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THE RESPONSE OF CULTURED MAMMALIAN CELLS TO  
DIPHThERIA TOXIN

I. AMINO ACID TRANSPORT, ACCUMULATION, AND INCORPORATION IN  
NORMAL AND INTOXICATED SENSITIVE CELLS\*

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PLATE 36

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Recent *in vitro* studies on the mode of action of diphtheria toxin have demonstrated that the earliest detectable, and presumed primary, effect is the inhibition of protein synthesis (1, 7, 8, 20). However, the sequence of events in this inhibition has not yet been determined. Since data have been obtained from whole cell and cell-free systems, there is question regarding the identity of the mechanism of action in both systems (3, 9, 10). It has been suggested that in whole cell systems the intact cell membrane prevents the toxin from reaching sites sensitive in cell-free systems.

Two mechanisms of inhibition of protein synthesis must be considered: (a) The toxin molecule penetrates the cell (12) and acts at cytoplasmic sites. The latter is inferred from the studies on cell-free systems (1, 7, 8) showing inhibition of protein synthesis. (b) The toxin molecule acts at the cell membrane. We reported a drop in the amino acid pool level of intoxicated KB cells, suggesting an alteration in membrane permeability producing a deficiency in intracellular amino acid levels (13). In addition, Strauss and Hendee (20) observed rapid alteration and disintegration of intoxicated HeLa cell membranes.

To clarify and extend our earlier work, we have devised an experimental system to serve for more detailed study of the mode of action of diphtheria toxin and the resistance to toxin displayed by certain cells. Data from microscopic observations have been correlated with data from biochemical experiments. We have investigated further the possibility that inhibition of protein synthesis in intact cells is caused by an alteration at the cell membrane, adversely affecting the accumulation of amino acids. The results indicate that the intracellular

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levels of amino acids are not altered by the action of diphtheria toxin. However, the cell membrane does become weakened and is rendered fragile to mechanical manipulation. These alterations appear to be independent of the inhibition of protein synthesis.

### *Materials and Methods*

*Cell Cultures.*—Two diphtheria toxin-sensitive human cell lines were used: (a) KB, human epidermoid carcinoma,<sup>1</sup> and (b) HeLa, human epithelial carcinoma, a subline of strain S-3.<sup>2</sup> Cells were grown on glass surfaces at 36°C. Milk dilution bottles, 16 × 150 mm tubes and 25 ml Erlenmeyer flasks were employed. Stock cultures were checked routinely for bacteria, fungi, and mycoplasma.

*Media.*—Cells were propagated on Eagle's minimum essential medium (MEM) (2), containing 10% calf serum. Penicillin at 50–100 units and streptomycin at 50–100 µg/ml were incorporated in stock culture medium during early work, but later were omitted from stock culture and experimental media.

*Radioactive Compounds.*—The following labeled compounds were used: α-aminoisobutyric acid-1-<sup>14</sup>C (6.02 mc/mole) from Calbiochem, Los Angeles, Calif.; <sup>35</sup>S-methionine (80 µc/mg) from Schwarz Bio Research, Orangeburg, N. Y.; <sup>14</sup>C-L-methionine (11.63 mc/mole) from New England Nuclear Corp., Boston, Mass.; purified amino acid-<sup>14</sup>C (UL) mixture (15 amino acids) (100 µc/ml), Schwarz Bio Research.

*Diphtheria Toxin.*—4 lots of crude toxin and 1 lot of recrystallized, purified toxin were used.<sup>3</sup> Of these, two of the lots of crude toxin were purified. A column 20 × 0.7 cm was packed with washed Selectacel ion exchange cellulose<sup>4</sup> in 0.01 M phosphate buffer at pH 6.9. The column was stabilized by washing with the phosphate buffer. Manipulations were performed at 4°C. Dialyzed crude toxin was then applied to the column and washed into the resin bed with the phosphate buffer. Fractions were eluted with concentrations of sodium chloride solution increasing from 0.02 M to 1.0 M in 0.02 M phosphate buffer at pH 6.9. The toxin appeared in the fraction released with 0.1 M sodium chloride. A typical batch of toxin purified by this procedure contained 41 MLD/Lf and 1 Lf was equal to 3.0 µg protein.

Toxin was tested for immunological specificity by neutralization with antitoxin, as determined by activity in cell culture and rabbit skin. The purified samples were also characterized by gel diffusion against antiserum, immunoelectrophoresis, and reactivity in guinea pigs and rabbit skin.

*Diphtheria Toxoid.*—1 lot of purified diphtheria toxoid was used.<sup>5</sup> It contained 117.6 Lf/ml, 10.36 µg protein/Lf, and 1658 Lf/mg nitrogen.

<sup>1</sup> Obtained from Dr. Charles Gitterman, Merck, Sharp & Dohme, Rahway, N. J.; from Microbiological Associates, Inc., Bethesda, Md., and from The Tissue Culture Laboratory, Oakland, Calif.

<sup>2</sup> Obtained from the Department of Pharmacology, Stanford University, Stanford, Calif.

<sup>3</sup> Crude toxin (ultrafiltered), 800 Lf/ml, 33 MLD (guinea pig) per Lf, preserved with Merthiolate; crude toxin, lot 932-4, 125 Lf units/ml, 33 MLD/Lf, no preservative. Both received from Dr. J. M. B. Corkell, Connaught Medical Research Laboratories, University of Toronto, Canada. Crude toxin, lot 1036, 125 Lf units/ml, 32 MLD/Lf, no preservative; crude toxin, lot 1051, 93Lf units/ml, 43 MLD/Lf, no preservative. Both received from Wyeth Laboratories, Inc., Marietta, Pa. Purified, 5 × recrystallized diphtheria toxin, lot RX 7328. 10 MLD/Lf; 1 Lf was equal to 3.1 µg protein. Received from Dr. D. C. Edwards, Wellcome Research Laboratories, Beckenham, England.

<sup>4</sup> No. 701 DEAE (Standard); exchange capacity, 0.9 mEq/g. Schleicher and Schuell Co., Keene, N. H.

