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Activity of Diphtheria Toxin

II. Early Events in the Intoxication of HeLa Cells

JAMES L. DUNCAN AND NEAL B. GROMAN

Department of Microbiology, Northwestern University Medical and Dental Schools, Chicago, Illinois 60611, and Department of Microbiology, School of Medicine, University of Washington, Seattle, Washington 98105

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The initial steps in the interaction of diphtheria toxin with HeLa cells were studied. It was demonstrated that lethal doses of toxin are rapidly adsorbed to the cell. The kinetics of uptake, as measured by lethality, indicated that a single toxin molecule is able to cause cell death. Studies on the effect of *p*H on intoxication showed that adsorption of toxin occurred over a wide *p*H range but was partially inhibited at high *p*H values. Experiments to determine the influence of the ionic environment on intoxication indicated that adsorption of toxin did not take place in the absence of salts and was partially inhibited in the presence of a polyanion. The evidence indicates that the initial binding of toxin to the cell is electrostatic in nature, involving positively charged surface groups. Attempts to demonstrate specific receptors for the attachment of toxin to cells were unsuccessful, suggesting that toxin adsorption may be a nonspecific process. The effect of energy inhibitors on intoxication was examined. Sodium fluoride, an inhibitor of glycolysis, almost completely prevented intoxication in HeLa cells, whereas inhibitors of respiration and oxidative phosphorylation had no effect. Sodium fluoride did not prevent adsorption of toxin but appeared to inhibit a later step in the intoxication process, perhaps the transport of toxin to subsurface or intracellular levels.

Recent studies on the mode of action of diphtheria toxin have shown that toxin inhibits the incorporation of amino acids into polypeptides in extracts of susceptible cells (4). This inhibition was dependent on a cofactor identified as NAD (nicotinamide adenine dinucleotide). Collier (3) used a cell-free protein-synthesizing system from rabbit reticulocytes, and found that toxin had no effect on ribosomes or polysomes and did not release nascent polypeptide chains, but inactivated an enzyme necessary for peptide synthesis, transferase II. These findings were subsequently confirmed in other laboratories (8, 17) and, in addition, Goor, Pappenheimer, and Ames (10) found that nicotinamide reversed the inactivation of transferase II when added to intoxicated extracts.

Honjo et al. (12) found that, in the presence of toxin, the adenosine diphosphate ribose (ADPR) portion of NAD was bound to transferase II, an event linked to the loss of transferase activity. The reaction was reversible, and they suggested that toxin acted as an enzyme in the reaction. Gill et al. (6) showed that in the presence of toxin NAD was hydrolyzed to yield free nicotinamide, and that transferase activity was concomitantly lost. Furthermore, when intact cells were treated with toxin, the inactive ADPR derivative of transferase II accumulated intracellularly.

It is known that exposure of susceptible cell cultures to only a few hundred molecules of toxin per cell is sufficient to kill the cells (19). Pappenheimer and Brown (18) studied the uptake of toxin labeled with ^{125}iodine by HeLa cells and found that only 25 to 50 molecules of toxin were bound per cell after exposure to a saturating dose of toxin (10^7 molecules). Furthermore, radioautographs indicated that most of the bound toxin molecules were fixed to the outer cell membrane. To explain the lethal effect of diphtheria toxin on intact living cells, Gill et al. (6) proposed that a few toxin molecules become fixed in the cell membrane. At these sites, the toxin molecules catalyze the formation of the inactive ADPR-transferase II complex, thus inactivating the cell's complement of transferase II.

Little is known about the initial interaction of toxin with susceptible cells. In this study, we examined the characteristics of toxin adsorption to HeLa cells and the subsequent step(s) leading to irreversible toxicity.

