

STUDIES ON THE MODE OF ACTION OF
DIPHThERIA TOXIN*

VI. SITE OF THE ACTION OF TOXIN IN LIVING CELLS

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It has been shown in previous communications that, in the presence of the specific cofactor nicotinamide adenine dinucleotide (NAD), diphtheria toxin inhibits the transfer of amino acids from aminoacyl-tRNA to the growing polypeptide chains on the ribosomes in cell-free extracts from mammalian cells (1). Although bacterial cell extracts are completely insensitive to inhibition (1), toxin and NAD inhibit amino acid incorporation in cell-free extracts from chick embryonic tissues (2) and from yeast (3). Collier (4) has demonstrated that toxin and NAD act specifically to cause inactivation of the labile peptide bond-forming enzyme, transferase II (5). Goor and Pappenheimer (6) also concluded that transferase II was the site of toxin action in cell extracts and noted that only the *soluble* form was inactivated; the ribosome-bound enzyme was not affected. It was further observed (3) that the inactivation of transferase II in cell-free systems could be prevented or reversed in the presence of a sufficient concentration of nicotinamide. Finally, it has been found that toxin and NAD interact mole for mole to form a relatively dissociable complex (7, 8). Assuming that this toxin-NAD complex interacts further with transferase II to form an enzymatically inactive product, an equation based on the mass law has been derived that accurately predicts the per cent inhibition of amino acid incorporation in cell-free systems at any given NAD and toxin concentration (3).

Although the model we have just described adequately explains the action of toxin in cell-free systems, serious difficulties arise when we attempt to use the model to explain the inhibition by toxin of growth of HeLa cells in culture (3). First, the action of toxin on living HeLa cells is *not* reversible in the presence of

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nicotinamide. Second, inhibition of amino acid incorporation in cell cultures is complete despite the fact that extracts from intoxicated cells are still capable of incorporating amino acids at a moderate rate.¹ Third, although a saturating dose may contain sufficient toxin to interact with all of the transferase II present in a cell suspension, experiments using toxin labeled with tritiated methionine have shown that only a small fraction of a saturating dose is actually taken up by the cells (9). Finally, very much smaller doses amounting to only a few hundred molecules per cell suffice to inhibit cell growth within 2 or 3 days (10, 11).

In view of these apparent discrepancies between the action of toxin *in vivo* and *in vitro*, it seemed important to determine, as precisely as possible, the maximum number of toxin molecules fixed per cell in the presence of a large excess of toxin. This has now been accomplished with HeLa cells, using highly purified diphtheria toxin labeled with carrier-free iodine-125 and the technique of radioautography. The results have shown that even at concentrations of toxin approaching saturation, only 25–50 molecules of the toxin protein are bound per cell. Radioautographs made from cell sections have located most, if not all, of the toxin molecules fixed to the outer cell membrane.

Materials and Methods

Cells and Cell Culture.—HeLa cells of strain S-3 were maintained in spinner cultures on Eagle's medium supplemented with 5–8% calf serum (Industrial Biological Laboratories, Rockville, Md.) or antitoxin-free horse serum. The latter was kindly supplied by the Antitoxin and Vaccine Laboratories of the Massachusetts Department of Health. A culture of mouse L cells from Dr. R. S. Chang was maintained in the same medium.

Diphtheria Toxin and Antitoxin.—The diphtheria toxin preparation, purified by ammonium sulfate precipitation, DEAE-cellulose, and Sephadex chromatography has been described previously (6). The stock solution which was nearly colorless, contained 11,000 Lf/ml, 27.5 mg of protein/ml and had an optical density of $276\text{ m}\mu = 26.6$. Its toxicity was 60–70 ML/D/Lf unit. It was stored in the frozen state.

Horse antitoxin 5353AD was a pepsin-digested globulin fraction showing only a single band on immunoelectrophoresis against purified toxin. A faint additional band has been detected against a component present in crude toxin (see Raynaud and Relyveld, reference 12).

Protein Synthesis in Normal and Intoxicated Cells.— $1.25\text{ }\mu\text{C}$ ¹⁴C-leucine was added per 20 ml of cell culture in small spinner vessels. At hourly intervals, 1 ml samples were collected on 0.6 μ Millipore filters and the cells were washed with Eagle's salt solution followed by 5% trichloroacetic acid (TCA). They were placed on planchets, dried, and counted.

Iodine-125 Labeled Toxin.—Carrier-free iodine-125 was obtained from New England Nuclear Corp., Boston, Mass., as Na¹²⁵I in 0.1 N NaOH. Toxin was iodinated by the method of Greenwood et al. (13). In a typical preparation: 10 mc Na¹²⁵I (4.6 m μ moles) in 0.1 ml NaOH was treated successively with rapid mixing at room temperature with 0.11 ml 0.5 M phosphate buffer, pH 7.6, 0.1 ml purified toxin containing a total of 2.2 m μ moles² (55 Lf) in 0.05 M phos-

¹ Recent observations by Mr. James Kurnick in this laboratory have shown that amino acid incorporation in crude extracts from intoxicated HeLa cells cannot be inhibited further by toxin.

² Assuming a molecular weight of 65,000 for toxin (14).

phate buffer, pH 7.6, and 200 μg chloramine-T freshly dissolved in 0.1 ml of the same buffer. After 6 min, 0.1 ml sodium metabisulfite (2.4 mg/ml) and 0.4 ml of 1% KI in the same 0.05 M buffer were added. The mixture was then passed through a small Sephadex G50 column ($V_0 = 4.5$ ml) equilibrated with 0.05% crystalline bovine serum albumin (BSA) in 0.1 M phosphate at pH 7.6. The labeled toxin was chased with a further 0.4 ml 1% KI and then eluted with buffered BSA. 1 ml fractions were collected from which 1 μl samples were taken for gamma counting in a Picker Liquimat β , γ scintillation counter. Of the total radioactivity, 95% emerged as a sharp peak with the protein, corresponding to 2.0 atoms ^{125}I per toxin molecule. In other experiments, the yield of bound iodine approached 99%.

