JOURNAL OF BACTERIOLOGY, July 1968, p. 61-69
Vol. 96, No. 1

Response of Cultured Mammalian Cells to Diphtheria Toxin

III. Inhibition of Protein Synthesis Studied at the Subcellular Level

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Received for publication 13 March 1968

Diphtheria toxin inhibited protein synthesis in intact KB cells. The action of the toxin upon the cell did not result in disaggregation of polyribosomes, or in impairment of their ability to function in protein synthesis. A reduction in single ribosomes and a concomitant increase in polyribosomes did result from the action of toxin. Nascent peptides were not cleaved from polyribosomes by the action of toxin, but treatment of fully intoxicated cells with puromycin resulted in cleavage of these peptides, and caused accelerated polyribosome breakdown. Our data indicated that the toxin must enter the cell to exert its effect. The component or components sensitive to toxin were localized in the 100,000 $\times$ g supernatant fraction of cytoplasmic extracts. When extracts from intoxicated cells were treated with nicotinamide, a significant proportion of their capacity to synthesize protein was restored. The specificity of this reaction suggested that nicotinamide adenine dinucleotide is involved in the action of toxin in the intact cell, and that one component inactivated by toxin is soluble transferase II.

Diphtheria toxin inhibits protein synthesis in intact sensitive cells (2, 9, 12, 14, 16) and in cell-free systems prepared from mammalian cells (3, 4, 6, 10). Significant progress has been made toward establishing the requirements for this action and for describing the actual mechanism of inhibition in cell-free systems (3, 4, 6–8, 14). Recent work of Collier (3) and of Goor et al. (6, 8) has shown that diphtheria toxin in the presence of nicotinamide adenine dinucleotide (NAD) inactivates soluble transferase II, thus preventing the incorporation of amino acids into polypeptide chains in cell-free systems. Although the mode of action may be the same in the intact cell, this has not been confirmed previously, nor has it been established that the action of the toxin in the intact cell is dependent upon NAD. On the basis of the calculated number of toxin molecules required per cell to inhibit protein synthesis fully, it has been proposed that the mode of action in intact cells could not be the same as it is in cell-free systems (8).

We have studied the inhibition of protein synthesis by toxin in intact cells, in order to elucidate the steps involved and to provide a basis for comparing this action in the two systems. The transport and accumulation of amino acids and their incorporation into protein in toxin-treated cells have been described (12). In this paper, we are reporting our examination of the subcellular components involved in protein synthesis in normal and intoxicated cells.

Experiments were designed to determine whether toxin disrupted either the structure or function of polyribosomes or other components associated with them in protein synthesis. Components of normal and intoxicated cell extracts were tested for their ability to function in a cell-free system. The ability of nicotinamide, reported by Goor et al. (8), to reverse the inhibition of protein synthesis in intoxicated cell-free systems was examined with extracts from intoxicated cells.

Our results show that the responses to toxin of the intact cell and cell-free system are identical in several respects. In addition, indirect evidence has been provided for the involvement of NAD in toxin action in the intact cell, and we conclude that diphtheria toxin enters into an NAD-mediated complex with transferase II. Further, we suggest that the inactivation of transferase II occurs within the cell and is not related to any essential function associated with the cell membrane.

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MATERIALS AND METHODS

Cell cultures. KB, human epidermoid carcinoma cells were obtained from Microbiological Associates, Inc., Bethesda, Md., and Grand Island Biological Co., Grand Island, N.Y. They were grown in antibiotic-free Eagle's minimum essential medium (MEM) containing 10% calf serum, in milk dilution bottles, as previously described (12).

Diphtheria toxin. Purified, five times recrystallized diphtheria toxin, lot RX 7328 (10 MLD/LF; 1 LF is equal to 3.1 μg of protein) was used in all experiments. This toxin was received from D. C Edwards, Wellcome Research Laboratories, Beckenham, England.

Reagents. Adenosine triphosphate, dipotassium salt (ATP), guanosine triphosphate (GTP), creatine phosphate (CP), creatine phosphokinase, and bovine pancreatic ribonuclease (five times crystallized) were obtained from Calbiochem, Los Angeles, Calif. Puromycin-dihydrochloride and 2-mercaptoethanol were supplied by Sigma Chemical Co., St. Louis, Mo. Nicotinamide was obtained from General Biochemicals, Chagrin Falls, Ohio.

Radioactive compounds. The following labeled compounds were used; purified ^{14}C-amino acid mixture (15 amino acids, 100 μc per ml) and ^{14}C-L-valine (190 mc per mmole). Both were from New England Nuclear Corp., Boston, Mass.