MATERIALS AND METHODS

Cells. Stock Hela cell cultures were maintained as monolayers in 16-oz (0.473 liter) prescription bottles. The cells were fed every 48 hr and were transferred at 5 to 7 days as previously described (5). To prepare the cell cultures used in the experiments, cells from one 16-oz (0.473 liter) bottle were transferred to twelve to fifteen 2-oz (59 ml) French square bottles. These cells received fresh medium after 2 days and were used on the 2nd or 3rd day after transfer.

Media. Cell cultures were maintained in Eagle's minimum essential medium or in Hanks balanced salts solution (HBSS) containing 0.1% yeast extract and 0.1% proteose peptone no. 3 (Difco). The media was supplemented with 8% calf serum and penicillin (100 units/ml), streptomycin (100 μg/ml), and nystatin (25 units/ml).

Toxin and antitoxin. Diphtheria toxin (5× crystallized) prepared at the Wellcome Research Laboratories, Beckenham, England, was provided by A. M. Pappenheimer, Jr. In some experiments, a partially purified preparation of toxin [1,520 flocculating units (Lf)/ml], obtained from J. M. B. Corkill (Connaught Medical Research Laboratories, University of Toronto, Canada), was used. Working stock solutions, from which fresh toxin was prepared for each experiment, contained 43.6 Lf per ml of sterile distilled water. Unless otherwise stated, toxin solutions were prepared by dilution in HBSS to give 0.3 Lf/ml. This toxin concentration was employed in most experiments, since it was the minimum concentration at which most (> 90%) of the cells on the monolayer would become intoxicated during the 1-hr exposure period. HBSS without toxin served as the control.

Diphtheria antitoxin was obtained from Wyeth Laboratories, Marietta, Pa. Antitoxin solutions contained approximately 5 units/ml in complete medium.

Determination of cytotoxicity. After exposure to toxin or control solutions and after appropriate washing procedures, the cultures received complete medium and were allowed to incubate for 3 to 4 days. During this time, almost all of the cells in the toxin-treated cultures rounded up and floated off of the glass surface into the medium; they were considered nonviable. To determine the viability of those cells remaining in the toxin-treated cultures and the control cells, the cultures were then washed with saline and exposed to 0.3 ml of 0.05% trypsin for 15 min and the dispersed cells were diluted in saline. A trypan blue solution (0.2 mg/ml) was added to the cell suspension to a concentration of 10% (v/v). After 2 min, samples were removed for counting. The cells were counted in a hemacytometer, and the number of viable (unstained) and dead cells in the cultures was calculated. All counts were made at least in duplicate. In the experiments reported here, cytotoxicity is expressed as the number of viable cells remaining in toxin-treated cultures, given as a per cent of viable cells in the controls.

Reagents. Diethylaminoethyl (DEAE)-dextran was obtained from Pharmacia, Uppsala, Sweden. Heparin (sodium) was purchased from Invenex, San Francisco, Calif.

The following enzymes were used: trypsin (1:250) from Difco; pancreatin, lipase (steapsin), chymotrypsin, and pepsin from Nutritional Biochemicals Corp., Cleveland, Ohio; lysozyme and hyaluronidase from Armour Pharmaceutical Co., Kankakee, Ill.; neuraminidase (from *Vibrio cholera*) from General Biochemicals, Chagrin Falls, Ohio; elastase from Mann Research Laboratories, New York, N.Y.

RESULTS

Adsorption of toxin. Although toxin appears to adsorb rapidly to susceptible cells (21, 23), no studies on the kinetics of this process have been reported. We attempted to study the rate at which cells in a population adsorb a lethal dose of toxin. To separate the adsorption process from subsequent steps in intoxication, ammonium chloride, an inhibitor of toxin action, was used. Kim and Groman (14) found that, when cells were exposed to toxin in the presence of various ammonium salts and amines, intoxication did not occur; they concluded that in the presence of ammonia toxin adsorbed to the surface of the cell, but subsequent steps leading to irreversible toxicity were inhibited (15).