Properties of Labeled Toxin.—Toxicity tests in guinea pigs showed approximately 40–50 MLD/Lf indicating that there had been little or no loss in toxicity after iodination. When small aliquots of $^{125}\text{I}_2$ -toxin were mixed with cold toxin at a concentration of 100 Lf/ml, 97.5% of the radioactivity was precipitable by 5% TCA and 96% by antitoxin. Therefore, approximately 98% of the TCA-precipitable label was specifically precipitable by antitoxin.

Preparation of Cells for Radioautography.—Freshly prepared $^{125}\text{I}_2$ -toxin was diluted to 16 Lf/ml in Eagle's medium containing 5% calf serum and passed through a 0.45 μ Millipore filter just before use. Cells in exponential growth were treated with 0.3–0.6 saturating doses (0.13–0.25 Lf/ml) of the filtered labeled toxin and incubated for 6 hr at 37°C in spinner cultures at a volume of 100 ml. At the same time ^{14}C -leucine uptake was followed in small spinners containing the same concentration of toxin. Incorporation of leucine into cell protein was arrested by the toxin within 3–5 hr in every case.

At the end of the incubation period, the intoxicated cells were collected in the International centrifuge by centrifugation at 800 rpm for 7 min. Supernates were saved for quantitative estimations of residual toxin in terms of radioactivity specifically precipitable by antitoxin or by TCA and for toxicity (MLD) in guinea pigs. The cells were washed three times by re-suspending them to 100 ml in Eagle's salt solution followed by centrifugation. The third and final washing contained less than 0.005% of the added radioactivity. The washed cells were suspended in 5 ml salt solution for counting and for radioautography. The iodine-125 collected with the cells amounted to less than 1 cpm per 300 cells (see Tables I and II).

Radioautography.—Drops containing about 10^7 cells were spread across clean microscope slides using a 1 cm wide glass spreader. Three samples were spread across each slide, one of which was always a nonradioactive untreated control cell suspension. The other two were labeled toxin-treated cell suspensions to be compared. After drying, the slides were dipped into fresh Eastman Nuclear Tract emulsion NTB-2³ at 45°C in the dark and allowed to drain in a vertical position in the dark for at least 1 hr until dry (15). Because this method of draining could result in a nonuniform layer of dried emulsion, three groups of slides were prepared in which the position of each smear was rotated from top to bottom. After drying they were placed in lightproof boxes and stored at -30°C . At intervals of 7, 14, 21, 28, and 60 days, slides were removed, developed, and stained through the emulsion with toluidine blue. A drop of Canada balsam was placed on each slide before overlaying with a cover slip.

Slides were examined under high power phase contrast with a grid placed in one ocular to divide the field into squares 20 μ on a side. Grain counts were made on about 50 squares containing cells and 50 squares without for each slide. Three slides were counted for each pair of suspensions compared so that each smear was counted when placed at the top, middle, and bottom of a slide. Background grain counts tended to be slightly higher at the bottom of the slide where the emulsion was thickest. In counting, care was taken to avoid regions near radioactive aggregates of denatured protein (see Fig. 1). Slides were coded so as to avoid any possible bias on the part of the observers.

³ The NTB-2 emulsions we have received have varied considerably in background count. It is important to check each lot before use.

Radioautographs of Cell Sections.—3 ml of a washed suspension containing a total of about 2.5×10^7 $^{125}\text{I}_2$ -toxin-treated HeLa cells were fixed with 10% formalin and the centrifuged pellet dehydrated and embedded in paraffin. We are greatly indebted to Mr. Fred Casella of the Polaroid Corp., Cambridge, Mass., for cutting sections 1–2 μ thick (i.e. 5–10 sections per HeLa cell). The sections were fixed to slides and the paraffin removed with xylene. They were rehydrated through a stepwise series of alcohol-water rinses. After thorough washing with water, the slides were coated with emulsion, dried, and stored in the usual manner.

TABLE I
Distribution of Radioactivity in Cultures of HeLa and of L Cells Treated for 6 hr at 37°C with ^{125}I -Labeled Diphtheria Toxin

	A	B	C
	HeLa	HeLa	"L"
Total No. of cells in 100 ml	3×10^7	3×10^7	3.5×10^7
$^{125}\text{I}_2$ -toxin added, moles	5×10^{-10}	5×10^{-10}	5×10^{-10}
Unlabeled toxin added, moles	—	4×10^{-8}	—
Total iodine-125 remaining in supernate, <i>cpm</i> *	1.61×10^9	1.54×10^9	1.45×10^9
Per cent of iodine-125 precipitable by antitoxin†	97.5	96.8	97.0
Iodine-125 in 3rd washing, <i>cpm</i>	6.2×10^4	8.6×10^4	7.3×10^4
Iodine-125 collected with washed cells, <i>cpm</i>	1.0×10^5	1.1×10^5	1.1×10^5
Apparent number of toxin molecules "collected" per cell	600	700	650

* The toxicity of the supernate was between 4 and 5 guinea pig MLD/ml. Within limits of error, this was exactly the amount of toxin added.

† Expressed as per cent TCA-precipitable counts precipitated by antitoxin.

RESULTS

Uptake of Labeled Toxin by Cells—Experiment 1.—

A 320 ml culture of HeLa cells was centrifuged at room temperature for 7 min at 800 rpm and the pellet resuspended in fresh medium containing 5% calf serum to a count of 3×10^5 cells/ml. Similarly, a 150 ml culture of mouse L cells was centrifuged and resuspended to give 3.5×10^5 cells/ml. The suspensions were then distributed among three spinner bottles so that each contained 120 ml of culture and labeled toxin as follows:

A. 3.6×10^7 HeLa cells + 6×10^{-10} moles $^{125}\text{I}_2$ -toxin.

B. 3.6×10^7 HeLa cells + 6×10^{-10} moles $^{125}\text{I}_2$ -toxin + 4.8×10^{-8} moles cold toxin.

C. 4.2×10^7 mouse L cells + 6×10^{-10} moles $^{125}\text{I}_2$ -toxin.

After mixing, 20 ml from each culture was withdrawn to small spinner vessels each containing 1.25 μC ^{14}C -leucine. All six cultures were then incubated for 6 hr at 37°C. Leucine uptake was followed in 1 ml samples taken from the small spinners at hourly intervals. Protein synthesis stopped in culture B containing excess cold toxin (11 Lf/ml) within 2 hr and within 5 hr in culture A with only 0.13 Lf/ml $^{125}\text{I}_2$ -toxin. Even 11 Lf/ml had no effect on leucine uptake by L cells within an 18 hr period.

