STUDIES OF THE ACTIVITY OF DIPHTHERIA TOXIN*

I. Poliovirus Replication in Intoxicated HeLa Cells

By J. L. DUNCAN, D.D.S., and N. B. GROMAN, Ph.D.

(From the Department of Microbiology, School of Medicine, University of Washington, Seattle)

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Although diphtheria toxin was discovered almost 80 yr ago, the mechanism of toxin action remained unknown until recent years, when improvement in tissue culture techniques made it possible to study toxins in homogenous cell populations. Placido Sousa and Evans (1) and Lennox and Kaplan (2) found that several types of mammalian cells were susceptible to diphtheria toxin and showed that these cell lines could be used for assaying toxin.

Strauss and Hendee (3) studied protein synthesis in intoxicated HeLa cells by following the incorporation of methionine-^{35}S into cell protein. They found that 1.5–2 hr after the addition of a saturating dose[1] of toxin, protein synthesis stopped completely. Virtually no inhibition of protein synthesis was observed when diphtheria antitoxin was added to cells within 30 min of toxin addition. With lower doses of toxin the time required to reach the stage of irreversible toxicity increased progressively.

Collier and Pappenheimer (4) used cell-free extracts to study the effect of toxin on protein synthesis. They demonstrated that toxin specifically inhibited the incorporation of amino acids into polypeptides in extracts of rabbit reticulocytes and HeLa cells. This inhibition was dependent on a cofactor, nicotinamide–adenine dinucleotide. Toxin did not inhibit the formation of aminoacyl sRNA, but did prevent the transfer of amino acids from sRNA to the growing peptide chain.

The present work is both a study of the effect of diphtheria toxin on poliovirus synthesis and a study of the mode of action of toxin using virus replication as a biological indicator of toxin action. The data speak to both viewpoints but the primary orientation of the work is toward the problem of toxin action.

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[1] A saturating dose of toxin is an amount of toxin which produces irreversible toxicity in the least possible time. This includes all doses above the minimum saturating dose. The latter corresponds to 0.3 Lf per ml, or approximately 0.8 μg of toxin protein. Irreversible toxicity is the stage of intoxication beyond which antitoxin can no longer rescue the cells.

Materials and Methods

Cells.—HeLa cells obtained from Dr. J. J. Holland were used in all experiments. Stock cultures of HeLa cells were maintained as monolayers in 16-oz prescription bottles under 50 ml of medium. The cells were fed every 48 hr and transferred every 10 days. Transfer was carried out by rinsing the cells twice with 0.15 M NaCl and incubating them with 2 ml of 0.05% trypsin (Difco Laboratories, Inc., Detroit, Mich.; 1:250) for 30 min at 37°C. The cells were dispersed by aspiration with a pipette and added to fresh medium. Cells from one monolayer were passed to three new 16 oz bottles. All cultures were grown at 37°C and gassed with 5% CO_2.

To prepare monolayers used in the experiments, cells from one 16 oz bottle were passed to 15 50 ml French square bottles. These cells received fresh medium after 2 days and were used on the 3rd day after passage. At that time a confluent monolayer had formed containing approximately 2×10^6 cells.

Media.—The basic medium (YEM) used for maintenance of all cell cultures consisted of Hanks' balanced salt solution (HBSS) containing 8% calf serum, 0.1% yeast extract, and 0.1% proteose peptone No. 3 (Difco). Addition of 10 ml of 4.2% $NaHCO_3$ per liter of medium brought the pH to approximately 7.4. The medium also contained 100,000 units of penicillin, 100,000 μg of streptomycin, and 25,000 units of nystatin per liter.

The overlay medium used in all virus plaque assays was prepared by adding 12 ml of 3% agar to 100 ml of YEM. In the overlay medium used in studies of infectious centers, 4 ml of 3% agar was added to 10 ml of YEM.

Diphtheria Toxin, Antitoxin, and Toxoid.—Diphtheria toxin (5 × crystallized) was provided by Dr. A. M. Pappenheimer, Jr. Stock solutions of toxin in sterile distilled water contained 436 Lf per ml, and a working stock, from which fresh toxin solution was prepared for each experiment, contained 43.6 Lf per ml. Diphtheria antitoxin was obtained from Wyeth Laboratories, Marietta, Pa., and toxoid, containing 30 Lf per ml was obtained from Parke, Davis & Co., Detroit, Mich.

