacterial mass nearly doubled. A net increase in bacterial dry weight of 4.7 mg. was associated with the liberation of approximately 250 µg. toxin protein (about 5% by weight).

Table 1. *Relation of optical density to dry weight during toxin production by Corynebacterium diphtheriae strain PW no. 8_r (Pd)*

Time (hr.)	Optical* density at 540 mμ	Dry weight* (mg./ml.)	Porphyrin* (m-mole × 10 ⁻³ /ml.)	Toxin* (Lf/ml.)
0.75	6.0	3.10	0.019	Not done
3.75	7.6	4.50	0.132	Not done
6.25	11.2	5.89	0.381	5-7.5
10.5	—	7.32	0.587	35
22.25	21.0	10.55	1.21	108
28.25	25.5	11.6	1.13	100
30.75	22.0	12.5	1.38	116

* Figures given average of determinations on duplicate flasks.

Fumarase synthesis during growth on iron-deficient medium

An overnight culture of *Corynebacterium diphtheriae* PW no. 8_r (Pd) was centrifuged, washed with iron-free medium and resuspended in the same medium containing 4% (w/v) maltose to OD = 5.0. The culture was incubated with shaking at 35°, samples were removed at intervals over a 22 hr. period and their OD determined. Samples (3 ml.) were diluted to exactly 15 ml. in 0.02M-phosphate buffer (pH 7.4) and disrupted for 10 min. in the 10 kc Raytheon sonic oscillator. It was observed that as the bacterial iron content decreased in successive samples, the organisms became progressively more fragile and more easily broken by ultrasonic disintegration. After 22 hr. the culture filtrate contained 70 Lf toxin/ml. Figure 1 shows that fumarase, an enzyme which does not contain iron, increased about fivefold during the 22 hr. period in proportion to the increase in bacterial mass as measured by OD. Thus the bacteria were able to form new cellular protein at a

constant differential rate of synthesis during toxin production. This result would hardly be expected if toxin were released either by autolysis or by phage lysis of a major proportion of the bacterial population.

Fig. 1. Fumarase formation in Fe-free medium by *Corynebacterium diphtheriae* PW no. 8_a strain. The final yield of toxin in this experiment was 70 Lf/ml.

Toxin production from ³⁵S-methionine-labelled bacteria

The SM-1 variant of *Corynebacterium diphtheriae* PW no. 8 is exacting for L-methionine. Organisms washed with methionine-free AMC medium (Yoneda, 1957*b*) were inoculated to OD = 0.1 in 200 ml. AMC medium containing 2% (w/v) maltose, 0.1 μ g. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ /ml. and 50 μ g. ^{35}S -L-Methionine (specific activity = 2520 counts/min./ μ g.) per ml. After growth for 15 hr. at 35°, the culture was harvested, centrifuged in the cold and washed twice with chilled iron-free medium containing no methionine. The labelled bacteria were then thoroughly resuspended in 125 ml. iron-free AMC medium containing 4% (w/v) maltose, and 200 μ g. unlabelled L-methionine/ml. The homogeneous bacterial suspension (OD = 4.3) giving 48,000 counts/min./ml. was distributed in 30 ml. amounts into four 300 ml. Erlenmeyer flasks and incubated at 33°. After 1, 2, 4 and 6 hr. of incubation a flask was removed from the shaking machine, the OD measured and the bacteria removed by centrifugation at 5000 rev./min. in the Servall SS-2 for 30 min. After determining

the flocculation titre of the supernatant fluid, the toxin was quantitatively precipitated by antitoxin, from each of two 10 ml. samples. To the first sample the calculated amount of antitoxin was added. To the second sample an amount of purified unlabelled toxin was added sufficient to bring the total to 400 Lf, followed by the addition of 1 ml. antitoxin 5353 AD (440 units/ml.). The flocculation mixtures were placed in the water bath at 40° for 1 hr. and then left overnight in the cold. The specific precipitates were collected by centrifugation and washed with chilled saline until no radioactivity could be detected in the supernatant fluids. The precipitates were then dissolved and made up to exactly 2 ml. in 0.25N-acetic acid and 0.1 ml. samples were placed on planchets, dried and counted.