After 6 hr incubation, the cells from bottles, A, B, and C (100 ml each) were collected and washed as described under Materials and Methods. The toxicity remaining in supernate A was determined by subcutaneous injection into guinea pigs. Within the limits of experimental error, all of the toxicity originally added was recovered in the supernate. The distribution of radioactivity in each culture is summarized in Table I. After three washings with Eagle's salt solution, a total of about 100,000 counts were recovered with the cells, corresponding to only 0.006–0.007% of the labeled toxin added and equivalent to only 600–700 toxin molecules per cell. Even this figure is high, however, since as later became evident from the radioautographs, only 5–10%

TABLE II
Effect of Antitoxin on Distribution of Radioactivity in Cultures of HeLa Cells Treated with ^{125}I -Labeled Diphtheria Toxin for 6 hr at 37°C

	No antitoxin	Antitoxin (4 units/ml)
Total number of cells in 100 ml	4.7×10^7	4.7×10^7
$^{125}\text{I}_{2,2}$ -toxin added, moles	1.0×10^{-9}	1.0×10^{-9}
Total iodine-125 remaining in supernate, <i>cpm</i>	3.64×10^9	3.49×10^9
Per cent precipitable by antitoxin*	97.3	97.5
Iodine-125 in 3rd washing, <i>cpm</i>	2×10^5	7.3×10^4
Iodine-125 collected with washed cells, <i>cpm</i>	3.0×10^5	5.3×10^4
Apparent number of toxin molecules "collected" per cell	1050	190

* Expressed as per cent of TCA-precipitable counts precipitated by antitoxin.

of the radioactivity collected with the cells was actually cell bound. The remaining 90–95% was in the form of insoluble highly radioactive aggregates of denatured protein (see Fig. 1). Since the toxin was passed through a Millipore filter immediately before the experiment, we must conclude that some surface denaturation occurred during the 6 hr period of stirring even in the presence of 5% calf serum.

Experiment 2—Effect of Antitoxin.—

A culture of HeLa cells (OD, 700 = 0.41) was centrifuged and resuspended in fresh medium containing 5% calf serum to a cell count of 4.7×10^5 /ml. The culture was divided into two 100 ml portions, to one of which was added 4 units/ml antitoxin 5353 previously passed through Sephadex G25 to remove traces of preservative. To each 100 ml was then added a total of 25 Lf labeled toxin containing 2.2 atoms ^{125}I per molecule of toxin protein. ^{14}C -leucine uptake was followed in small spinners containing the same concentrations of toxin and antitoxin. Protein synthesis was completely arrested within 4 hr at 37°C in the absence of antitoxin. After 6 hr at 37°C, the two cultures were centrifuged, washed, and the distribution of radioactivity determined as in the preceding experiment. The results are summarized in Table II.

As in Experiment 1, less than 0.008% of the added radioactivity was col-

lected with the washed cells. It is of interest to note that antitoxin reduced the recovered radioactivity still further, to the equivalent of less than 190 molecules per cell. Since the radioactive debris seen in the radioautographs was also greatly reduced in the presence of antitoxin, it seems almost certain that the insoluble radioactive material collected in the absence of antitoxin consisted mainly of aggregated and denatured toxin itself.

Radioautographs.—The cell-associated grain counts from the radioautographs of Experiment 1 are summarized in Table III. HeLa cells treated with 0.13 Lf/ml $^{125}\text{I}_2$ -toxin were compared with identically treated mouse L cells

TABLE III
*Fixation of $^{125}\text{I}_2$ -Toxin by Mammalian Cells**

Time	^{125}I decay	Average number of grain counts per cell†		
		HeLa	Mouse "L"	HeLa + excess unlabeled toxin
<i>days</i>	<i>%</i>			
7	7.5	6.9	1.9	1.6
14	15	12.1	6.0	2.6
21	21	15.1	7.8	5.0
28	28	22.0	11	10
60	50	(40)§	(20)	(18)

* Cells treated 6 hr with 0.13 Lf/ml $^{125}\text{I}_2$ -toxin at 37°C before washing and preparing for radioautography.

† Each figure represents average of more than 100 cells corrected for background counts.

§ Estimated counts in parentheses.

and with HeLa cells treated with the same amount of $^{125}\text{I}_2$ -toxin to which an 85-fold excess of unlabeled toxin had been added. The table gives the average number of grain counts per cell after subtracting background counts made on slides developed after 7, 14, 21, and 28 days of exposure to the photographic emulsion. The table also gives a predicted estimate of 40 cell-associated counts per HeLa cell at 60 days when 50% of all the iodine-125 atoms had decayed.⁴ Since the toxin preparation contained an average of 2.0 iodine-125 atoms per molecule, the grain count at 60 days should roughly approximate the number of toxin molecules bound per cell. As a first approximation, this would seem to be a reasonable assumption (i.e., 40 toxin molecules per cell). Although some decays cannot be recorded because they do not pass through the emulsion, others may give rise to tracks of multiple grain counts (Ada et al., reference 16). Furthermore, it seems probable that the HeLa cell-associated grain count may include some nontoxin-labeled material that is also bound to L cells, the binding of

⁴ At 60 days, there were too many grains for reliable counting.

which is unaffected by excess unlabeled toxin. Taking all of these considerations into account, we estimate that there are probably no more than 50 molecules of toxin fixed per cell.