Toxin solutions were prepared by dilution in HBSS containing $NaHCO_3$ to give 0.3 Lf per ml (a saturating dose). HBSS plus $NaHCO_3$ without toxin served as the control. Antitoxin solutions contained 3 Lf per ml in YEM.

Virus.—Type 1 poliovirus (Mahoney) was provided by Dr. Carl E. Cords, Jr. Pools of virus stock were obtained by allowing 2 ml of a high-titered virus inoculum to absorb to monolayers in 16-oz bottles for 1 hr at 37°C. 20 ml of YEM was added to each bottle and incubation continued. 9 hr after infection the cells were frozen and thawed to release intracellular virus and the virus solutions from each bottle were pooled.

Infection and Assay of Mature Virus.—Monolayers were infected by adding 0.2 ml of virus inoculum (multiplicity of infection > 10) to each bottle. The cells were incubated at 37°C for 30 min with periodic gentle agitation. After 30 min the monolayers were washed three times with YEM and incubated in 5 ml of YEM.

9 hr after infection, medium was removed from the monolayers and 2 ml of fresh YEM added. The cells were frozen and thawed three to four times to release intracellular virus. Mature virus was assayed by the monolayer plaque technique. Serial 10-fold dilutions of the virus samples were made, and 0.2 ml of each dilution was allowed to adsorb to HeLa cell monolayers for 30 min at 37°C. The monolayers were washed three times with YEM to remove unadsorbed virus, and 5 ml of agar overlay was added to each bottle. After incubation for 48 hr the overlay was poured off and the cells stained with 1% crystal violet solution (5). Excess dye was washed off and the plaques counted.

Extraction and Assay of Infectious Viral Ribonucleic Acid.—Infectious viral ribonucleic acid (I-RNA) was extracted using a modification (6) of the method of Gierer and Schramm (7). 5 ml of 0.02 M phosphate buffer containing 5×10^{-4} ethylenediamine sodium tetraacetate, pH 7.2, and 5 ml of water-saturated phenol were added to infected monolayers. The bottles

were shaken for 5 min and the mixture was centrifuged for 5 min at 2000 rpm. The aqueous phase was removed and extracted three times with equal volumes of ether. Residual ether was removed by bubbling nitrogen through the solution.

Infectious RNA was assayed (6) by making serial 10-fold dilutions in 2.0 M $MgSO_4$ containing 10^{-2} M tris(hydroxymethyl)aminomethane, pH 7.2. HeLa cell monolayers were washed with 0.15 M NaCl and 0.3 ml of each dilution was added. After incubation for 15 min at room temperature, the monolayers were washed three times with NaCl. 5 ml of agar overlay was added to the infected monolayers and they were incubated at 37°C for 48 hr. The overlay was then poured off and the monolayers stained as before.

Procedure for Intoxication of Cells.—Unless otherwise stated the monolayers received 5 ml of a saturating dose of toxin (0.3 Lf/ml) or control solution and were incubated for the appropriate time at 37°C. The cells were washed once with antitoxin (3.0 Lf/ml) and once with YEM. In some experiments in which the cells were exposed to toxin for 1 hr or more, they were washed three times with YEM. In control experiments the yield of virus was the same whether the cells were washed with antitoxin or with YEM.

In all experiments, parallel uninfected monolayers received toxin or control solution. After incubation for 1 hr at 37°C they were washed with antitoxin and 5 ml of fresh YEM was added. The cells were observed over a period of 5 days, during which time the toxin-treated monolayers were destroyed, indicating irreversible toxicity had occurred.

Other Reagents.—Prolamine and glycamine were obtained from the Calbiochem Company, Los Angeles, Calif.

RESULTS

The Effect of Toxin on Virus Replication.—

Preliminary experiments were performed to determine whether toxin did, in fact, have any effect on virus replication. HeLa cell monolayers were infected and then exposed to toxin (0.3 Lf/ml) or control solution for 1 hr. After washing with antitoxin, incubation continued to the 9th hr of infection, at which time mature virus and infectious viral RNA were assayed.