Intoxication of cells. Log-phase cultures, 48 hr old, were used in all experiments. When extracts from intoxicated and normal cells were compared, by sucrose gradient analysis or in cell-free systems, cell sheets were washed twice in warm Hanks' balanced salt solution (HBSS) and were suspended in serum-free MEM or MEM containing amino acids at 1:20 the normal concentration. Cell concentration was from 6.5×10^5 to 7.5×10^5 cells per ml. Equal portions of the cell suspensions were distributed to capped Erlenmeyer flasks which were placed in a water-bath shaker. All procedures were carried out at 36 C. After the cells had equilibrated for 20 min, a saturating concentration of diphtheria toxin and ^{14}C-labeled amino acids, when required, were added to the appropriate cultures. [A saturating level of toxin in cell cultures is defined as the lowest concentration of toxin which will produce a detectable effect in the minimum time. A saturating concentration of toxin for KB cells is 10 guinea pig MLD per ml (12).] The cultures were incubated for various periods, as described for each experiment.

Even with the standardized conditions indicated above, there may be a difference of several minutes, from one experiment to another, between the time of the first detectable inhibition of protein synthesis and complete inhibition (12). So that we might know exactly from which stage of intoxication samples were obtained, a "shut-off curve" was run with each experiment. Small samples of ^{14}C-labeled intoxicated and normal cells, to be used in gradient analyses or cell-free studies, were removed at intervals during the incubation period. These points were then plotted as incorporation of radioactive amino acids into protein over time (*see* Fig. 1d) to show the progressive intoxication of the cells in each experiment. At the end of the incubation period, the cultures were chilled in an ice bath and transferred to cold centrifuge tubes for further processing.

When normal cell extracts were needed for use in cell-free systems, the log-phase cultures were washed with and suspended in chilled HBSS for further processing.

Preparation of subcellular fractions. The chilled cells, in suspension, were collected by centrifugation at $150 \times g$ for 10 min. They were washed twice in cold HBSS, containing an excess of nonradioactive amino acid corresponding to the ^{14}C-amino acid used in the labeling procedure. The cells were then suspended in a hypotonic buffer (HB) containing 0.01 M tris(hydroxymethyl)aminomethane (Tris)-chloride (*p*H 7.4), 0.01 M KCl, 0.0015 M magnesium acetate, and 0.006 M 2-mercaptoethanol. The cells were then disrupted with 3 to 5 strokes of a tight-fitting, all-glass Dounce homogenizer. When extracts were to be used in cell-free systems, enough 1.25 M sucrose in HB to give a final concentration of 0.25 M sucrose was added. The resulting homogenate was always a product of 40 million cells per ml. This homogenate was then centrifuged at $800 \times g$ for 10 min. The $800 \times g$ supernatant fluid, containing sucrose, was centrifuged again at $13{,}000 \times g$ for 15 min. Ribosomes were prepared by centrifuging the $13{,}000 \times g$ supernatant fluid at $100{,}000 \times g$ for 1 hr. The $100{,}000 \times g$ supernatant fluid was removed and stored in ice. The pellets were rinsed twice with medium A [0.25 M sucrose, 0.05 M Tris-chloride (*p*H 7.5), 0.025 M KCl, 0.005 M magnesium acetate] and then were resuspended gently in this medium for further purification. This was accomplished by centrifugation through 10% sucrose in the same buffer solution as medium A, at $100{,}000 \times g$ for 3 hr. This pellet was then combined with supernatant fractions for use in cell-free incorporation studies.

Density gradient analysis. To obtain ribosomal sedimentation patterns by density gradient centrifugation, the $800 \times g$ supernatant fractions were treated with sodium deoxycholate (final concentration, 0.5%), and 0.3-ml samples were layered onto 15 to 30% (w/w) linear sucrose gradients in Spinco SW-39 tubes. The gradient medium contained 0.01 M Tris-chloride (*p*H 7.4), 0.01 M KCl, and 0.0015 M magnesium acetate. The gradients were centrifuged at either 25,000 or 28,000 rev/min for 2 hr in a Spinco model L ultracentrifuge.

After centrifugation, the bottoms of the tubes were punctured and two-drop fractions were collected. The fractions were brought to 1 ml with distilled water and the optical densities (OD) at 260 and 235 mμ were recorded. If the cells had been labeled with ^{14}C-amino acids, the distribution of radioactive protein among the fractions was then determined. Bovine serum albumin (500 μg) was added to the samples; each sample was then precipitated with trichloroacetic acid at a final concentration of 5%. After at least 1 hr at 4 C, fractions were heated at 90 C for 15 min, collected on filter discs, washed three times with 5% trichloroacetic acid, and dried. The dried discs were placed in scintillation vials with 15 ml of a solution of 0.5% 2,5-diphenyloxazole (PPO) and 0.03% 1,4-

bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) in toluene and counted in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) with a counting efficiency of 84% for ^{14}C. The specific activity (counts/min per OD 260) was calculated and compared for samples within each experiment.