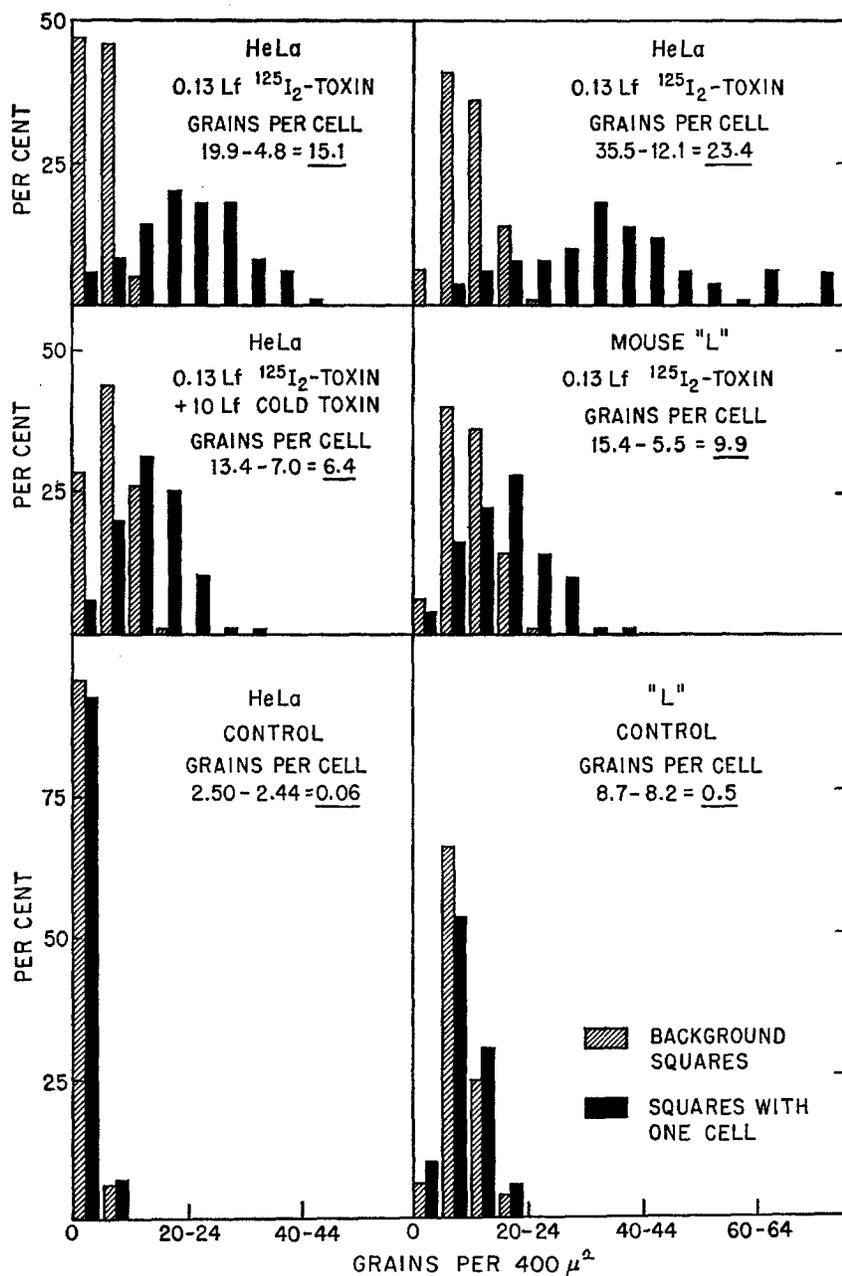
Table III shows that the toxin-resistant L cells fix less than half as much label as do HeLa cells under identical conditions. Moreover, it is clear that when HeLa cells are exposed to $^{125}\text{I}_2$ -toxin in the presence of a large excess of unlabeled toxin, the cell-associated grain count is reduced to about the same level as observed with L cells, suggesting, as mentioned above, that the residual activity may be nonspecific.

Although the radioautographs developed after 60 days of exposure showed too many grains for reliable counting, even a cursory inspection of the photographs shown in Figs. 2 and 3 will confirm the results given in Table III. Fig. 2 *d* shows the background of developed grains after 60 days of exposure of photographic emulsion to normal untreated HeLa cells. Figs. 2 *a*, 2 *b*, and 2 *c* show typical radioautographs of the $^{125}\text{I}_2$ -toxin-treated HeLa and L cell suspensions of Experiment 1.

In Text-fig. 1 the results of radioautographs developed after 28 days of exposure have been expressed in a different way. Two slides were studied in detail: one comparing $^{125}\text{I}_2$ -toxin-treated HeLa cells with similarly treated L cells and an untreated L cell control; the other showing the effect of excess unlabeled ("cold") toxin on $^{125}\text{I}_2$ -toxin by HeLa cells. The control for the latter slide was a smear of normal untreated HeLa cells. In each case, 100 successive $400\ \mu^2$ squares containing a single cell and 100 squares with no cells were counted. The per cent of squares with grain counts 0-4, 5-9, 10-14, etc., are shown graphically in the figure. In each case, the distribution of grain counts is close to that expected from a normal distribution curve. It will be noted from the figure that the background counts were not the same for both slides. In general, background counts tended to be higher in treated suspensions, probably because of the presence of minute traces of soluble labeled "toxoid," denatured toxin, or radioactive breakdown products. It is of interest that the background tended to be less with antitoxin-treated HeLa cells and there was almost no uptake of label by HeLa cells exposed to $^{125}\text{I}_{2,2}$ -toxin in the presence of excess antitoxin. This is clearly shown in Figs. 3 *a* and 3 *b*.

Location of Cell-Bound Toxin.—Figs. 3 *c* and 3 *d* show radioautographs of 1-2 μ sections of the same $^{125}\text{I}_{2,2}$ -toxin-treated HeLa suspension seen in Fig. 3 *a*. The emulsion was exposed for 30 days in each case. It will be seen at once that the developed grains lie almost exclusively at the periphery of the cells. Clearly, the bulk of the cell-bound toxin is fixed to the cell membrane and never reaches the cell interior at all.