HeLa cell cultures were exposed to toxin or control solutions in the presence of ammonium chloride (0.2 mg/ml) at 37 C for various lengths of time. The solutions were removed, and the cells were washed once with warm HBSS. Complete medium was added to the cells; they were incubated for 3 to 4 days at 37 C and then counted.

With a concentration of 1.0 Lf of toxin per ml, 80% of the cells adsorbed a lethal dose of toxin within 1 min (Fig. 1). Exposure for 4 to 5 min was sufficient to kill greater than 90% of the cells. When exposed to 0.436 Lf of toxin per ml, approximately 20 min was required for 80% of the cells to adsorb a lethal dose. In some experiments, a second set of controls received antitoxin after exposure to toxin plus ammonium chloride for 30 min. No cytotoxic effects were observed, indicating that in the presence of ammonium chloride the toxin had remained accessible to antitoxin.

Effect of pH on intoxication. Little is known about the environmental factors involved in intoxication. The toxin molecule and the cell both carry a net negative charge at physiological *p*H values, and one would predict that ionized groups on the cell membrane and on the toxin molecule itself might participate in the attachment of toxin to the cell.

To study the effect of *p*H on intoxication, cell cultures were exposed for 1 hr to toxin in HBSS or control solutions adjusted to various *p*H values with NaOH or HCl. Toxin was removed and the cells were washed with antitoxin. Complete

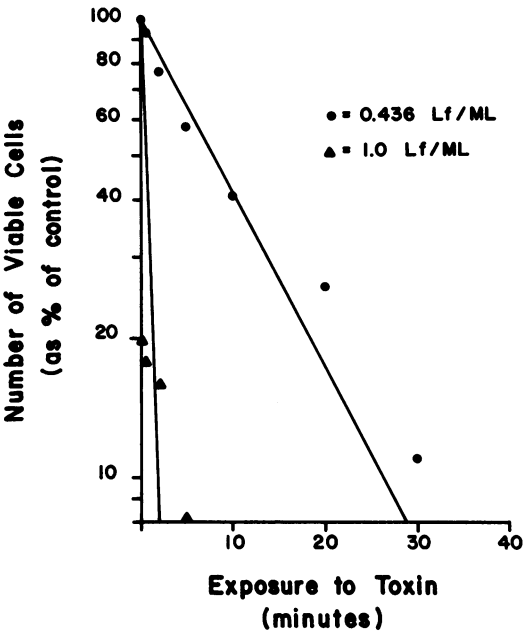

FIG. 1. *Rate of adsorption of a lethal dose of toxin to HeLa cells.*

medium was added, and the cells were counted after 4 to 5 days (Table 1).

Intoxication occurred over a wide *p*H range but was inhibited at high *p*H values. There was no effect on the control cultures at these *p*H values; the monolayers were intact and the cells exhibited normal morphology. Control monolayers were destroyed at *p*H 4.5 and 10. In an additional experiment at *p*H 9.7, no antitoxin was given; the monolayers were washed twice with HBSS at *p*H 9.7 after toxin exposure. These monolayers were only partially intoxicated, suggesting that toxin attachment is inhibited at this *p*H, and that electrostatic forces may be involved in the attachment of toxin to the cell.

Effect of the ionic environment on intoxication. To study further the importance of ionized surface groups in the adsorption of toxin, cells were exposed to toxin in the presence of various substances having an effect on the ionic environment. After incubation for 1 hr, the cells were washed with antitoxin or with the agent present in the toxin solution. Complete medium was added, and the cells were observed for 4 to 5 days for evidence of cytopathic effects (Table 2).

It appears that adsorption of toxin does not take place in the absence of ions; toxicity was not observed when the cells were exposed to toxin in sucrose, even though no antitoxin was given. Toxin adsorption was partially inhibited in the presence of the polyanion heparin. Adsorption and intoxication occurred normally in the presence of DEAE-dextran, a polycation. The results tend to support the hypothesis that positively charged groups are involved in the attachment of toxin to the cell.