Table 2. *Toxin production by ³⁵S-methionine-labelled Corynebacterium diphtheriae strain PW no. 8 SM-1 variant*

Time (hr.)	Growth* (OD 590)	Total toxin in 10 ml. sample (Lf)	Unlabelled toxin added (Lf)	Specifically precipitable methionine (counts/min.)	³⁵ S- (μg.)	Total methionine precipitated† (μg.)
1	5.9	50	350	412	0.16	1.77
		50	0	412	0.16	1.77
2	8.3	100	300	800	0.32	3.54
		100	0	368	0.15	3.54
4	10.0	200	200	595	0.24	7.08
		200	0	440	0.17	7.08
6	12.5	350	50	595	0.24	13.2
		350	0	453	0.18	13.2

* Washed bacteria inoculated at time zero to OD = 4.3 and 48,000 counts ³⁵S-methionine/min./ml. into Fe-free medium containing 200 μg. unlabelled L-methionine.

† Calculated, assuming 2.44 μg. toxin protein/Lf and a methionine content of 1.45 %.

The results are given in Table 2. It will be noted that from 10 ml. of culture of ³⁵S-labelled bacteria giving a total of 480,000 counts/min. (190 μg. methionine) only 400–600 counts/min., equivalent to 0.16–0.24 μg. methionine, were specifically precipitable from the supernatant by antitoxin. The methionine content of diphtheria toxin is approximately 1.45 % (Pappenheimer & Yoneda, 1957). Assuming 2.44 μg. protein /Lf, it can be calculated that of the 5 Lf toxin/ml. liberated during the first hour, only 0.45 Lf/ml. or 9 % was labelled. This amount of toxin might well have been preformed and carried over with the inoculum, or formed from an ³⁵S-methionine pool carried over with the inoculum. Over the next few hours, there was little or no increase in the specifically precipitable ³⁵S and of the 35 Lf toxin/ml. released during the entire 6 hr. period only 2 % or less contained the label.

Toxin production from ¹⁴C-labelled bacteria

The SM-1 variant of *Corynebacterium diphtheriae* PW no. 8 strain does not require phenylalanine for its growth. However, in the presence of an excess of ¹⁴C-phenylalanine, bacterial synthesis of this amino acid is repressed and the organisms take up, during growth, an amount of ¹⁴C equivalent to a phenylalanine content of about 2.5 %.

A culture of the SM-1 strain was inoculated to OD = 0.1 in 400 ml. AMC medium containing 0.1 μg. FeSO₄·7H₂O/ml., 2 % (w/v) maltose and 24 μg. ¹⁴C-L-phenylalanine

(specific activity = 350 counts/min./ μ g.)/ml. After shaking for 16 hr. at 34°, the culture had reached OD = 1.9. It was centrifuged and washed once with 100 ml. iron-free AMC medium containing no phenylalanine. The washed organisms were homogenized and resuspended in chilled iron-free medium containing 4% (w/v) maltose and divided into two equal portions, A and B, of 86 ml. each. To A was added 3 ml. of 0.8% (w/v) ^{14}C -L-phenylalanine (350 counts/min./ μ g.) and the culture distributed into four 125 ml. Erlenmeyer flasks: 25 ml. in A1 and A2, 20 ml. in A3 and A4. To B was added 3 ml. of 1% (w/v) unlabelled phenylalanine and the culture distributed as in series A. All eight flasks were placed on the shaking machine and incubated at 34°. At 1, 2, 4 and 7 hr., one flask from each series was removed. After determining the OD, duplicate 0.5 ml. samples were filtered through Millipore filters and the bacteria washed thoroughly on the filter with ice-cold 5% (w/v) trichloroacetic acid containing 150 μ g. unlabelled phenylalanine/ml. The filters were glued to planchets, dried and counted. The remainder of each culture was centrifuged at 5000 rev./min. for 20 min. in the cold and the supernatant fluid filtered through a Millipore filter. Duplicate 0.1 ml. samples of filtrates from series B were placed on planchets, dried and counted. Flocculation tests on the 4 hr. filtrates showed 10 Lf/ml. (A3 and B3) and at 7 hr., 22 Lf/ml. (A4 and B4).