Amino Acid Pool Size in Normal and Intoxicated Cells.—In the preceding paper (3), we suggested the possibility that toxin might interfere with peptide bond formation at certain critical sites on the cell membrane involved in trans-



TEXT-FIG. 1. Distribution of grain counts in radioautographs of normal and ¹²⁵I₂-toxin-treated HeLa and L cells after a 28 day exposure time. For each smear, 100 squares containing one cell (solid bars) and 100 squares containing no cell (hatched bars) were counted. The height of each bar gives the per cent of squares with counts of 0-4, 5-9, 10-14, etc. It is clear that the envelope closely approximates a normal distribution curve in each case.

port of an essential amino acid. Such a possibility now appears to be extremely unlikely. Moehring et al. (17) have recently shown that transport of the amino acids methionine and α -aminoisobutyrate across KB or HeLa cell membranes continues even after all protein synthesis has ceased. We have confirmed their observations by determining the pool size of the individual amino acids in normal and intoxicated HeLa cells.

3 ml of packed normal cells and 3 ml of packed intoxicated cells (3 Lf/ml \approx approximately 10 saturating doses of toxin for 5 hr), each from about 1500 ml culture, were washed twice with

TABLE IV
Free Amino Acids in Normal and Intoxicated HeLa Cells

Amino acid	Pool size		Medium concentration
	Normal	Intoxicated*	
	<i>μmoles/ml packed cells</i>		<i>μmoles/ml</i>
Glutamic acid	2.23	2.10	2.0
Ammonia	2.05	1.72	—
Proline	0.45	0.46	—
Glycine	0.305	0.37	—
Alanine	0.51	0.46	—
Valine	0.58	0.55	0.2
Isoleucine	0.53	0.53	0.4
Leucine	0.52	0.53	0.4
Tyrosine	0.42	0.42	0.2
Phenylalanine	0.32	0.32	0.2
Lysine	0.06	0.14	0.4
Histidine	0.34	0.28	0.2
Arginine	0.12	0.185	0.6

* Intoxicated cell concentrations were normalized by a factor of 1.06.

50 ml changes of Eagle's salt solution and then extracted with 8 ml 5% TCA at 0° overnight. The cells were centrifuged and washed once with 3 ml cold 5% TCA. The combined supernate and washing, measuring 10 ml in each case, was extracted five times with 20 ml changes of ether. The aqueous layers were evaporated to dryness, redissolved in 1 ml water and analyzed in the Spinco amino acid analyzer. Table IV compares the pool sizes of individual amino acids extracted by cold TCA from packed normal and intoxicated HeLa cells with the amino acid composition of the culture medium.

It will be noted that the only differences of possible significance are seen with the two basic amino acids, lysine and arginine, where the results seem to indicate a somewhat higher concentration in the intoxicated than in the normal cells. For these two amino acids, however, an excess was added to the medium to compensate for destruction by cellular enzymes. In addition to the amino acids listed in Table IV, the analysis showed that the combined totals of threonine + serine + glutamine + asparagine + aspartic acid + methionine

sulfoxide amounted to 7.7 and 8.0 μ moles/ml of packed normal and intoxicated cells, respectively. These amino acids are not readily separable in the amino acid analyzer.

DISCUSSION

It has been demonstrated that in the presence of just under one saturating dose of highly purified diphtheria toxin labeled with two atoms of carrier-free iodine-125 per molecule, HeLa cells in spinner culture take up no more than 0.0005% of the added label within a period of 6 hr at 37°C. The maximum number of toxin molecules fixed, as estimated from the radioautographs, is probably less than 50 out of some 10^7 molecules added per cell. Moreover, radioautographs of sectioned cells clearly show that the toxin molecules are bound at the surface membrane. Thus it is unlikely that any significant number of toxin molecules ever succeed in reaching the cytoplasm or nucleus of the cell. Even this small number of toxin molecules attached to the cell membrane suffices to bring all protein synthesis by the cell to a complete standstill within 3 hr.

The labeled toxin used in our experiments was of high purity as evidenced by the fact that 98% of the TCA-precipitable radioactivity was also precipitable by a highly specific antitoxin. Nevertheless, it could be argued that the small number of cell-associated grain counts seen in the radioautographs may not be attributable to toxin itself but rather to minor traces of some labeled impurity with a high affinity for cell membranes. Even the fact that a highly specific antitoxin prevents fixation of radioactivity by the cells cannot be taken as proof that the grains seen are due to toxin. The action of antitoxin might conceivably be due to the presence of small amounts of antibody (not detected by immunoelectrophoresis) directed against the impurity. In our opinion, the strongest evidence against such an unlikely interpretation is our observation that mouse L cells take up relatively little labeled toxin. Although amino acid incorporation in crude extracts from L cells is just as sensitive to inhibition by toxin as in similar extracts from HeLa cells (3), it has long been known that living cultures of L cells are not affected by toxin except at very high concentration (11, 18). While it is true that radioautographs of $^{125}\text{I}_2$ -toxin-treated L cells did show some increase above background in cell-associated grain counts, the increase was no more than that observed with HeLa cells treated similarly in the presence of an 85-fold excess of unlabeled toxin. The failure of toxin to react with mouse cell membranes provides a satisfying explanation for the natural resistance of that species to diphtheria toxin.

The finding that only a few molecules are actually fixed by sensitive cells even when treated with a saturating dose of toxin, suggests that its mode of action is probably the same at all concentrations. Presumably, when cells are exposed to concentrations of toxin that are less than a saturating dose (i.e. $<10^{-8}$ M), diffusion becomes rate-limiting and more time is required for all of

the receptor sites to become saturated. Even at a toxin concentration of 10^{-13} M (approximately 300 molecules/cell) growth of certain sensitive mammalian cell lines stops within 3 days (9-11).