One would expect that an electrostatic interaction between toxin and cell would be influenced by the salt composition of the medium. To examine the possibility that divalent salts are required to form bridges between the toxin molecule and the cell, cultures were exposed to toxin in HBSS containing ethylenediaminetetraacetic acid (EDTA; 0.2 mg/ml), in HBSS containing no Ca^{++}, and in Ca^{++}-free HBSS containing EDTA. After a 30-min exposure period, the cells were washed with the same medium lacking toxin and were incubated in complete medium for 3 to 4 days. In all cases, intoxication occurred, indicating that divalent cations are not required for the adsorption of toxin.

The influence of the salt environment on toxin adsorption was examined in more detail. Isotonic

TABLE 1. *Effect of pH on intoxication*[a]

*p*H	Cytotoxicity[b]
4.7	++++
5.0	++++
6.4	++++
7.1	++++
8.0	++++
9.2	+++
9.7	−

[a] HeLa cells were exposed to toxin in HBSS at each *p*H for 1 hr.

[b] Symbols: ++++, 75 to 100% destruction; +++, 50 to 74% destruction; ++, 25 to 49% destruction; +, 5 to 24% destruction; −, 0 to 5% destruction.

TABLE 2. *Effect of the ionic environment on intoxication*[a]

Toxin in	Concn (in water)	Cytotoxicity[b]	
		With antitoxin	Without antitoxin
Sucrose	0.3 M	−	−
Heparin	150 units/ml	±	±
DEAE-dextran	300 μg/ml	+	+
HBSS		+	+

[a] HeLa cells were exposed to toxin in each solution for 1 hr.

[b] Symbols: ±, approximately 50% destruction of monolayer; +, total destruction of monolayer; −, 0 to 5% destruction.

sucrose solutions (0.3 M) containing various concentrations of NaCl, $CaCl_2$, or $AlCl_3$ were prepared. Cell cultures were washed twice with one of the solutions and exposed to toxin in the same solution. After incubation for 30 min at 37 C, toxin was removed and the cultures were again washed two times with the sucrose-salt solution. Complete medium was added, and the cells were incubated and counted at 3 to 4 days.

Toxin adsorption took place in the presence of each of the salts (Table 3). It appears that on a concentration basis trivalent cations are more effective in mediating adsorption than are divalent tcations, and divalent cations are more effective than monovalent cations. At the highest concentration of $CaCl_2$, toxin adsorption apparently decreased.

Effect of enzyme treatment on intoxication. The adsorption of enteroviruses to susceptible host cells is mediated by specific virus-binding receptor material (11), and certain enzymes can selectively inactivate these receptors on HeLa cells without affecting cell viability (27). Schaeffer, Gabliks, and Calitis (22) found that trypsin treatment of human embryonic intestine cells rendered them resistant to staphylococcal entertoxin B for 48 hr. The following experiment was performed in an attempt to demonstrate a similar "receptor" phenomenon in the diphtheria toxin-HeLa cell system.

HeLa cell monolayers in 16-oz (0.473 liter) bottles were washed twice with saline and exposed to the proteolytic enzymes trypsin, pancreatin, Pronase, chymotrypsin, or elastase for various times at 37 C. The cells were dispersed by pipetting and added to complete medium. Monolayers in French square bottles were prepared in the usual manner. After a 2-hr incubation period, during which most of the cells became attached to the glass, the complete medium was gently removed and the cells were washed with saline. Toxin or control solution was added for 1 hr and then removed, and the cells were washed with antitoxin. Complete medium was added, and the cells were observed for several days for monolayer formation. We found that treatment with proteolytic enzymes was unable to prevent intoxication of the cells; no monolayer was formed (Table 4).