<sup>5</sup> Received from Cutter Laboratories, Berkeley, Calif.

*Antitoxin:* Three antitoxins were used:

(a) Diphtheria plasma<sup>6</sup> (horse 2771) containing 230 units/ml and preserved with 500 units of penicillin and 500  $\mu$ g of streptomycin per milliliter.

(b) Diphtheria plasma<sup>7</sup> (horse 5517) containing 625 units/ml and preserved with 0.4% phenol.

(c) Diphtheria antitoxin<sup>8</sup> (lot 21208), refined and concentrated to contain 2000 units/ml and containing no preservative.

Toxins and antitoxins containing preservatives were thoroughly dialyzed before use.

*Characterization of Cellular Response to Diphtheria Toxin.*—

*Microscopic observation of cytopathogenicity:* Log phase cells growing in culture tubes were washed with balanced salts solution (BSS) (4) and the appropriate dilutions of toxin were applied in 1 ml of MEM. The cells were observed at half-hourly or hourly intervals and the cytopathic effects scored. Changes in morphology observed were extreme granulation, retraction of cell processes, and appearance of swellings or "blebs" in cell membranes. Once the intoxicated state was reached, most cells separated from the glass within a few hours. Many swollen cells were seen in the medium. The per cent of cells affected was recorded as: 0, no reaction; +, 0-25%; ++, 26-50%; +++, 51-75%; +++++, 76-100%.

*Dye exclusion:* Cells in the log phase were washed with BSS and removed from glass by incubation with 0.05% trypsin. After dispersing and washing, they were suspended to a density of approximately  $5 \times 10^5$  per milliliter in MEM containing either a saturating level of toxin<sup>9</sup> or an equal amount of heat-inactivated toxin. 4 ml amounts of cell suspension were placed in small serum bottles. The bottles were closed with rubber stoppers and shaken gently in a reciprocal water bath shaker. Samples were withdrawn periodically with a hypodermic syringe. 0.2 ml samples were mixed with 0.2 ml amounts of 0.04% Erythrocin B in BSS and the proportions of stained and unstained cells recorded per 1000 cells (16). The per cent of cells excluding dye at 0 time was determined (initial viable count). Counts of cells excluding dye during the period of the experiment were expressed as a per cent of the initial per cent of viable cells.

*Radioactive tracer experiments:*

(a) Test systems: The effect of diphtheria toxin on amino acid uptake, accumulation and incorporation into protein was tested with cells attached to glass or in suspension. Cell sheets were washed, and toxin and/or radioactively labeled amino acids in serum-free medium were added to the cultures. Alternatively, cells to be tested in suspension were grown in suspended culture or lifted and dispersed from sheets by treatment with 0.05% trypsin. Suspended cells were centrifuged at 150 g and washed. The cells were then exposed to serum-free medium containing toxin and/or radioactively labeled amino acids. Incubation was at 36°C. Suspension cultures were incubated at 36°C for 30 min before the addition of toxin and/or radioactively labeled amino acids. The interaction was allowed to proceed for various periods of time and then stopped by chilling in an ice bath. When bottles with cell sheets were tested, they were first chilled, then the cells were suspended by scraping with a rubber policeman.

Maintaining chilled conditions, the test suspensions were centrifuged at 900 g for 30 seconds and washed. Chilled 75% ethanol or 5% trichloroacetic acid (TCA) or water was added to the cell pellets. When only amino acid incorporation into protein was being determined, the reactions were stopped by adding trichloroacetic acid to 5% concentration directly to the cell culture.

<sup>6</sup> Received from Dr. J. M. B. Corkell, Connaught Medical Research Laboratories.

<sup>7</sup> Received from Lederle Laboratories, Pearl River, N. Y.

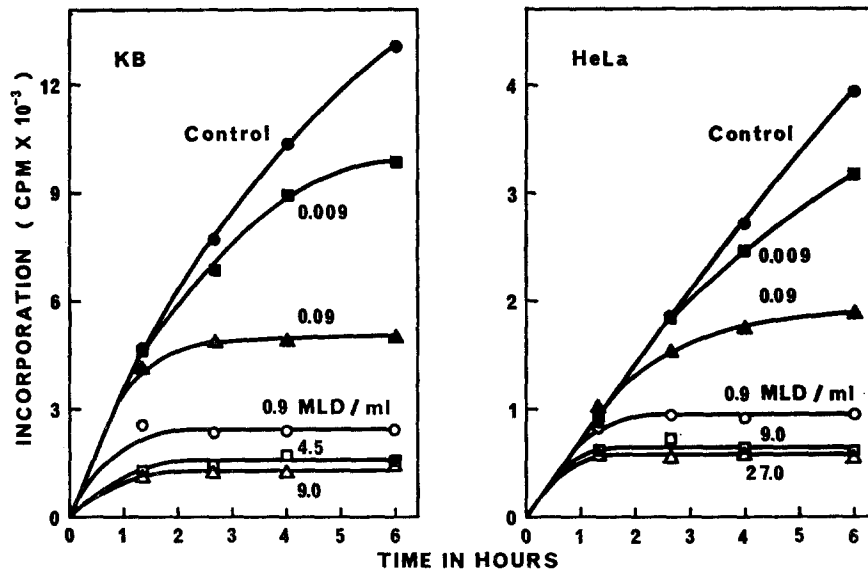
<sup>8</sup> Received from Wyeth Laboratories, Marietta, Pa.

<sup>9</sup> The saturating level of toxin is defined as the lowest concentration of toxin which produces a detectable toxic effect in the minimum time.

## (b) Chemical procedures:

(1) The  $\alpha$ -aminoisobutyric acid (AIB) was extracted from chilled, washed cell pellets by boiling the pellet in 1 ml of distilled water for 10 min. The cells were sedimented by centrifugation and washed with distilled water. The original supernatant and the wash were deposited on planchets for determination of radioactivity.