At the present time we can do no more than speculate as to how so few molecules of toxin bound at the cell surface can bring about the complete and irreversible cessation of protein synthesis by the whole cell. We suggested earlier that toxin might interact with transferase II located at certain strategic sites on the cell membrane where peptide bonds are formed that are critical for membrane function. It was suggested that transport of a particular amino acid across the membrane might be the essential function involved. However, this is clearly not the case, as we have now shown that the individual amino acid pool sizes of normal and intoxicated cells are identical within limits of experimental error, and Moehring et al. (17) have found that intoxicated KB and HeLa cells continue to transport certain amino acids across the cell membrane long after protein synthesis has been arrested. We now suggest that the postulated critical receptor sites on the cell membrane might be the location of those particular polysomes involved in the synthesis of transferase II, the enzyme known to be inactivated by toxin in cell extracts (4, 6). Interaction of toxin with nascent transferase II might cut off, at its source, the supply of this enzyme, which is essential for synthesis of all cellular proteins.

The action of diphtheria toxin on mammalian cells is strikingly reminiscent of the action of certain colicines on their bacterial hosts. In particular, Nomura (19) has shown that only a few colicine E3 molecules fixed to specific receptor sites on the bacterial cell surface suffice to inhibit protein synthesis completely by a sensitive strain of *Escherichia coli* without affecting either DNA or RNA synthesis. The failure of colicine to penetrate to the cell interior was proved by the finding that even when bound to the cell, colicines K and E3 could be destroyed by trypsin and the inhibition of protein synthesis reversed. Like colicine E3, diphtheria toxin is extremely sensitive to inactivation by trypsin. Unfortunately, our attempts to reverse the action of diphtheria toxin by treatment of washed intoxicated cells with trypsin have been unsuccessful.⁵

Our failure to obtain any evidence for penetration of toxin molecules into the cell cytoplasm raises the interesting question of whether HeLa or L cells are capable of protein uptake by pinocytosis as has been reported in the literature (20, 21). We have observed that iodine-125-labeled toxin added to crude extracts from normal HeLa cells undergoes fairly rapid proteolysis with the splitting off of low molecular weight radioactive fragments. No such degradation is seen when toxin and whole cells are incu-

⁵ The activity of the trypsin used in these experiments was checked by adding 20 saturating doses of toxin to a suspension of HeLa cells already containing the same amount of trypsin and incubating under the identical conditions used with intoxicated cells. Amino acid incorporation proceeded normally for more than 5 hr, indicating that at least 98% of the toxin had been rendered ineffective by trypsin before it could become fixed to the cells.

bated together for many hours. Ehrenreich and Cohn (22) have recently reported experiments similar to our own on the uptake of iodinated human serum albumin (HSA) by cultured macrophages. HSA is a protein of about the same size and charge as diphtheria toxin. In their experiments, about 0.002% of the label remained with the cells after a 24 hr "pulse" of 5×10^7 cpm ^{125}I -HSA. It is not entirely clear from their paper whether Ehrenreich and Cohn ruled out the presence of small amounts of insoluble radioactive aggregates of denatured HSA such as we have seen in our experiments with labeled toxin (Fig. 1). Thus it is not certain whether the radioactivity was taken up by their macrophages through a process primarily of phagocytosis or of pinocytosis. Somewhat similar experiments were carried out by Mesrobian et al. (23), who reported on the pinocytosis of fluorescein-labeled diphtheria toxin by guinea pig peritoneal leukocytes. In our opinion, their experiments are not convincing since they used very high concentrations of labeled toxin and apparently failed to consider the possibility of phagocytosis of particulate fluorescent aggregates of denatured toxin that were almost certainly present.

Chapman-Andersen (24) and Holter (25) have pointed out that it is becoming increasingly difficult to distinguish between phagocytosis and pinocytosis. So far as we are aware, no one has observed active phagocytosis of particles by HeLa cells or by L cells in spinner cultures, and the evidence for pinocytosis by these strains rests mainly on morphological observations of vesicle formation. Toxin does not appear to affect vesicle formation. On the other hand, most studies on pinocytosis have been carried out with actively phagocytic cells such as amebae and macrophages. The present experiments have raised the question as to whether pinocytosis can take place at all in the complete absence of particulate material, that is, in the absence of any phagocytosis.

SUMMARY

Using the technique of radioautography, it has been shown that a probable maximum of only 25–50 molecules iodine-125-labeled toxin per cell is bound by human HeLa cells treated with approximately 10^7 molecules of toxin per cell, or just under one saturating dose. Radioautographs of sections from labeled cells locate most if not all of the toxin molecules fixed to the outer cell membrane. Under identical conditions far less label is taken up by mouse L cells. It is probable that the resistance of this species to diphtheria toxin can be accounted for in terms of the failure of mouse cells to bind the toxin protein.

The irreversible inhibition of protein synthesis in a living cell culture by a few molecules of toxin located at the cell surface is discussed in relation to the known interaction between toxin, NAD, and transferase II in mammalian cell extracts.

We are grateful to Mr. Fred Casella of the Polaroid Corporation for his expert preparation of 1–2 μ sections of intoxicated HeLa cells. We are also indebted to Dr. Guido Guidotti, Biology Department, Harvard University, for helpful discussions and for analysis of the amino acids in pools extracted from normal and intoxicated cells.

Addendum.—After this paper was already in press, we were informed by Dr. Ronald Goor of his finding that diphtheria toxin can exist in the form of a fully toxic dimer

(Goor, R. S. 1968. *Nature*. 217:1053). We, therefore, examined in the ultracentrifuge the behavior of the preparation used in the present studies. The toxin sedimented as a single 6.6S component instead of the expected 4.3S. Examination of several preparations has revealed the presence of a 6.6S component *only* when merthiolate was used as preservative. Although our preparation showed no mercury when tested in Dr. Bert Vallee's laboratory, it seems probable that merthiolate does catalyze the formation of dimers. It is thus possible that the number of toxin binding sites per HeLa cell may be even lower than we have estimated.