The enzymes neuraminidase, lysozyme, and hyaluronidase were added to cell monolayers for 1 hr at 37 C. The monolayers were washed twice with saline and exposed to toxin or control solution for 1 hr. The cells were washed with antitoxin, complete medium was added, and the monolayers were observed for 5 days for cytopathic effects. Table 4 shows that treatment of cells with these enzymes did not prevent intoxication. Lipase (0.1%) and pepsin (0.1%) proved to be toxic for the cells.

Effect of energy inhibitors on intoxication. If toxin molecules are actually transported into the cell, one would predict that this penetration would be an energy-requiring process; however, no energy requirement for intoxication has been shown (15, 23). Because specific inhibitors of glycolysis were not used in earlier investigations, the effect of energy inhibitors on intoxication was re-examined.

HeLa cell monolayers were exposed to various inhibitors in HBSS for 1 hr and then exposed to toxin plus inhibitor for an additional hour. The cells were washed with HBSS and incubated in complete medium containing antitoxin. The cells were observed for cytotoxicity and counted at 4 to 5 days (Table 5). Potassium cyanide, an agent which inhibits respiration by blocking electron transport, and 2,4-dinitrophenol, an agent which uncouples oxidative phosphorylation, gave no protection. Iodoacetate, an inhibitor of glycolysis, appeared to give no protection at 1.3×10^{-4} M; higher concentrations were tested, but interpretation of the results was com-

TABLE 3. *Effect of salt concentration on toxin adsorption*[a]

Concn	No. of viable cells (as per cent of control)		
	NaCl	$CaCl_2$	$AlCl_3$
M			
10^{-1}	22	46	ND[b]
10^{-2}	92	<5	ND
10^{-3}	98	30	<5
10^{-4}	100	86	26

[a] HeLa cells were exposed to toxin for 30 min in isotonic sucrose solutions containing various salt concentrations.

[b] Not done.

TABLE 4. *Effect of enzyme treatment on intoxication*

Enzyme	Concn	Time of exposure	Cytotoxicity[a]
	%	*min*	
Trypsin	1.0	60	+
Pancreatin	0.1	30	+
Chymotrypsin	1.0	60	+
Elastase	0.5	60	+
Pronase	0.01	30	+
Neuraminidase	50[b]	60	+
Lysozyme	0.5	60	+
Hyaluronidase	0.25	60	+

[a] Symbol: +, total destruction of intact monolayers or inhibition of monolayer formation.

[b] Expressed as units per milliliter.

plicated by the fact that iodoacetate itself is somewhat toxic for HeLa cells. Sodium fluoride, an inhibitor of glycolysis, was not itself toxic for HeLa cells at approximately 10^{-2} M and provided almost complete protection against diphtheria toxin. In an additional experiment, HeLa cells were exposed to NaF and toxin as before. The toxin solution was removed and the cells were washed three times with NaF in HBSS; no antitoxin was given. These monolayers subsequently showed cytopathic effects and were destroyed. Controls with NaF alone were normal.

To determine whether glycolysis was indeed inhibited under the experimental conditions described above, cell cultures were exposed to 3 ml of NaF (10^{-2} M) in HBSS or HBSS alone and were incubated at 37 C. The cultures were removed at various times, and glycolytic activity was measured by determining the amount of lactic acid formed, by the method of Barker and Summerson (1). An appreciable inhibition of lactic acid formation in the cultures treated with NaF is shown in Table 6.

The results of these experiments show that sodium fluoride does not prevent adsorption of toxin, but appears to inhibit a later step in intoxication, perhaps the penetration of toxin into the cell.

TABLE 5. *Effect of energy inhibitors on intoxication*[a]

Inhibitor	Concn	No. of viable cells (as per cent of control)
	M	
KCN	5.0×10^{-4}	<5
2,4-Dinitrophenol	6.0×10^{-5}	<5
Iodoacetic acid	1.3×10^{-4}	<5
NaF	2.0×10^{-2}	93

[a] HeLa cells were exposed to inhibitor for 1 hr, and then incubated in inhibitor plus toxin for 1 hr.