(2) Cellular components precipitated with ethanol or trichloroacetic acid reacting overnight were separated by centrifugation. The supernatants containing free amino acids were transferred and the sedimented precipitates were washed with the original precipitating reagents. The washings were added to the first supernatants. The ethanol extracts were deposited on



TEXT-FIG. 1. The effect of diphtheria toxin concentration on incorporation of  $^{14}\text{C}$ -amino acids into KB and HeLa cell protein. Replicate, 48 hr cell sheets in 25 ml Erlenmeyer flasks were washed with BSS containing MEM amino acids before receiving 3 ml of serum-free MEM or MEM with the indicated concentration of reconstituted crystalline toxin. All solutions were warmed to  $36^\circ$  before use. Each KB culture contained  $0.33 \mu\text{C}/\text{ml}$ , and each HeLa culture contained  $0.11 \mu\text{C}/\text{ml}$  of  $^{14}\text{C}$  reconstituted amino acid mixture.

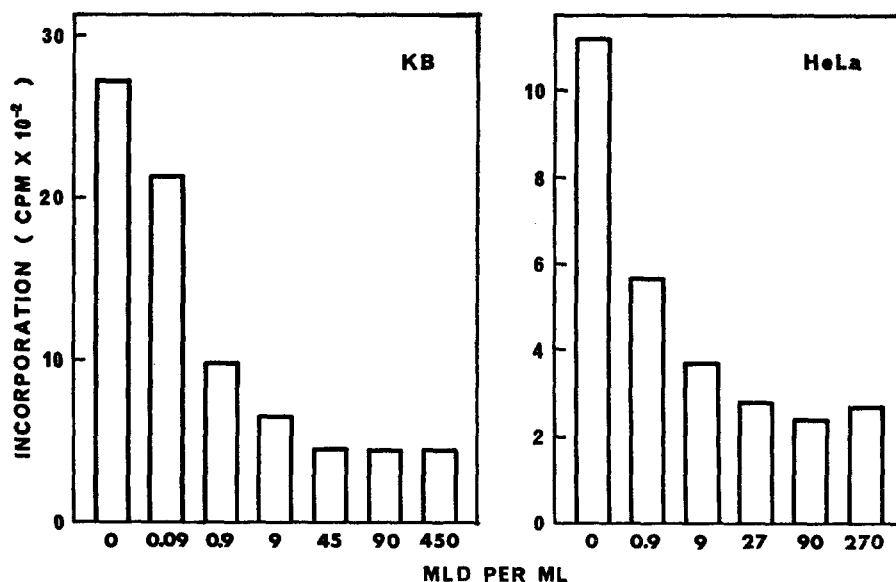
planchets. The TCA extracts were separated three times with 10 volume amounts of ethyl ether. The aqueous phases free of TCA were combined and deposited on planchets. The residual precipitated cellular components were dissolved in  $0.025 \text{ N}$  sodium hydroxide and transferred to planchets. In some experiments, the cell pellet in 5% TCA was heated at  $90^\circ\text{C}$  for 10 min. The precipitated cellular components were then collected on a Millipore filter disc and washed with three 3 ml washes of 5% TCA. The filter discs were transferred to planchets for determination of radioactivity.

Radioactivity was determined by assay in a thin-window gas flow counter. Appropriate corrections for self-absorption were made using a standard weight calibration curve.

## RESULTS

As shown in Text-fig. 1, the incorporation of  $^{14}\text{C}$ -labeled amino acids in both KB and HeLa cells was inhibited within 6 hr by toxin concentrations of 0.009

MLD/ml and higher. The rate of incorporation declined with increasing concentration of toxin. An optimal rate of inhibition was obtained with 9 MLD/ml. Inhibition was observed within 1 hr with the higher concentrations of toxin. The existence of a saturating concentration is shown in Text-fig. 2. For both KB and HeLa cells, 9 MLD was a saturating dose, producing the same inhibition as 30- to 60-fold concentrations of this dose. The earliest times for inhibition of protein synthesis were 15 min in the KB cell, and 30 min in the HeLa cell. The appro-

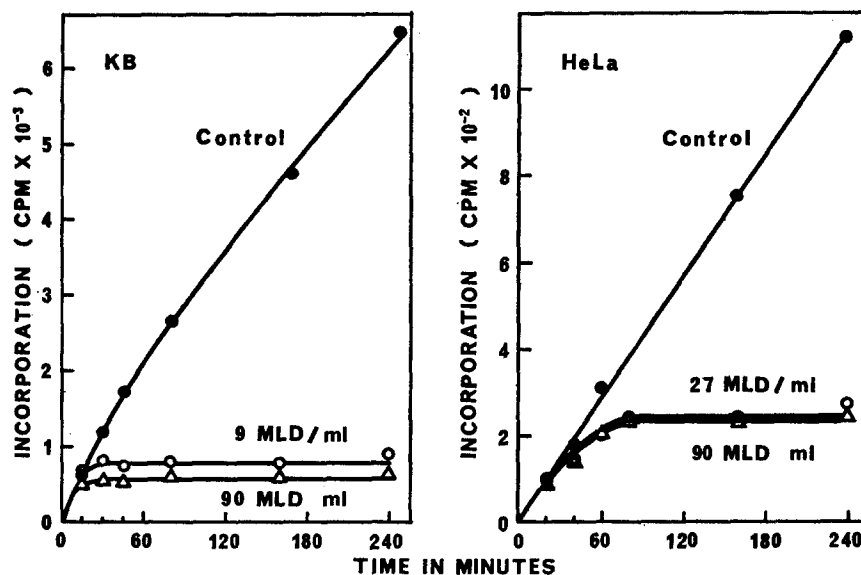


TEXT-FIG. 2. Saturating levels of diphtheria toxin in KB and HeLa cells. Replicate 48 hr flask cultures received 3 ml of serum-free MEM or MEM with the indicated concentration of diphtheria toxin, as described in Fig. 1. Dialyzed toxin (lot 1051, Wyeth) was used in KB cell cultures. HeLa cultures received reconstituted crystalline toxin. <sup>14</sup>C reconstituted amino acid mixture was added at 0.1  $\mu$ c/ml in KB cultures and 0.05  $\mu$ c/ml in HeLa cultures. All cultures were incubated for 4 hr.

ropriate data are shown in Text-figs. 3 and 4. These results are in contrast to the rapid action of saturating levels of puromycin, another inhibitor of protein synthesis, under similar conditions (Text-fig. 5). The addition of heat-denatured toxin preparations or toxoid in concentrations equal to the highest tested levels of toxin did not affect protein synthesis. The action of the toxin was neutralized by specific antitoxin (Table I).