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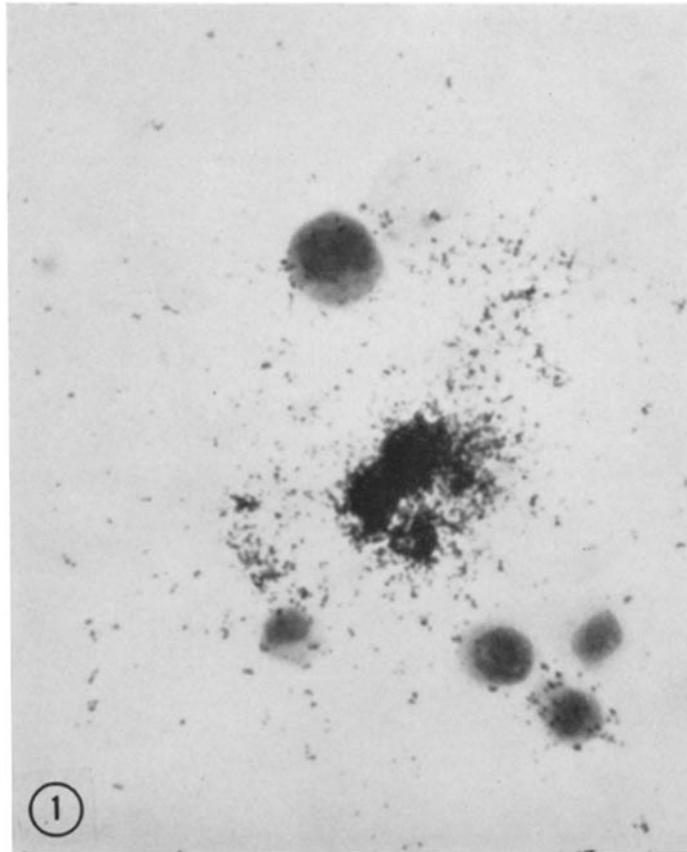
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EXPLANATION OF PLATES

PLATE 103

FIG. 1. $^{125}\text{I}_2$ -toxin-treated HeLa cells showing radioactive aggregate of denatured toxin. About 90–95% of all the radioactivity "collected" with the washed intoxicated cells was present in such aggregates. Areas containing radioactive debris were avoided in making grain counts. Exposure time, 28 days. $\times 1050$.



(Pappenheimer and Brown: Mode of action of diphtheria toxin. VI)

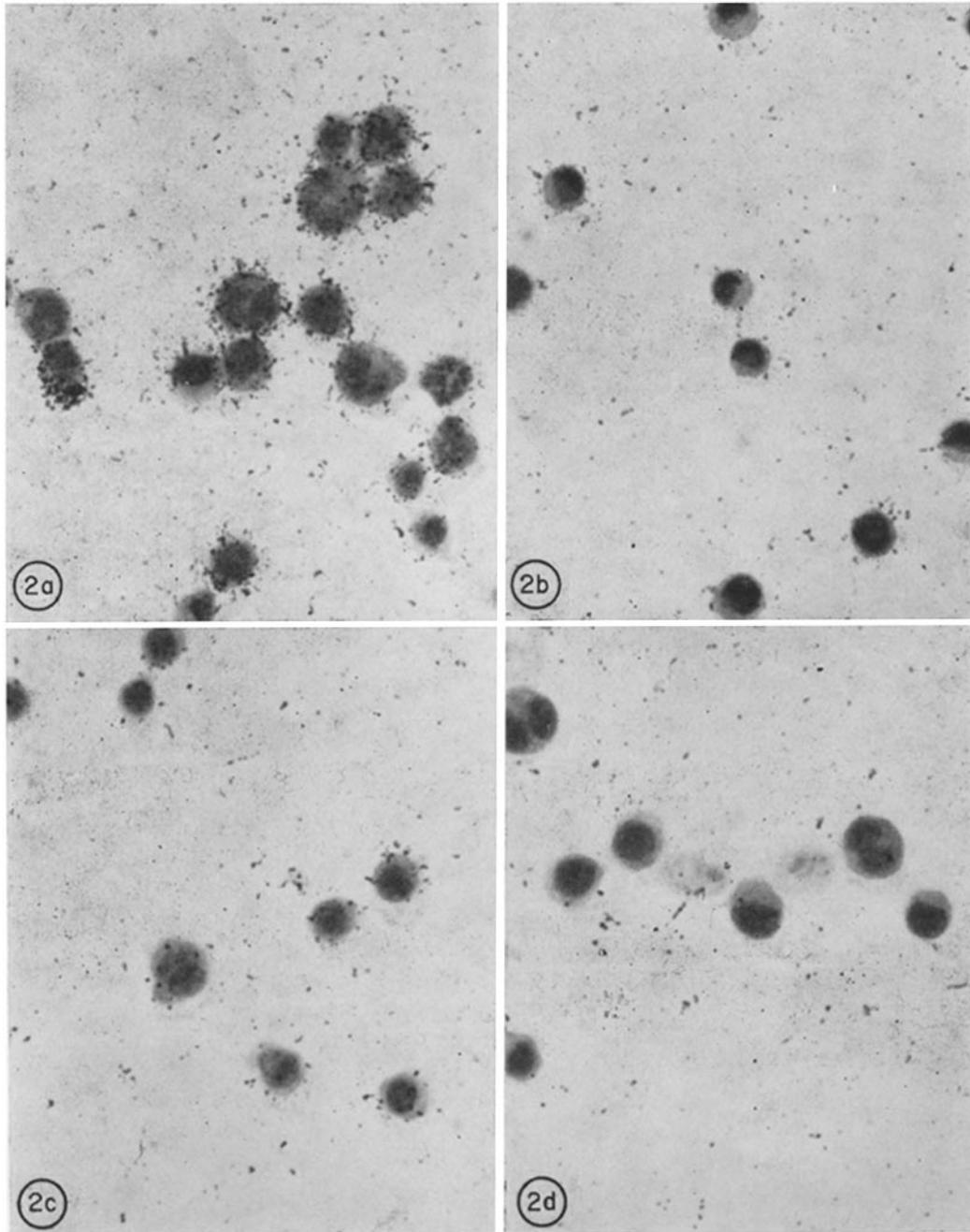
PLATE 104

FIG. 2 *a*. HeLa cells treated with $^{125}\text{I}_2$ -toxin (0.13 Lf/ml). Average cell-associated grain count was about 40 above background. Exposure time, 60 days. $\times 790$.