Amino acid incorporation by cell-free preparations. The amino acid incorporating systems contained either 0.4 ml of the 13,000 × *g* supernatant fluid or 0.4 ml of the recombined 100,000 × *g* supernatant fluid and washed ribosomes per ml of reaction mixture. Reaction mixtures contained a standard amount of ribosomal and supernatant material, verified by measurement of the OD of these fractions at 260 and 280 mμ. The 13,000 × *g* extracts contained from 5.5 to 7.5 mg of protein per ml. The 100,000 × *g* supernatant extracts contained from 1.15 to 1.40 mg of protein per ml, and the washed ribosomes contained from 0.60 to 0.75 mg of protein per ml when resuspended in a volume of buffer equal to the volume of extract from which they were centrifuged. The complete reaction mixture also contained 50 μmoles of Tris-chloride buffer (*p*H 7.5), 5 μmoles of magnesium acetate, 50 μmoles of KCl, 6 μmoles of 2-mercaptoethanol, 1 μmole of ATP, 0.25 μmole of GTP, 10 μmoles of CP, 120 μg of creatine phosphokinase, and 2 μc of ^{14}C-L-valine, supplemented with 0.05 μmole per ml of each of 19 other amino acids. All components of the system, except the extracts, were combined in the cold and the reaction was initiated by addition of the extract. Samples were incubated at 36 C, and 0.3-ml portions were collected in 7% perchloric acid containing 20 mg of Casamino Acids per ml. The samples were processed for radioactive counting as reported elsewhere (11).

RESULTS

To determine whether toxin caused any structural alteration of the polyribosomes and to investigate the fate of nascent peptides attached to polyribosomes in intact, toxin-treated cells, sucrose gradient profiles of extracts prepared from intoxicated and normal cells were examined. Figure 1 shows the gradient profiles and associated activity obtained when three replicate cultures of toxin-treated cells were sampled at different times during the course of intoxication (i.e., inhibition of protein synthesis). The 25-min sample appeared as a normal cell profile. The 50-min sample represented middle to late intoxication, and the 70-min sample was obtained when inhibition of protein synthesis was complete (*see* Fig. 1d). The amount of polyribosomal material, found in fractions 5 through 25, increased and the amount of single ribosomes, mostly in fractions 29 through 33, decreased as intoxication proceeded to completion. The radioactivity associated with the polyribosomes did not decrease, indicating that there was no cleavage of nascent peptides.

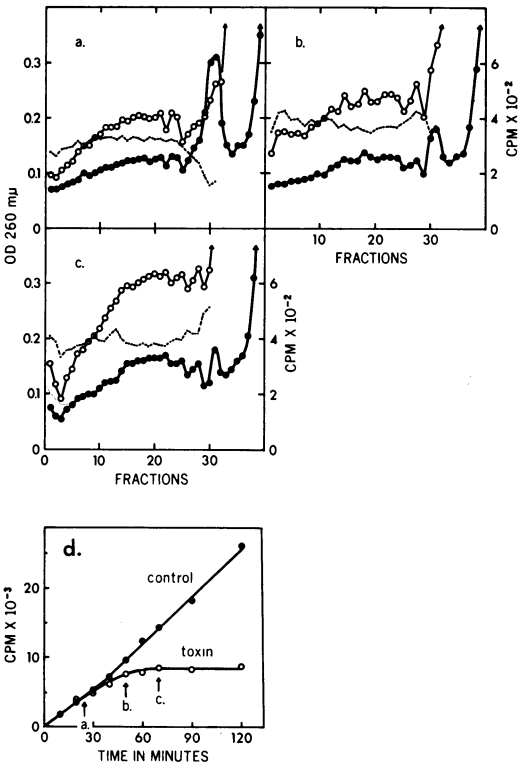

FIG. 1. *Sucrose gradient profiles of progressively intoxicated KB cells. Three suspension cultures were prepared as described. At zero-time, diphtheria toxin was added to a final concentration of 15 MLD per ml and ^{14}C-valine was added to a concentration of 0.33 μc per ml. The incubation was terminated: (a) after 25 min; (b) after 50 min; and (c) after 70 min. Cell extracts were prepared, and 800 × g supernatant fluid was layered on 15 to 30% sucrose gradients. Gradients were centrifuged for 2 hr at 25,000 rev/min. Symbols: ●, OD at 260 mμ; ○, counts/min of ^{14}C-valine incorporated; dotted and dashed curve, specific activity, counts/min per OD 260. Specific activity is read from the radioactivity scale × 10^{-3}. Direction of sedimentation is from right to left. (d) The shutoff curve for this experiment (Fig. 1a, b, c) shows the progressive inhibition of protein synthesis in the cells.*