With all doses of toxin, inhibition of incorporation of amino acids preceded visible cytopathic effects. When saturating levels were used, the former began at 15-30 min and was complete at 30-80 min, while the latter began at 2-3 hr and was complete at 6-24 hr. These data are summarized in Table II.

The effect of the toxin on protein synthesis could be ascribed to impairment of the transport and accumulation of amino acids. To test this hypothesis, the effect of the toxin on the transport and accumulation of AIB was studied. AIB is a nonutilizable amino acid and would pass easily through a damaged membrane. Initially,  $^{14}\text{C}$ -labeled AIB or  $^{35}\text{S}$ -methionine and toxin were added to cultures of KB cells in sheets. At subsequent intervals the cells in these cultures were suspended by scraping and tested for the intracellular concentrations of



TEXT-FIG. 3. Earliest evidence of diphtheria toxin action. Replicate 48 hr flask cultures received 3 ml of serum-free MEM or MEM plus indicated concentrations of reconstituted crystalline toxin, as described in Fig. 1. KB cultures contained  $0.11 \mu\text{c}/\text{ml}$  and HeLa cultures contained  $0.05 \mu\text{c}/\text{ml}$  of  $^{14}\text{C}$  reconstituted amino acid mixture.

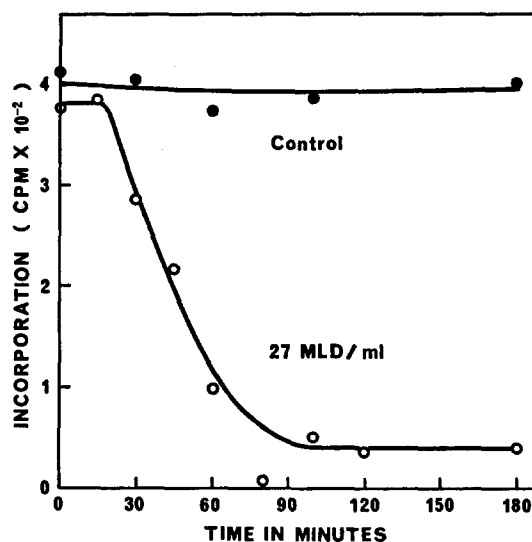
AIB or the amount of  $^{35}\text{S}$ -methionine incorporated into protein. The AIB was lost from intoxicated cells when protein synthesis was inhibited (Text-fig. 6).

However, when toxin and AIB were added to cells already in suspension, AIB was not lost, indicating that under these experimental conditions the cell membranes were not damaged. These data are shown in Text-fig. 7. When packed cell volumes were determined along with accumulation of AIB, the distribution ratios<sup>10</sup> could be calculated. In a typical experiment, the distribution ratio for cultures incubated in the presence or absence of toxin was 6. This value greater than 1 indicates a sustained concentration of amino acids.

<sup>10</sup> The distribution ratio is defined as the ratio of the concentration of AIB in the cell water to the concentration in the medium.

To confirm the absence of membrane damage, a dye exclusion test was used. HeLa and KB cells were suspended and high concentrations of toxin, 27 and 41 MLD/ml, were added. Periodically, samples of cells were removed and tested for their ability to exclude Erythrocin B. As predicated, an equally high percentage of normal and intoxicated cells excluded the dye (Text-fig. 8).

When intoxicated cells attached to glass were observed by phase-contrast microscopy (Fig. 1), they were first seen to retract their cell processes and to



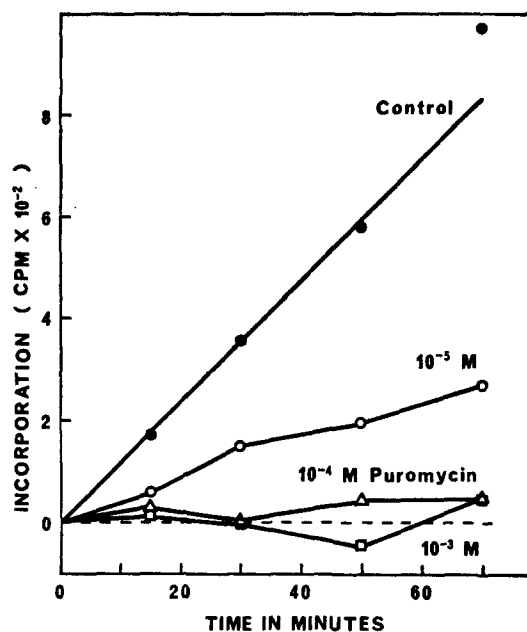
TEXT-FIG. 4. Detection of early action of diphtheria toxin by pulse labeling of cell protein. Replicate 48 hr flask cultures of HeLa cells received 3 ml of serum-free MEM or MEM with 27 MLD of reconstituted crystalline toxin, as described in Fig. 1. At indicated times, each culture was pulse-labeled for 3 min by addition of  $0.8 \mu\text{C}$  of  $^{14}\text{C}$  reconstituted amino acid mixture. Incorporation was stopped by addition of TCA to 5%.

round up. Blebs in the membrane developed, and subsequently the cells detached from the glass and floated in the medium. Disintegration of the cells was not observed, but cells swelled and displayed a distended intact membrane.

The nonutilizable amino acid, AIB, was retained in suspended intoxicated cells while protein synthesis was inhibited. Similarly, utilizable amino acids were retained while protein synthesis was inhibited. This effect was demonstrated with a variety of amino acids, singly and in groups. A representative experiment employing  $^{14}\text{C}$ -methionine and toxin at 66 MLD/ml is shown in Text-fig. 9.