Table 3. *Growth and lysis of bacteria labelled with ^{14}C -phenylalanine in Fe-free medium containing phenylalanine*

Organisms: *Corynebacterium diphtheriae*, PW no. 8 strain, SM-1 variant

Flask no.*	Time (hr.)	Bacterial growth (mg. dry wt./ml.)†	Bacterial ^{14}C		Supernate‡ (Counts/min./ml.)
			(Counts/min./ml.)	(Counts/min./mg. dry wt.)	
A ₀	0	(2.0)§	(7400)	(3700)	94,000
B ₀		(2.0)	(7400)	(3700)	(59)
A ₁	1	2.40	9970	4170	—
B ₁		2.40	7225	3000	107
A ₂	2	2.86	11,000	3850	—
B ₂		2.86	7030	2460	151
A ₃	4	3.41	15,000	4400	—
B ₃		3.41	6630	1940	261
A ₄	7	4.14	19,300	4650	—
B ₄		4.14	6630	1600	392

* A, 270 μ g. ^{14}C -L-phenylalanine (9.4×10^4 counts/min./ml.)/ml. B, 340 μ g. unlabelled L-phenylalanine/ml.

† Calculated from OD assuming 0.47 mg. dry wt./OD unit.

‡ Corrected for self absorption; actual count 32% lower.

§ Zero time values in parentheses by extrapolation.

Table 3 shows that from a bacterial inoculum containing 7400 counts/min./ml., only 285 counts/min./ml. (corrected for self absorption) were released into the supernatant fluid during the 6 hr. which elapsed between the first and last samples in series B, equivalent to the lysis of less than 4% of the initial bacterial suspension. In other words, if toxin were released by lysis of the initial bacterial suspension, the 55 μ g. unlabelled toxin released into the external medium during the course of the experiment (see Table 4) must have been derived from only 80 μ g. labelled organisms. Toxin, therefore, is not a product of cell lysis.

Toxin was precipitated quantitatively and specifically from duplicate 10 ml. samples of culture filtrates from flasks incubated for 1 and 2 hr. and from duplicate 5 ml. samples from the 4 and 7 hr. filtrates. An amount of purified unlabelled toxin (370 Lf/ml.; 2.5 μ g. protein/ml.) was added to each sample so as to bring the total Lf content to approximately 200 Lf, and then 200 units of antitoxin added. In the case of the 7 hr. filtrates (A4, B4) unlabelled toxin was added only to one sample: the other 5 ml. sample was precipitated by 100 units of antitoxin without blending. The flocculation mixtures were placed in the water bath at 40° for 1 hr. and then left overnight in the cold. They were centrifuged and washed 3 times with chilled saline containing 0.1 % (w/v) unlabelled phenylalanine. The washed floccules were suspended in 1 ml. saline and the precipitate completely dissolved by addition of

Table 4. *Toxin production by bacteria labelled with ^{14}C -phenylalanine*

Organism: *Corynebacterium diphtheriae*, strain PW, no. 8, SM-1 variant. The inoculum was equiv. 2.1 mg. dry wt. ^{14}C -bacteria/ml. giving 7400 counts/min./ml. in Fe-free medium. A, 270 μ g./ml. ^{14}C -phenylalanine (9.4×10^4 counts/min./ml.) B, 340 μ g./ml. unlabelled phenylalanine.

Time (hr.)	Bacterial growth (mg. dry wt. organism/ml.)	Total toxin (Lf/ml.)	Specifically precipitable ^{14}C (counts/min./ml.)		^{14}C -Toxin* (Lf/ml.)	
			A	B	A	B
1	2.40	—	19	2.3	0.9	0.11
2	2.86	—	78	3.5	3.7	0.17
4	3.41	10	217	6.9	10.3	0.33
7	4.14	22	458	6.9	21.8	0.33

* Calculated assuming 21 counts/min./Lf.