In another experiment (Fig. 2), replicate cell cultures were incubated with ^{14}C-valine in the presence and absence of toxin until incorporation of amino acids into protein was stopped by the toxin. One intoxicated and one control sample were then treated with puromycin. The profiles obtained confirmed the ribosome-polyribosome shift in intoxicated cells. Nascent peptides were not released from polyribosomes by the action of the toxin. Further, it was noted that puromycin could cleave peptides from the polyribosomes of these fully intoxicated cells. One control culture

(Fig. 2e) was treated with an excess of ^{12}C-valine to "chase" activity from the nascent peptides. In this case, and when cells were treated with puromycin for only brief periods, it was observed that an increasing amount of radioactivity not removed by puromycin appeared in the material which sedimented slightly ahead of the single ribosomes, probably indicating incorporation of ^{14}C activity into ribosomal protein. This increase became more apparent with longer labeling times.

Inhibition of protein synthesis began some minutes prior to the 60-min sampling time; thus, comparison of intoxicated cell profiles with those of control cells, which continued to incorporate linearly for 60 min, may be misleading. The higher ^{14}C activity in the polyribosome region of control cell extracts could be interpreted to mean peptides were cleaved in intoxicated cells. To clarify this point, we designed experiments in which the gradient profiles of toxin-treated samples incubated for 50 min were compared with those of control samples incubated for 30 and 50 min. The activity found in the nascent peptides of the toxin-treated sample was less than that of the 50-min control and slightly greater than that of the 30-min control. A decreased rate of incorporation of ^{14}C-amino acids by intoxicated cells was first observed between 20 and 25 min. Inhibition of incorporation was complete by 50 min. Little activity was incorporated into nascent peptides of intoxicated cells after 30 min.

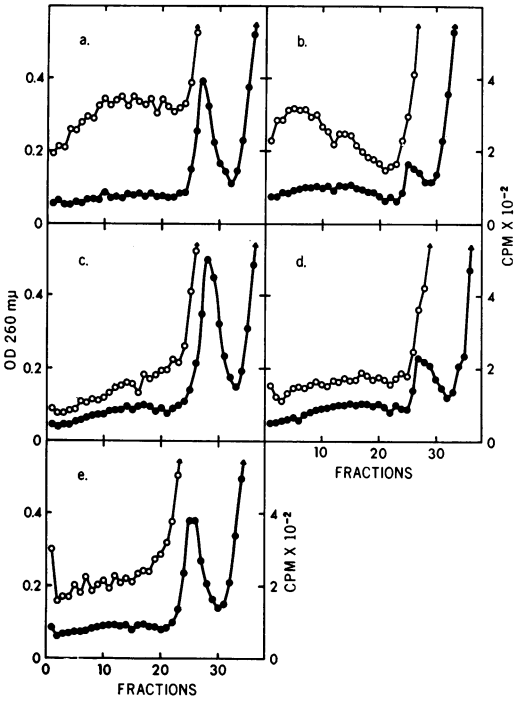

FIG. 2. *Sucrose gradient profiles reflecting the effects of toxin and puromycin on polyribosomes in intact KB cells. At zero-time, five suspension cultures received ^{14}C-valine to equal 0.27 μc per ml. Cultures (b) and (d) received toxin to a final concentration of 15 MLD per ml. After incubation for 60 min, the cultures were treated as follows: (a) control, incorporation terminated; (b) toxin, incorporation terminated; (c) puromycin, puromycin-HCl was added to a final concentration of 10^{-4} M and incubation was terminated after 30 sec; (d) toxin plus puromycin, puromycin was added to 10^{-4} M and incubation was terminated after 30 sec; (e) a 200-fold excess of ^{12}C-valine was added and incubation was terminated after 15 min. In each case, incubation was terminated by rapid chilling of the cell suspension in an ice bath. Cell extracts were prepared, and 800 × g supernatant fluid was layered on 15 to 30% sucrose gradients. Gradients were centrifuged for 2 hr at 28,000 rev/min. Symbols: ●, OD at 260 mμ; ○, counts/min of ^{14}C-valine incorporated.*

Accelerated polyribosome breakdown, as well as cleavage of peptides, occurs in normal cells incubated with puromycin for longer periods of time (19). We tested the ability of puromycin to carry out this function in a fully intoxicated population of KB cells (Fig. 3). Duplicate sets of toxin-treated and control cultures were incubated for 60 min in the presence of ^{14}C-valine; one set was then incubated for 10 min in the presence of puromycin. In both puromycin-treated cultures, puromycin caused accelerated breakdown of polyribosomes, an increase in single ribosomes, and removal of a significant amount of activity from the polyribosome region. The specific activities calculated from these data make it clear that the loss of activity from this region reflects not only a loss of polyribosomes, but a stripping of labeled peptides as well.