The transport and accumulation of amino acids was not affected until long after inhibition of protein synthesis. Toxin was added to KB cells in suspension

and  $^{14}\text{C}$  amino acid mixture was added at the same time as toxin, or 2 hr afterward. Cells were tested for the presence of intracellular amino acids and amino acid incorporation into protein at various intervals up to 6 hr. The results are shown in Text-fig. 10. In both toxin-treated and control cell cultures, identical levels of  $^{14}\text{C}$ -labeled amino acids were reached after 1 to 2 hr. During this time, protein synthesis was severely inhibited, apparent after 30 min when the  $^{14}\text{C}$ -



TEXT-FIG. 5. The effect of puromycin on incorporation of  $^{14}\text{C}$  amino acids into KB cell protein. Replicate 48 hr flask cultures of KB cells received 3 ml of serum-free MEM or MEM with the indicated concentration of puromycin. Test medium contained  $^{14}\text{C}$  reconstituted amino acid mixture at  $0.1 \mu\text{c/ml}$ . Experimental design was as described in Fig. 1.

labeled amino acids were added with toxin and immediately apparent when they were added 2 hr after the addition of toxin.

#### DISCUSSION

The mode of action of diphtheria toxin can be studied in cell-free systems or in living cells. In this study whole cells have been used. Both cell lines, KB and HeLa, were highly sensitive to low levels of toxin. The activity of the toxin, determined by its effect on protein synthesis, is related to the MLD content of the participating toxin. The different toxin preparations used in this study gave comparable results when compared on an MLD basis.

Studies with both cell-free and whole cell systems, including those reported



here, support inhibition of protein synthesis as an important component of the mode of action. The data presented are consistent with the hypothesis that diphtheria toxin enters the cell and acts at cytoplasmic sites. During this time, the cell membrane is morphologically intact, but we have found evidence of

TABLE I  
*Effect of Toxin and Altered Toxin on KB and HeLa Cells*

	CPM	Inhibition
		%
Experiment 1*		
Control—no toxin	658	—
Toxin‡—3 Lf/ml + antitoxin§—4 units/ml	640	3
Toxin‡—3 Lf/ml	13	98
Experiment 2		
Control—no toxin	1250	—
Purified toxoid—5 Lf/ml	1236	1
Toxin‡—0.5 Lf/ml	151	88
Experiment 3¶		
Control—no toxin	2287	—
Heat-denatured toxin‡—4 Lf/ml	2270	—
Crude toxin**—2.1 Lf/ml	596	74
Toxin‡—10 Lf/ml	389	83

\* HeLa cells, 180 min exposure to toxin. Experimental design as in Text-fig. 4.

‡ Purified, Wellcome Research Laboratories. See footnote 3.

§ Lot 21208, Wyeth Laboratories. See footnote 8.

|| KB cells, 150 min exposure to toxin. Experimental design as in Text-fig. 1.

¶ KB cells, 120 min exposure to toxin. Experimental design as in Fig. 1.

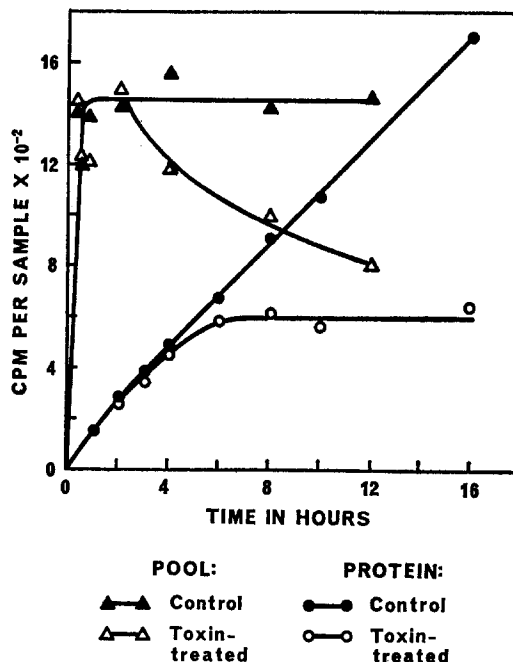
\*\* Lot 1051, Wyeth Laboratories. See footnote 3.

TABLE II  
*The Action of Diphtheria Toxin on KB and HeLa Cell Lines*

	Cell line	
	KB	HeLa
Microscopic observations		
Saturating dose	0.9–4.5 MLD	0.9–4.5 MLD
First cytopathic effect*	2 hr	3 hr
Time to reach 4 <sup>+</sup>	6 hr	24 hr
Incorporation of <sup>14</sup> C-amino acids	9–45 MLD	9–27 MLD
Saturating dose	15 min	30 min
First inhibition of amino acid incorporation	30 min	80 min
Complete inhibition of amino acid incorporation		

\* All times presented are for a saturating dose of toxin.

alterations in the membrane. The membranes of susceptible cells exposed to toxin become fragile and can be disrupted by manipulations that do not affect control cells. Thus, studies utilizing whole cells must be designed to avoid such



TEXT-FIG. 6. The effect of diphtheria toxin on amino acid accumulation and incorporation into protein by monolayer cultures.

*Pool:* Replicate cultures of KB cells were washed with serum-free MEM. 10 ml of serum-free MEM or MEM plus 0.26 MLD/ml of dialyzed toxin (ultrafiltered from Dr. Corkell, see footnote 3) was added to each culture. <sup>14</sup>C-AIB was included at 0.014  $\mu$ c/ml.