The results of numerous experiments showed that the yield of mature virus or infectious RNA obtained from toxin-treated cultures was 90% less than that obtained from control cultures. Similar experiments showed that toxoid (0.3 Lf/ml) was without effect.

Direct Effect of Toxin on Virus Particles and I-RNA.—

Experiments were performed to determine whether mature virus particles or I-RNA were directly inactivated by toxin. A mixture consisting of equal volumes of a poliovirus suspension (1×10^8 PFU/ml) and 0.43 Lf of toxin in HBSS was incubated with shaking at 37°C. Control mixtures contained no toxin. Samples were removed at 30, 60, and 120 min and assayed. Similarly, a suspension of infectious RNA plus toxin was incubated at 37°C and samples removed at 30 and 120 min and assayed.

The results showed that incubation of virus particles or I-RNA with toxin caused no decrease in the number of plaques formed. It appears that toxin has no direct effect on virus particles or I-RNA.

Intoxication of Cells Prior to Virus Infection.—

Monolayers were given toxin or control solution 5, 3, or 1 hr before infection. After 1 hr, toxin was removed, the cells washed three times with medium, and incubation continued. At

0 time the cells were infected and 9 hr later the yields of mature virus and I-RNA were assayed.

A reduction of at least 90% in the yield of I-RNA and mature virus in intoxicated cells was observed at each time tested (Table I). Only a slight difference in yield was observed at the various times of toxin addition, suggesting

TABLE I

Intoxication of HeLa Cells before Poliovirus Infection

Time of toxin addition* before infection	Virus yield	
	Mature virus	I-RNA
hr	*% of control*	*% of control*
5	5	2
3	4	4
1	9	9

* Cells exposed to toxin (0.3 Lf/ml) for 1 hr.

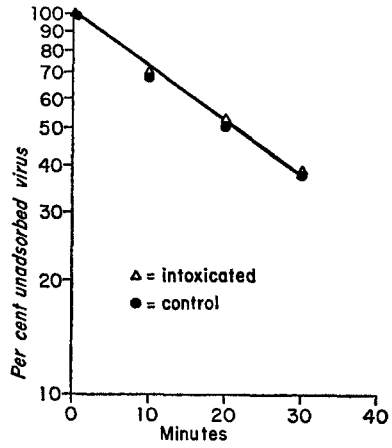

FIG. 1. Rate of poliovirus adsorption in intoxicated and nonintoxicated HeLa cells.

that toxin given to cells 1 hr before infection is as effective in preventing replication as is toxin given several hours before infection.

In one experiment toxin was added at the same times, but was not removed until the cells were infected. The virus yield from these cells was only slightly less than the yield observed when toxin was removed after 1 hr, indicating that incubation for 1 hr in toxin is sufficient to produce maximum inhibition of virus replication.

Effect of Toxin on Virus Adsorption.—

To determine what effect intoxication had on the ability of cells to adsorb virus particles, cells were treated with toxin or control solution for 1 hr and then infected, using a multiplicity

of approximately 0.01. 10, 20, and 30 min after addition of virus, the inoculum was washed off and unadsorbed virus assayed.

The results in Fig. 1 show that the rate of virus adsorption in intoxicated and control cells is the same, at least up to 90 min after toxin addition.

Effect of Toxin on Viral Eclipse.—Holland (8) demonstrated that poliovirus eclipse occurs in two steps: a reversible, temperature independent step, and an irreversible, temperature dependent step. Virus particles which have not reached the second stage can be recovered as infectious virus by a number of agents including 8 M urea, 6 M LiCl, and low pH conditions.