TABLE 6. *Effect of sodium fluoride on lactic acid formation in HeLa cells*[a]

Exposure time (hr)	Culture	Amt of lactic acid/ml
0		0.7
1	Control	1.6
	NaF added	0.9
2	Control	2.6
	NaF added	1.6

[a] HeLa cells were exposed to 10^{-2} M NaF in HBSS or to HBSS alone.

DISCUSSION

The experiments just described indicate that toxin adsorbs rapidly to HeLa cells and suggest that the adsorption process is mediated by an electrostatic interaction between the toxin molecule and the cell surface. The diphtheria toxin molecule is strongly electronegative at physiological *p*H values, with an isoelectric point at *p*H 4.1 (25). The cell also bears a net negative charge (20, 24). Several lines of evidence suggest that electrostatic forces are involved in the initial adsorption of toxin to the cell. The adsorption rate of toxin at various temperatures is unknown, but Strauss and Hendee (23) observed that toxin did adsorb to HeLa cells at 10 C. The results of the present investigation indicate that the ionic state of surface groups is important in the adsorption of toxin to the cell. Thus, toxin adsorption does not occur in the presence of isotonic sucrose and is partially inhibited in the presence of a polyanion. Toxin adsorption occurs in the presence of a polycation and at various salt concentrations in isotonic sucrose.

One explanation for these results is that in the absence of salts (or cations) the repulsive force between the negatively charged toxin molecule and the cell is too high to permit contact. Adding cations would reduce the repulsive potential and permit contact. Evidence from the *p*H studies also lends support to this explanation. Adsorption of toxin occurred over a wide *p*H range in HBSS, but was partially inhibited at around *p*H 9.7. At this *p*H, positively charged amino groups are neutralized and an increase in the net negative charge occurrs. It is also possible that the neutralization of positive charges causes an electrostatic or configurational change, or both, to take place; this prevents the correct "fit" necessary for toxin-cell interaction. Finally, antitoxin prevented any cell killing in cultures treated with toxin at *p*H 9.7, although some adsorption did occur. This suggests that an additional step(s) leading to irreversible toxicity is *p*H-sensitive.

Attempts to demonstrate specific receptors for the attachment of toxin were unsuccessful. Specific receptor sites are known to be involved in the attachment of some animal viruses and perhaps in the intoxication of cell cultures by staphylococcal enterotoxin B (11, 22). There is little evidence, however, that specific receptors are necessary for the adsorption of proteins to cells. Indeed, many proteins are known to be nonspecifically adsorbed by cells. The evidence presented here indicates that the adsorption of diphtheria toxin to cells may be nonspecific.

It appears that the adsorption of toxin to a

susceptible cell is an electrostatic process which requires an appropriate ionic environment for its success. An electrostatic "fit" between the toxin molecule and the cell surface is inferred. There is no direct evidence for the presence of specific toxin receptors on the cells; nevertheless, the fact that only small numbers of toxin molecules are adsorbed (18) suggests that there are only a limited number of areas on the cell surface to which toxin can successfully adsorb. Our observation that the kinetics of toxin adsorption, as measured by lethality, are first order suggests that a single toxin molecule is sufficient to cause cell death.

The data presented here show that NaF, an inhibitor of glycolysis, almost completely prevents intoxication in HeLa cells, whereas inhibitors of respiration and oxidative phosphorylation have no effect. Glycolysis is an important source of energy in HeLa cells when glucose is present (7), and the addition of NaF results in a marked inhibition of glycolysis from both endogenous substrate and glucose (26; Table 6). One interpretation of these findings, discussed below, is that some step in the intoxication process requires energy derived from the glycolytic pathway. A second interpretation is that NaF, through its effect on glycolysis and energy metabolism, inhibits the reaction of toxin, NAD, and transferase II, a finding which Gill et al. (6) have reported. Thus, if fluoride treatment resulted in trapping of NAD in its reduced form, NADH, the inactivation of transferase II would not proceed (9). Finally, it is possible that NaF acts in a direct manner to prevent toxin molecules from reaching their site of action.