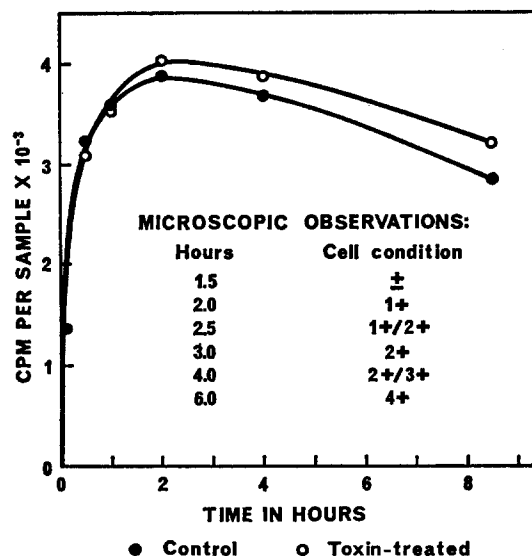
*Protein:* Replicate cultures of KB cells were washed with serum-free MEM. One ml of serum-free MEM or MEM plus 0.26 MLD per ml of dialyzed toxin (ultrafiltered from Dr. Corkell, see footnote 3) was added to each culture. <sup>35</sup>S-methionine was included at 0.017  $\mu$ c/ml.

effects. If cells in suspension are desired or required, they must be suspended prior to addition of toxin.

The transport of amino acids continues even after intoxicated cells cease to synthesize protein, and the levels accumulated are as high as in control cells. Even after 2 hr's exposure to saturating levels of toxin, KB cells can still effectively transport and accumulate amino acids in amounts sufficient to sustain protein synthesis. These results support the view that the physiological alteration to cell membranes is not associated with the mechanisms for transport of

amino acids. Although the membranes of intoxicated cells are rendered more fragile than normal cells, the integrity of the membrane persists for many hours after cessation of protein synthesis. This observation is in contrast to the rapid disintegration of cells noted by Strauss and Hendee (20).

The inhibition of protein synthesis, measured by incorporation of labeled amino acids, constitutes the earliest detectable effect of diphtheria toxin on cells. The period following application of toxin and preceding this inhibition has



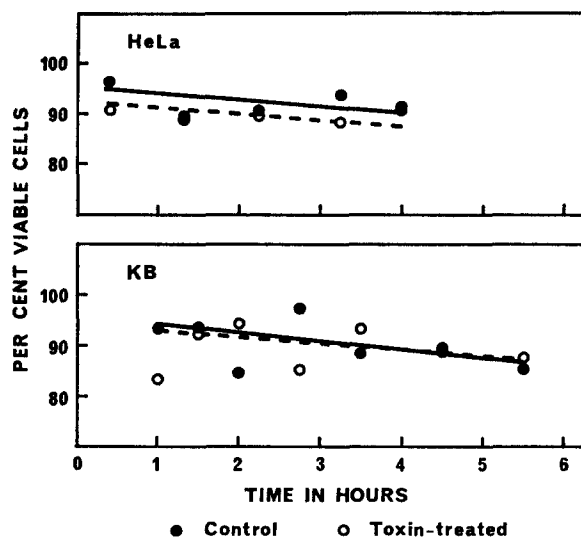
TEXT-FIG. 7. The effect of diphtheria toxin on transport and accumulation of  $^{14}\text{C}$ -AIB into KB cells suspended in MEM. Each test suspension contained  $30\ \mu\text{l}$  of packed cells in 3 ml of MEM. AIB was included in each sample at a concentration of  $0.33\ \mu\text{c}/\text{ml}$ . Purified toxin was included in appropriate samples to 123 MLD/ml. The corresponding monolayer cultures, observed microscopically, contained 24.6 MLD/ml.

been referred to as the latent period. A number of workers have described and made use of the latent period in studies of toxin-cell interactions (6, 9, 20). It has been divided into reversible and irreversible periods. Pappenheimer et al. (15) summarized the factors affecting the duration of the latent period. These were concentration of toxin, cell strain, and temperature. More recently, Kim and Groman (9) reported that inhibitors present in crude toxin filtrates alter toxin activity and that ammonia affords sensitive cells a reversible protection. Our study suggests that the conditions of culture and age of the cells also have an effect on the response of sensitive cells to toxin.

When cells were cultured and exposed to toxin while still attached to glass, the latent period was significantly reduced. As shown in Table II, the effect of

toxin was seen in 15 min with KB cells and 30 min with HeLa cells. These latent periods are shorter than those previously reported (8, 15, 19, 20). It was not possible to show this rapid action of toxin with cells suspended by trypsinization or scraping, or with cells grown in suspension according to the method of McLimans et al. (11).

When suspended HeLa cells were exposed to saturating levels of toxin by methods similar to those used by Strauss and Hendee (20), Strauss (19), or Kato and Pappenheimer (8), the values for the duration of the latent period and the

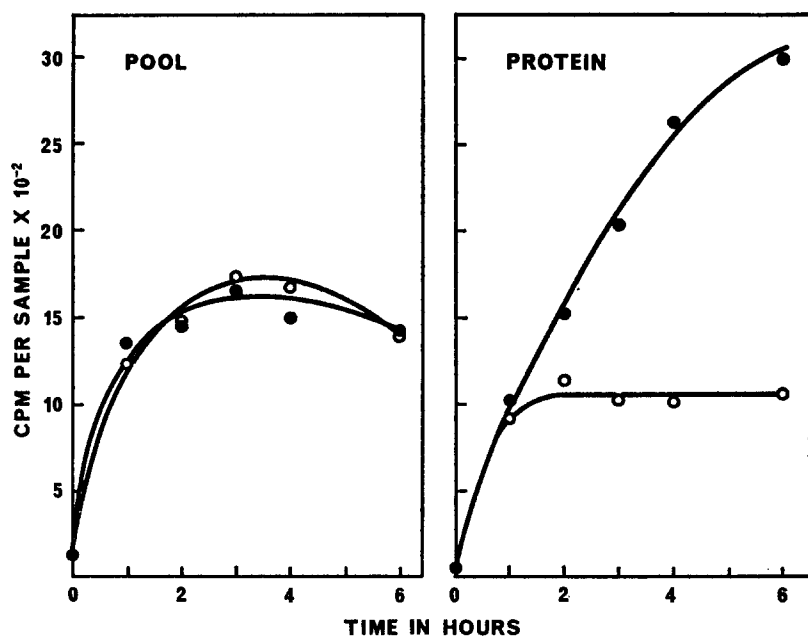


TEXT-FIG. 8. Exclusion of dye by intoxicated cells. HeLa cell suspensions were prepared in MEM or MEM with 41 MLD/ml of reconstituted crystalline toxin. KB cell suspensions were prepared in MEM or MEM with 27 MLD/ml of the same toxin. (See text for details.)

level of saturation were in good agreement with those previously reported. The latent period was between 60 to 90 min and inhibition was complete in 2 hr. Saturation was reached at a concentration of 10 MLD/ml. These values are as reported by Strauss and Hendee (20). The KB cell, a more sensitive cell, responded similarly to the MK, human kidney cell, of Kato and Pappenheimer (8). Saturation was the same as for HeLa cells. The latent period was shorter (40–60 min) and complete inhibition was reached more rapidly than was the case in HeLa cells.