To obtain further information regarding the toxin-sensitive component or components of the intact cell, cell-free systems were developed from KB cells and their response to toxin was investigated. First, the normal system was tested to evaluate its performance and its response to different levels of toxin added directly to the in vitro system. This provided a basis for comparing the sensitivity of the KB system with that of the HeLa and reticulocyte systems (4, 6, 8), and for evaluating the activity of the toxin. The time course of amino acid incorporation by the system in the presence and absence of 100 MLD per ml of toxin, together with the effect of four other toxin concentrations (0.1 to 500 MLD per ml), is shown in Fig. 4. A concentration-dependent inhibition of amino acid incorporation was evident. Saturation was reached at a concentration of toxin between 10 and 100 MLD per ml. A concentration of 500 MLD per ml did not further

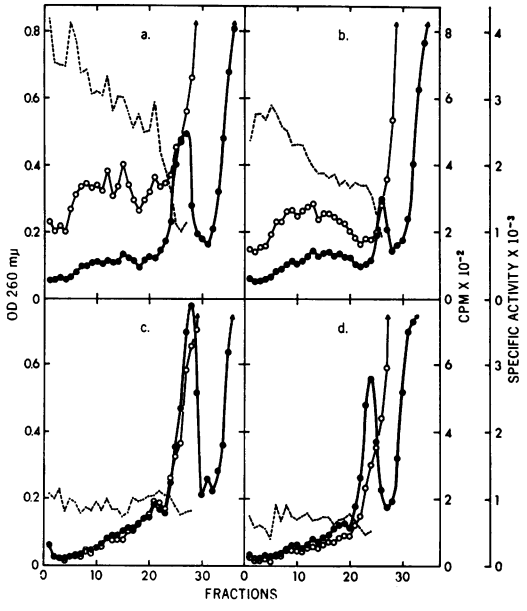

FIG. 3. *Effects of 10-min incubation with puromycin on normal and intoxicated KB cells. At zero-time, four suspension cultures received ^{14}C-valine to equal 0.34 μc per ml. Cultures (b) and (d) received toxin to a concentration of 15 MLD per ml. After an incubation period of 60 min, cultures were treated as follows: (a) control, incubation was terminated; (b) toxin, incubation was terminated; (c) puromycin, puromycin was added to 10^{-4} M and incubation was terminated after 10 min; (d) toxin plus puromycin, puromycin was added to 10^{-4} M and incubation was terminated after 10 min. Cell extracts were prepared and 800 × g supernatant fluid was layered on 15 to 30% sucrose gradients, which were centrifuged for 2 hr at 28,000 rev/min. Symbols: ●, OD at 260 mμ; ○, counts/min of ^{14}C-valine incorporated; dotted and dashed curve specific activity, counts/min per OD 260.*