2.5 ml. cold 5 % (w/v) trichloroacetic acid containing 150 μ g. unlabelled phenylalanine/ml. After a few minutes at room temperature, the precipitate reformed and after a further 30–60 min. was collected on Millipore filters, washed with 5 % (w/v) trichloroacetic acid, dried and counted. The results given in Table 4 are averages of duplicate determinations agreeing within 5 % in every case. From the radioactivity specifically precipitable by antitoxin from the 4 and 7 hr. samples, it is easily calculated that the toxin formed in the A series contained 21 counts/min./Lf. Assuming that phenylalanine synthesis was completely repressed by the excess labelled phenylalanine, a specific activity of 350 counts/min./ μ g. phenylalanine and 2.44 μ g. protein/Lf, the phenylalanine content of diphtheria toxin is calculated to be 2.5 %. (The concentration of ^{14}C -phenylalanine (24 μ g./ml.) used to prepare the labelled inoculum was sufficient to repress bacterial synthesis of this amino acid by only 40–50 %. The presence of a large excess of phenylalanine in the iron-free medium, giving almost complete repression, accounts for the increasing ^{14}C content of the bacteria in series A during the course of toxin production; column 5, Table 3). From the counts specifically precipitated from A1 and A2 filtrates, the toxin synthesized after growth for 1 and 2 hr. is calculated to be 0.9 and 3.7 Lf/ml., respectively.

Turning to the B series, we find that from a bacterial inoculum containing 7400 counts phenylalanine/min./ml., only 2.3 counts specifically precipitable ^{14}C /min./ml. was released into the culture filtrate during the first hour of growth. This small

amount of labelled toxin (equivalent to only 0.1 Lf in series A) might well have been transferred with the inoculum. After 7 hr. the culture filtrate still contained only 6.9 counts specifically precipitable $^{14}\text{C}/\text{min.}/\text{ml.}$ Thus, at most, 1.5–3 % of the toxin released during the experiment was labelled, and the remainder must have been synthesized from unlabelled phenylalanine by bacteria actively growing on the iron-free medium. This result agrees well with that obtained in the previous experiment with bacteria labelled with ^{35}S -methionine.

DISCUSSION

It is clear from the results presented that in an iron-free culture medium, diphtheria toxin is produced by growing bacteria. During the period when toxin is being released, the bacterial mass increases several fold, whether measured by optical density, dry weight or by the synthesis of a protein enzyme, fumarase. Whether or not the fourfold to fivefold increase in cell mass observed in these experiments or in those of others (Pope & Healy, 1933; Pappenheimer, 1947; Raynaud, Alouf & Mangalo, 1959; Edwards, 1960) is accompanied by actual multiplication of bacteria remains uncertain. Nishida (1954) and Barksdale *et al.* (1961) reported that the viable count decreased to 20 % or less while toxin was being formed. Edwards (1960), on the other hand, reported an experiment in which the number of viable bacteria increased fivefold during toxin formation, although in other experiments he was unable to obtain any consistent relationship between viable count and toxin yield. It should be stressed that the tendency of *Corynebacterium diphtheriae* strain PW no. 8, (Pd) to form clumps at the high population densities required (about 10^{10} organisms/ml.) makes reproducible and reliable viable counting difficult.

Barksdale *et al.* (1961) showed that when u.v.-irradiated cultures of toxigenic strains were placed in media of relatively low iron content, toxin release began somewhat earlier than in unirradiated cultures. They suggested that under the usual conditions for toxin production, as the bacterial iron content is decreased, phage multiplication is induced (autoinduction) and lysis of a proportion of the bacteria takes place. Whether or not a low bacterial iron content induced the change from prophage to vegetative phage, the present studies show conclusively that lysis occurs in too small a fraction of the bacteria to account for the amount of toxin released. In an experiment with bacteria labelled with ^{14}C -phenylalanine, producing toxin in a medium containing unlabelled phenylalanine, less than 4 % of the label, equivalent to only 80 $\mu\text{g.}$ dry wt. bacteria, was found in the supernatant fluid at a time when 55 $\mu\text{g.}$ unlabelled toxin had been liberated. It should be recalled that Yoneda & Pappenheimer (1957) were unable to detect appreciable amounts of nucleic acid in culture filtrates of *Corynebacterium diphtheriae* strain C7 (β) until after toxin production was maximal and bacterial growth had ceased. When bacteria labelled with either ^{35}S -methionine or ^{14}C -phenylalanine are placed in iron-free media containing unlabelled amino acids, the toxin formed is unlabelled except for a small fraction (probably preformed) released during the first hour. Thus diphtheria toxin is synthesized *de novo* from amino acids as has been shown to be the case with the β -galactosidase of *Escherichia coli* following addition of inducer (Hogness, Cohn & Monod, 1955).

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