When we compared our MLD values for a  $TCD_{50}$  using the method of Gablicks and Falconer (3), the data were in good agreement. They obtained values of 0.001 and 0.01 MLD/ml respectively for KB and HeLa cells. Our values were 0.0009 MLD for KB and 0.009 MLD for HeLa.

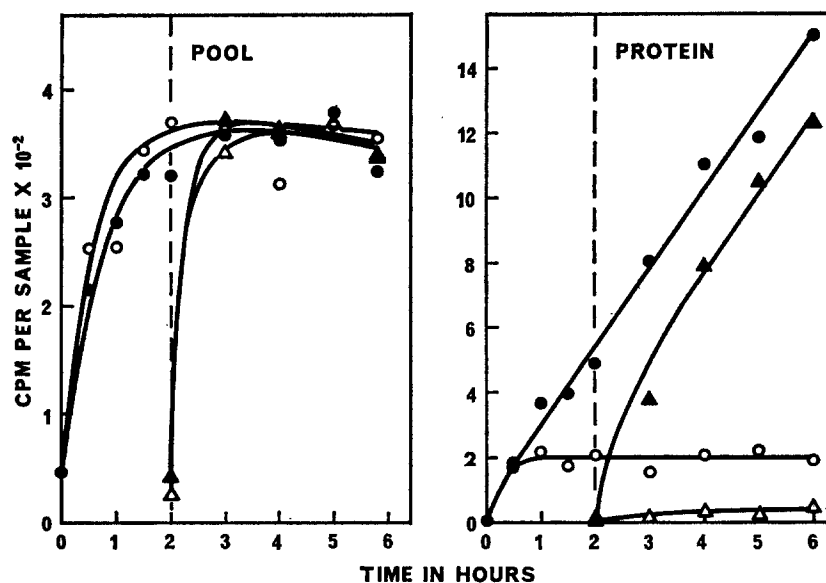
It thus appears that, with the use of early log phase cells attached to glass and handled as described, the latent period may be reduced. It is never completely eliminated, however, and instantaneous inhibition of protein synthesis, such as is apparent when puromycin is applied, is not possible with diphtheria toxin. These findings may be related to the mechanism by which toxin gains entry to the cytoplasm of the cell.



TEXT-FIG. 9. The effect of diphtheria toxin on amino acid accumulation and incorporation into protein by KB cell suspensions. Each test suspension contained 26  $\mu$ l of packed cells in 3 ml MEM. Dialyzed toxin (lot 1036, Wyeth) was added to a final concentration of 66 MLD/ml.  $^{14}$ C-methionine was included at a concentration of 0.049  $\mu$ c/ml. Samples were processed using the 75% ethanol method.  $\bullet$ — $\bullet$ , control;  $\circ$ — $\circ$ , toxin-treated.

To understand fully the mode of action of diphtheria toxin, we should know the mechanism by which the toxin enters cells and the number of active molecules of toxin required to kill a cell. It has been suggested that pinocytosis is not a likely route of entry, based on the size and negative charge of the molecule at physiological pH (10, 19). Toxin entry must be inferred from the accumulation of negative data which fail to ascribe any significant membrane activity to the toxin, and from the data showing that toxin inhibits protein synthesis in cell-free systems. Toxin does not affect the  $\text{Na}^+$ - $\text{K}^+$  balance, since intoxicated cells do not swell or shrink during the critical period of intoxication. High intracellu-

lar  $K^+$  levels are maintained for at least 2 hr (8). The transport of phosphate is affected only as the rate of protein synthesis is slowed (19). Diphtheria toxin does adsorb rapidly to the surfaces of sensitive cells. It is eluted only slowly by repeated washing, and then only for a finite time prior to inhibition of protein



TEXT-FIG. 10. Transport, accumulation and incorporation of  $^{14}\text{C}$ -amino acids in fully intoxicated KB cells. 48 hr cultures of KB cells were removed from glass, washed, and pooled in MEM. All manipulations were carried out at  $36^\circ\text{C}$ . Approximately  $7\ \mu\text{l}$  of cells were distributed to replicate tubes in 2 ml of MEM. Samples were equilibrated for 15 min. 1 ml of MEM containing  $^{14}\text{C}$  protein-labeling mixture at a concentration of  $0.11\ \mu\text{c}/\text{ml}$ , and reconstituted crystalline toxin where appropriate, was added to part of the tubes ( $\bullet$ — $\bullet$ , control;  $\circ$ — $\circ$ , toxin-treated). Final concentration of toxin was 9 MLD/ml. The rest of the tubes received MEM or MEM with toxin only, at the same concentration ( $\blacktriangle$ — $\blacktriangle$ , control;  $\triangle$ — $\triangle$ , toxin-treated). After 2 hr's incubation,  $^{14}\text{C}$  protein-labeling mixture was added to the second group of tubes, to a concentration of  $0.11\ \mu\text{c}/\text{ml}$ . Samples were processed using the 5% TCA method.

synthesis. It may be that toxin enters cells after altering or being altered by components of the cell surface (3).

We have calculated that as few as 150 to 250 molecules of toxin available per cell are sufficient to bring about cell death (18). It may be inferred that even fewer molecules, given enough time under proper conditions of cell culture, could irreversibly intoxicate a cell.