FIG. 2 *b*. HeLa cells treated with $^{125}\text{I}_2$ -toxin (0.13 Lf/ml) + unlabeled toxin (11 Lf/ml). Exposure time, 60 days. $\times 790$.

FIG. 2 *c*. L cells treated with $^{125}\text{I}_2$ -toxin (0.13 Lf/ml). Exposure time, 60 days. $\times 790$.

FIG. 2 *d*. Normal untreated HeLa cells showing a background of grains *not* associated with cells. Exposure time, 60 days. $\times 850$.



(Pappenheimer and Brown: Mode of action of diphtheria toxin. VI)

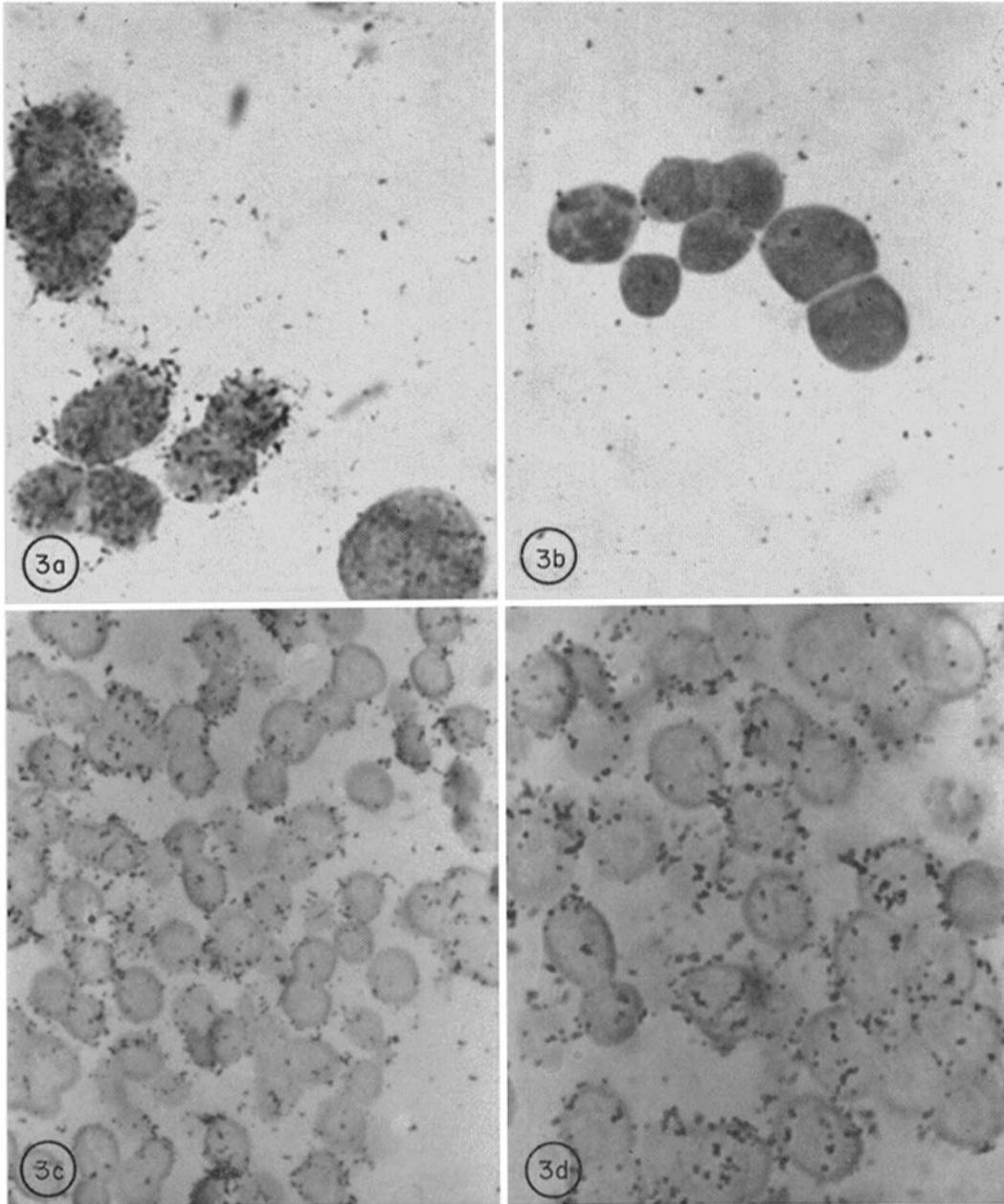
PLATE 105

FIG. 3 *a*. HeLa cells treated with $^{125}\text{I}_{2,2}$ -toxin (0.25Lf/ml). Exposure time, 30 days. $\times 1300$.

FIG. 3 *b*. HeLa cells treated with $^{125}\text{I}_{2,2}$ -toxin (0.25 Lf/ml) in the presence of 4 units/ml antitoxin. Exposure time, 30 days. $\times 1300$.

FIG. 3 *c*. Sections ($2\ \mu$) of the same $^{125}\text{I}_{2,2}$ -toxin-treated cell suspension shown in Fig. 3 *a*. Exposure time, 30 days. $\times 790$.

FIG. 3 *d*. Sections ($2\ \mu$) of the same $^{125}\text{I}_{2,2}$ -toxin-treated cell suspension shown in FIG. 3 *a*. Exposure time, 30 days. $\times 1300$.



(Pappenheimer and Brown: Mode of action of diphtheria toxin. VI)