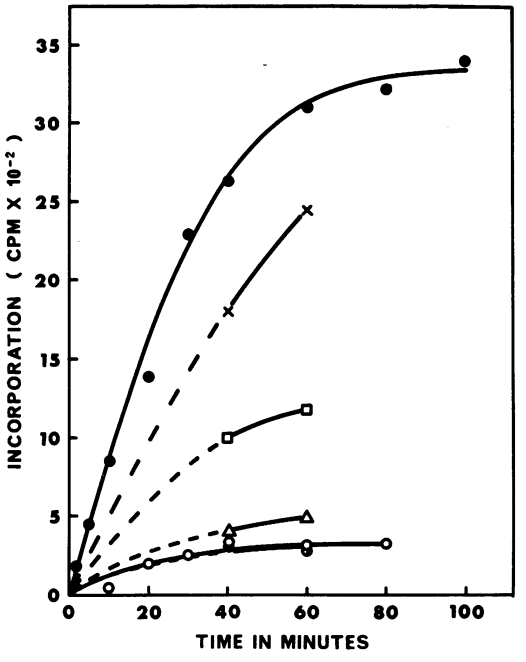

FIG. 4. *Effects of toxin on incorporation of ^{14}C-valine by cytoplasmic extracts from KB cells. Crude extracts of normal KB cells were prepared as described. All components of the cell-free system, except the extract, were combined in the cold. The reaction was initiated by addition of the extract. Samples were incubated at 36 C, and 0.3-ml samples were collected at the times indicated. Toxin was included in the reaction mixture at concentrations of: 0.1 MLD per ml, ×; 1.0 MLD per ml, □; 10.0 MLD per ml, △; 100.0 MLD per ml, ○; 500.0 MLD per ml, ◑; control, ●. The dashed lines are extrapolations to zero-time and can be used to estimate the rate at which amino acid incorporation decreases with increasing concentrations of toxin. The values plotted are counts/min per 0.3 ml of complete reaction mixture.*

reduce the rate of incorporation, and complete inhibition was not achieved. The per cent inhibition at each toxin concentration has been calculated for the 40- and 60-min values (Table 1). The effect of puromycin, ribonuclease, and the omission of the energy-generating system upon amino acid incorporation is also shown in Table 1. These reaction mixtures were part of the experiment described in Fig. 4.

This system was then used to evaluate the ability of washed ribosomes and supernatant fractions from fully intoxicated cell populations to participate in the incorporation reaction. Although toxin did not cause polyribosome breakdown or release nascent peptides from polyribosomes, it was still possible that functioning of the polyribosomes was impaired in some less obvious manner. To investigate this as well as the effect of toxin on supernatant components, fractions from toxin-treated and control cells were recombined. Cells were incubated for 60 min in the presence and absence of 15 MLD per ml of toxin. The shut-off curve showed complete inhibition of protein synthesis after 45 min. Washed ribosomes and supernatant fractions were prepared, combined, and incubated in reaction mixtures as described. Supernatant fractions from intoxicated cells were tested for the presence of toxin by intracutaneous injection into rabbit skin, and on KB monolayers. No detectable toxin activity was found in either case. Figure 5 presents the data obtained for both homologous and heterologous systems, as well as for incubation of ribosomes without supernatant fractions. It had been determined previously that supernatant fractions alone

TABLE 1. *Effect of various inhibiting agents on incorporation of ^{14}C-valine by cytoplasmic extracts of KB cells*[a]

Inhibitors	40 min		60 min	
	Counts/min	Per cent inhibition	Counts/min	Per cent inhibition
Control	2,635	—	3,100	—
Toxin				
0.1 MLD/ml	1,805	32	2,453	21
1.0 MLD/ml	1,002	62	1,179	62
10.0 MLD/ml	408	84	472	85
100.0 MLD/ml	339	87	320	90
500.0 MLD/ml	309	88	280	91
Puromycin (10^{-4} M)	72	97	—	—
Ribonuclease (10.0 μg/ml)	30	99	—	—
No energy[b]	36	99	24	99

[a] Cell-free system as described for Fig. 4.
[b] ATP, GTP, CP, and creatine phosphokinase were omitted.

could not incorporate amino acids. The supernatant fraction from intoxicated cells was inactive. The ribosomal fraction from intoxicated cells showed increased incorporation in all cases when combined with normal supernatant fractions. We attribute this to the buildup of polyribosomes in intoxicated cells. Ribosomes from control or intoxicated cells, with no supernatant fraction added to the reaction mixture, showed a low but consistent incorporation.

It has been shown that the inhibition of peptide synthesis in cell-free systems intoxicated with diphtheria toxin plus NAD can be reversed by the addition of nicotinamide (8). It was found that this restorative effect could not be demonstrated in intact, fully intoxicated HeLa cells (8). We have been unable to demonstrate it in intact KB cells. To learn more about the similarities of toxin action in intact cells and cell-free systems, we tested the ability of nicotinamide to reverse the inhibitory effect of toxin upon extracts from intoxicated cells. Crude extracts were prepared from normal KB cells and cells which had been incubated for 70 min with 15 MLD per ml of toxin. The cells were thoroughly washed after intoxication, and extracts were prepared as described in Materials and Methods. The extracts were then tested for incorporation of ^{14}C-valine in cell-free systems with and without 8×10^{-3} M nicotinamide. Table 2 shows the results of such an experiment. Extracts of intoxicated cells were inhibited from 85 to 94% in their ability to incorporate ^{14}C-valine. Extracts of intoxicated cells