It is clear that adsorption of toxin in and of itself is not lethal to the cell. Cells remain viable when exposed to toxin in the presence of NH_4Cl or NaF or at *p*H 9.5, conditions under which toxin adsorbs but remains accessible to the neutralizing effect of antitoxin. Intoxication therefore depends on the subsequent step or steps. The data presented in this paper and by others is compatible with the idea that, subsequent to adsorption, toxin molecules are actively transported to subsurface or intracellular levels. Moehring and Moehring (16) observed that poly-L-ornithine, which stimulates uptake of protein by mammalian cells, enhanced the lethality of toxin for normally resistant mouse L cells. We have observed (*unpublished data*) that bovine serum albumin increases the speed with which intoxication occurs in HeLa cells exposed to low doses of toxin. Bovine serum albumin and other proteins are known to increase the rate of pinocytosis in mouse macrophages (2). The experiments with energy inhibitors reported here support the idea that toxin is actively transported into the cell; active transport and pinocytosis are energy-requiring processes (13). We have found that the uptake of another macromolecule, that is, the infection of HeLa cells with poliovirus ribonucleic acid (RNA), is also inhibited by 10^{-2} M NaF (J. Duncan, *unpublished data*). Thus, by inhibiting the glycolytic production of adenosine triphosphate, NaF may prevent the transport of toxin (or viral RNA) into the cell.

On the basis of their studies with ^{125}iodine-labeled toxin and radioautographs, Pappenheimer and Brown (18) concluded that toxin remained at the outer cell membrane. However, their attempts to reverse toxin action by the addition of trypsin to intoxicated cells were unsuccessful. We think it is possible that, after adsorption of toxin to the cell membrane, the toxin molecules may be actively transported (pinocytosed) to subsurface or intracellular levels, perhaps just beneath the cell membrane. There, toxin would catalyze the formation of the ADPR-transferase II complex, resulting in the inactivation of the cell's complement of transferase II.

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LITERATURE CITED

1. Barker, J. B., and W. H. Summerson. 1941. The colorimetric determination of lactic acid in biological material. J. Biol. Chem. 138:535–554.
2. Cohn, Z., and E. Parks. 1967. The regulation of pinocytosis in mouse macrophages. II. Factors inducing vesicle formation. J. Exptl. Med. 125:213–230.
3. Collier, R. J. 1967. Effect of diphtheria toxin on protein synthesis: inactivation of one of the transfer factors. J. Mol. Biol. 25:83–98.
4. Collier, R. J., and A. M. Pappenheimer, Jr. 1964. Studies on the mode of action of diphtheria toxin. II. Effect of toxin on amino acid incorporation in cell-free systems. J. Exp. Med. 120:1019–1039.
5. Duncan, J. L., and N. B. Groman. 1967. Studies of the activity of diphtheria toxin. I. Poliovirus replication in intoxicated HeLa cells. J. Exp. Med. 125:489–500.
6. Gill, D. M., A. M. Pappenheimer, Jr., R. Brown, and J. T. Kurnick. 1969. Studies on the mode of action of diphtheria toxin. VII. Toxin-stimulated hydrolysis of nicotinamide adenine dinucleotide in mammalian cell extracts. J. Exp. Med. 129:1–21.
7. Goldberg, E. B., H. M. Nitowsky, and S. P. Colowick. 1965. The role of glycolysis in the growth of tumor cells. IV. The basis of glucose toxicity in oxamate-treated, cultured cells. J. Biol. Chem. 240:2791–2796.
8. Goor, R. S., and A. M. Pappenheimer, Jr. 1967. Studies on the mode of action of diphtheria toxin. III. Site of toxin action in cell-free extracts. J. Exptl. Med. 126:899–912.
9. Goor, R. S., and A. M. Pappenheimer, Jr. 1967. Studies on the mode of action of diphtheria toxin. IV. Specificity of the co-