Using saturating levels of labeled toxin, Pappenheimer et al. (15) were unable to detect a significant increase in radioactive counts in intoxicated cells. We have not been able to detect toxin in association with cells, using the fluorescent

antibody technique (18), and attempts to demonstrate loss of toxin activity from medium after contact with sensitive cells have not been successful (18). Thus it is reasonable to assume that the small number of molecules of toxin necessary for a lethal effect could be taken in by pinocytosis.

The uptake of protein molecules by cultured cells has been clearly shown using Sarcoma 180 (17), HeLa (5), and human sarcoma cells (14). Diphtheria toxin itself was shown to be actively taken into peritoneal exudate cells of the guinea pig by pinocytosis (12). In view of this evidence, one cannot exclude the possibility that toxin enters sensitive cells by a mechanism such as pinocytosis.

#### SUMMARY

The response to diphtheria toxin of two sensitive cell lines, KB and HeLa, was investigated. Inhibition of the incorporation of radioactively labeled amino acids into protein was the earliest detectable effect of diphtheria toxin. It was observed that, during the period of intoxication, the cell membrane was morphologically intact and retained its semi-permeable character, although it was rendered fragile and more easily disrupted by mechanical manipulations than the normal cell. The transport of amino acids continued even after intoxicated cells had ceased to synthesize protein, and the levels accumulated were equal to those of control cells. It was observed that cultural conditions, age, and handling of cells affected their response to toxin. In early log phase cells subjected to a minimum of handling before application of the toxin, the normally observed latent period preceding detectable effects was reduced to 15 min for KB cells and 30 min for HeLa cells, shorter times than previously reported. The data are consistent with the hypothesis that diphtheria toxin enters susceptible cells, possibly by pinocytosis, and there acts upon cytoplasmic sites of protein synthesis.

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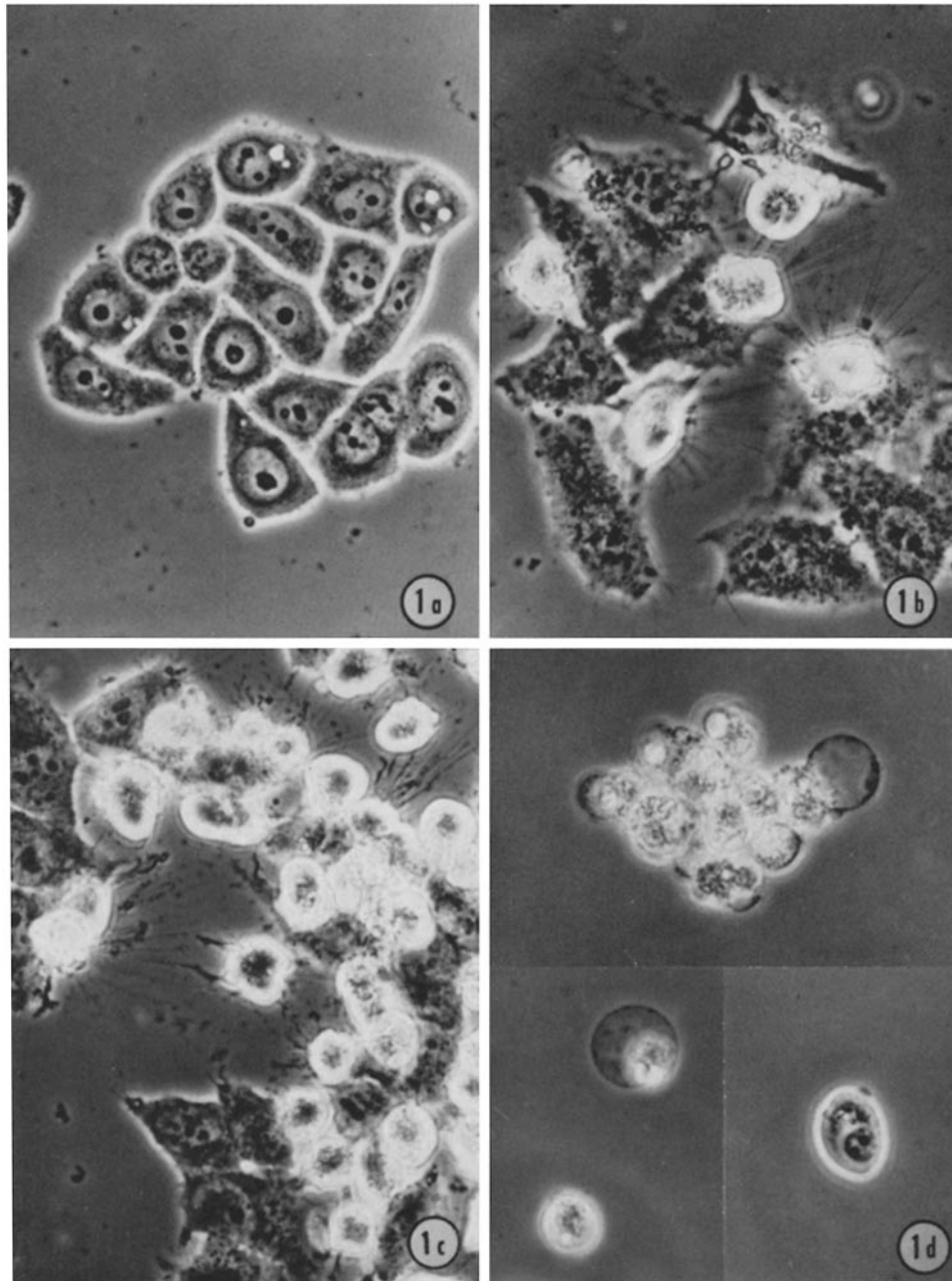
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## EXPLANATION OF PLATE

## PLATE 36

Fig. 1. Phase-contrast micrographs showing progressive intoxication of KB cell sheets exposed to 20 MLD/ml of reconstituted crystalline diphtheria toxin.  $\times$  1500.  
 (a) Normal KB cells; (b) KB cells after 3 hr exposure; (c) after 5 hr exposure; (d) after 8 hr exposure.





(Moehring et al.: Mammalian cell response to diphtheria toxin)