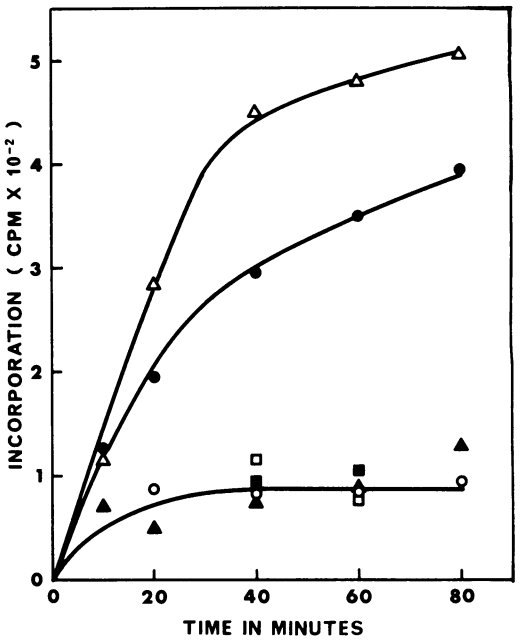

FIG. 5. *Incorporation of ^{14}C-valine by ribosomes and supernatant fractions from normal and intoxicated KB cells. Ribosomal and supernatant fractions were prepared as described, from normal and fully intoxicated cells. These fractions were recombined as indicated and reacted in cell-free system as described for Fig. 4. Symbols: ●, normal ribosomes, normal supernatant fractions; ○, normal ribosomes, intoxicated supernatant fractions; △, intoxicated ribosomes, normal supernatant fractions; ▲, intoxicated ribosomes, intoxicated supernatant fractions; □, normal ribosomes alone; ■, intoxicated ribosomes alone.*

TABLE 2. *Effect of nicotinamide on incorporation of ^{14}C-valine by extracts of normal and intoxicated KB cells*[a]

Extract	40 min		60 min		90 min	
	Counts/min	Per cent inhibition	Counts/min	Per cent inhibition	Counts/min	Per cent inhibition
Normal extract	1,091	—	1,205	—	1,229	—
Normal extract + nicotinamide, 8×10^{-3} M	1,212	—	1,276	—	1,336	—
Intoxicated extract[b]	13	99	67	94	184	85
Intoxicated extract + nicotinamide, 8×10^{-3} M	393	68	675	47	928	31

[a] Cell-free system as described for Fig. 4. Above values are the average of duplicate samples.
[b] KB cells were incubated for 70 min in the presence of 15 MLD per ml of toxin, and the extract was prepared as described in the text.

in the presence of nicotinamide were inhibited only 31 to 47%. This represented an increase in incorporating activity of 47 to 54% and showed that nicotinamide partially restored activity to extracts of pre-intoxicated intact cells, as it can to intoxicated cell-free systems.

DISCUSSION

We have studied the response of intact sensitive cells to diphtheria toxin and have come to the following conclusions regarding the action of toxin on the subcellular components involved in protein synthesis. Polyribosomes are not disaggregated; rather, they increase at the expense of the single ribosomes. Nascent or growing peptides are not cleaved. The accumulated polyribosomes of intoxicated cells are potentially functional, with an active messenger ribonucleic acid (RNA), as demonstrated by the fact that when ribosomes from intoxicated cells were isolated, washed, and recombined with normal supernatant fractions, they incorporated amino acids in a cell-free system even more efficiently than did an equal amount of normal cell ribosomes. Using cell-free systems and the extracts of normal and intoxicated KB cells, we found that the toxin-sensitive component was in the 100,000 $\times$ g supernatant fraction. When fully intoxicated cells, in which there was no longer a net synthesis of protein, were treated with puromycin, peptides were cleaved from the polyribosomes, and an accelerated breakdown of polyribosomes to single ribosomes occurred.

When we compared these data with the findings of Collier (3), who employed a reticulocyte cell-free system to study toxin action, we noted a number of points of similarity in the response of the intact cell and the cell-free system. Polyribosome buildup and blockage of peptide release are observed in both, as is the ability of ribosomes from intoxicated systems to function when provided with the normal supernatant enzymes. It has been reported that toxin plus NAD inactivates soluble transferase II in cell-free systems, but transferase II bound to the ribosomes is not inactivated (6). Our observation that puromycin is able to cause peptide release and accelerated polyribosome breakdown in intoxicated cells provides evidence that the transferase II which is in association with the ribosomes of intact cells is also insensitive to toxin, since evidence indicates that formation of the peptide bond between puromycin and the carboxyterminal amino acid of the nascent peptide chain requires the presence of the enzyme peptide synthetase (i.e., transferase II) (13, 17, 19).