factor (NAD) requirement for toxin action in cell-free systems. J. Exptl. Med. 126:913–921.
10. Goor, R. S., A. M. Pappenheimer, Jr., and E. Ames. 1967. Studies on the mode of action of diphtheria toxin. V. Inhibition of peptide bond formation by toxin and NAD in cell-free systems and its reversal by nicotinamide. J. Exp. Med. 126:923–939.
11. Holland, J. J., and L. C. McLaren. 1959. The mammalian cell-virus relationship. II. Adsorption, reception, and eclipse of poliovirus by HeLa cells. J. Exptl. Med. 109:487–504.
12. Honjo, T., Y. Nishizuka, and S. Hayaishi. 1968. Diphtheria toxin-dependent adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis. J. Biol. Chem. 243:3553–3555.
13. Karnovsky, M. L. 1962. Metabolic basis of phagocytic activity. Physiol. Rev. 42:143–168.
14. Kim, K., and N. B. Groman. 1965. In vitro inhibition of diphtheria toxin action by ammonium salts and amines. J. Bacteriol. 90:1552–1556.
15. Kim, K., and N. B. Groman. 1965. Mode of inhibition of diphtheria toxin by ammonium chloride. J. Bacteriol. 90: 1557–1562.
16. Moehring, J. M., and T. J. Moehring. 1968. The response of cultured mammalian cells to diphtheria toxin. II. The resistant cell: enhancement of toxin action by poly-L-ornithine. J. Exp. Med. 127:541–554.
17. Moehring, T. J., and J. M. Moehring. 1968. Response of cultured mammalian cells to diphtheria toxin. III. Inhibition of protein synthesis studied at the subcellular level. J. Bacteriol. 96:61–69.
18. Pappenheimer, A. M., Jr., and R. Brown. 1968. Studies on the mode of action of diphtheria toxin. VI. Site of the action of toxin in living cells. J. Exp. Med. 127:1073–1086.
19. Pappenheimer, A. M., Jr., R. J. Collier, and P. A. Miller. 1965. The effect of diphtheria toxin on the metabolism of mammalian cells in tissue culture, p. 21–35. *In* M. Solotorovsky (ed.), Cell culture in the study of bacterial disease. Rutgers Univ. Press, New Brunswick, N.J.
20. Philipson, L. 1963. The early interaction of animal viruses and cell. Progr. Med. Virol. 5:43–78.
21. Placido Sousa, C., and D. G. Evans. 1957. The action of diphtheria toxin on tissue cultures and its neutralization by antitoxin. Brit. J. Exp. Pathol. 38:644–649.
22. Schaeffer, W. I., J. Gabliks, and R. Calitis. 1967. Interference by trypsin in the interaction of staphylococcal enterotoxin B and cell cultures of human embryonic intestine. J. Bacteriol. 93:1489–1492.
23. Strauss, N., and E. D. Hendee. 1959. The effect of diphtheria toxin on the metabolism of HeLa cells. J. Exp. Med. 109: 145–163.
24. Tolmach, L. J. 1957. Attachment and penetration of cells by viruses. Advan. Virus Res. 4:63–110.
25. Wilson, G. S., and A. A. Miles. 1964. Principles of bacteriology and immunity, Vol. 1, 5th ed., P. 591. The Williams & Wilkins Company, Baltimore.
26. Wu, R. 1959. Regulatory mechanisms in carbohydrate metabolism. V. Limiting factors of glycolysis in HeLa cells. J. Biol. Chem. 234:2806–2810.
27. Zajac, I., and R. L. Crowell. 1965. Effect of enzymes on the interaction of enteroviruses with living HeLa cells. J. Bacteriol. 89:574–582.