The similarities between the response to toxin of protein-synthesizing components in intact cells and of cell-free systems indicated that the same mode of action may be involved in each. Goor et al. have demonstrated that NAD combines with diphtheria toxin in cell-free systems (7, 8), and this complex combines with transferase II to inactivate it. The addition of nicotinamide causes this complex to dissociate and release active transferase II (8). We found that 64% of the lost protein synthesizing activity can be restored to extracts prepared from intoxicated cells by the addition of nicotinamide to these extracts before they are used in cell-free systems. Therefore, it appears that the toxin-NAD-transferase II complex is formed in the intact cell. It is probable that the intoxication of the intact cell involves inactivation of transferase II, and that the cofactor NAD is required for this inactivation.

It has been proposed that, in the intact cell, the toxin might act at the membrane, inactivating only membrane-associated transferase II and inhibiting the formation of an enzyme essential to the transport of some required amino acid (8). This attractive hypothesis agrees with the two major arguments against the mode of action of toxin in the intact cell being the same as it is in cell-free systems: first, the large (65,000 molecular weight; 7) toxin molecule would not have to gain access to the interior of the cell; and second, inhibition of only some of the transferase II of each cell would explain how only 100 to 250 molecules of toxin per cell could fully inhibit protein synthesis (8, 15). There would be no need for a 1:1 toxin to transferase molecule relationship as was demonstrated in cell-free systems (7). We have studied the accumulation and transport of amino acids in intoxicated cells, however, and found that the ability of cells to carry out these functions was not impaired even 2 hr after complete inhibition of protein synthesis (12). In addition, cell membranes were able to exclude the dye erythrocin B for more than 5 hr following complete intoxication. More recently, it was shown (5) that HeLa cell membranes are not primarily affected by toxin; poliovirus adsorption and eclipse proceed normally following inhibition of protein synthesis.

If protein synthesis in the intact cell was inhibited due to the lack of an essential amino acid and all of the free transferase II was not inactivated, then the ability of the intoxicated cell extract to synthesize protein should be restored when the extract is reacted in a cell-free system with a full complement of amino acids. Instead, we found that the intoxicated cell extract was

consistently inhibited from 85 to 96% when fresh, crude extracts were compared in vitro. The residual activity in intoxicated cell extract appeared to be due only to ribosome-bound enzyme, since when supernatant fractions from these cells were combined in vitro with washed ribosomes from normal cells, they still incorporated no more amino acids than did washed ribosomes to which no supernatant fraction had been added. This suggested that the toxin inactivates all of the soluble transferase II and is active within the cell rather than at the surface.

In both cell-free and intact cell systems, some amino acid incorporation was observed even in the presence of saturating levels of toxin. In the intact cell, there was a short period following application of the toxin during which protein synthesis proceeded at a normal rate, then at a depressed rate, and finally there was complete inhibition of synthesis. In the cell-free system, a sharp depression in the rate of amino acid incorporation occurred from zero-time. This suggested that, in the intact cell, time is required for toxin to gain entry to the cell and proceed to the sites of protein synthesis.

In addition, our studies on the toxin-resistant L cell (11) have shown that resistance to toxin is linked to the process of macromolecular uptake. Cell-free systems prepared from L cells were sensitive to toxin, whereas the intact cells were highly resistant. Artificial stimulation of protein uptake by poly-L-ornithine was found to render this cell sensitive to toxin, giving us further reason to believe that toxin must enter the cell to exert its effect.

Although we have gained some understanding of how and where toxin acts in the intact cell, the situation is certainly more complex than in the cell-free system. The data suggested that the action of the toxin may not be limited to inhibition of transferase II in the intact cell. We still question how a buildup of polyribosomes can occur in cells where presumably the peptide synthetase enzyme is inactive and no synthesis or release of peptides is taking place. It has been shown that the movement of messenger RNA in relation to the ribosome requires peptide bond formation (1). It has also been shown (18, 19) that ribosomes can attach only at the point on the messenger which corresponds to the N-terminal end of the peptide chain, and that for normal release of a peptide chain a ribosome must reach the end of the genetic message. It appears that in the intoxicated cell the movement of ribosomes in existing polyribosomes is halted before they reach the end of the message, even though there is active enzyme still associated with the ribosomes. In addition, it seems that the increase in polyribosomes and decrease in single ribosomes occurs as newly formed polyribosomes are blocked in their normal function. It appears that puromycin, when introduced, makes some contact which, in the intact cell, transfer RNA cannot, and causes resumed movement of ribosomes (19), release of peptides, and breakdown of polyribosomes. It is possible that a second factor concerned with release of ribosomes and peptides from polyribosomes is also affected by the toxin. Further studies are required to clarify these points.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Training Grant AI-82 and Fellowship Grant 5-F2-AI-31, 089 from the National Institute of Allergy and Infectious Diseases.

We thank Sidney Raffel for discussion and helpful suggestions.

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