Monolayers were exposed to toxin or control solution for 1 hr. Virus inoculum containing a multiplicity of 0.3 was added, and the monolayers were incubated at 37°C. After 10 min

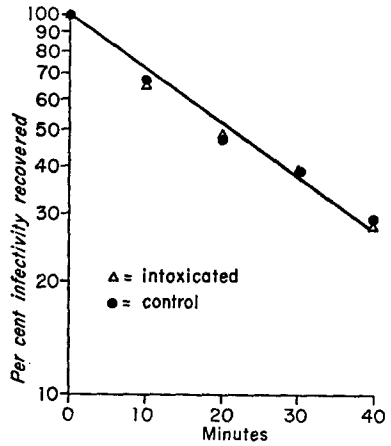

FIG. 2. Rate of poliovirus eclipse in intoxicated and nonintoxicated HeLa cells.

the inoculum was washed off, and the number of adsorbed virus particles was calculated by assaying the unadsorbed virus in the wash. At 10, 20, 30, and 40 min of infection, the cells received 2 ml of 8 M urea for 3 min. This treatment disrupted the cells. The samples were then assayed to determine the amount of adsorbed virus released by the urea treatment.

The results in Fig. 2 show no difference in the rate of viral eclipse in intoxicated or control cells.

Intoxication of Cells Infected with Viral RNA.—It has been shown that viral adsorption and eclipse proceed normally in intoxicated cells. It is possible, however, that the release of viral RNA from its protein coat is impaired in intoxicated cells and that this accounts for the inhibition of viral replication.

To bypass the uncoating step in virus infection, the cells were infected with poliovirus infectious RNA and given toxin or control for 1 hr. 9 hr after infection, mature virus was assayed, and the results showed a 90% decrease in virus yield from intoxicated cells.

This experiment does not rule out the possibility that the uncoating of virus in intoxicated cells is faulty but does show that this is not the only step in virus replication inhibited by toxin. Evidence to be presented in the next section also suggests that faulty uncoating cannot alone account for the inhibition.

Effect of Toxin Given after Infection.—To clarify the nature of the inhibiting mechanism of toxin, studies were made on the effects of toxin given at various times after infection.

Monolayers were infected and at various intervals after infection the cells were exposed to toxin or control medium for 1 hr. Infectious RNA and mature virus were assayed 9 hr after infection. The results are shown in Fig. 3.

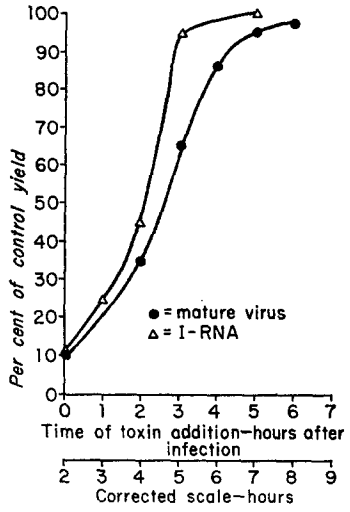

FIG. 3. Effect of toxin given after poliovirus infection. All assays were performed 9 hr after infection. The lower scale is corrected for the estimated time required for toxin action.

The yield of I-RNA and mature virus from cells receiving toxin immediately after infection was 90% less than the control yield. However, when the addition of toxin was delayed until the 3rd hr of infection, the yield of I-RNA was only 5% less than the control yield. Likewise, toxin addition at the 4th–5th hr of infection caused only a 5–15% decrease in the yield of mature virus. Thus, the extent to which toxin inhibits viral replication depends on the time afer infection of toxin addition. Toxin added early in the viral reproduction cycle inhibits nearly all virus replication. Toxin added late in the cycle has very little effect.

Strauss and Hendee (3) showed that 0.3 Lf of toxin inhibits cell protein synthesis after a delay of 90–120 min. When the time scale shown in Fig. 3 is corrected for a 2 hr delay the curves correspond quite well with the known time course of synthesis of poliovirus RNA and mature particles (9).

Effect of Toxin on the Viral Latent Period.—One-step growth curves of poliovirus in HeLa cells indicate that maximal amounts of intracellular virus are produced by the 6th–7th hr of infection. In previous experiments mature virus was assayed by disrupting the cells at 9 hr after infection.

To determine whether toxin inhibited viral replication irreversibly, cells were infected and toxin or control solution added. After 1 hr the cells were washed with antitoxin and incubation continued. At 9, 16, and 26 hr after infection the mature virus yield was assayed.

In all cases the virus yield was 3–4% of the control. It appears that toxin action is irreversible and that the decrease in virus yield obtained from intoxicated cells cannot be accounted for by a delay in the viral replication cycle.

Studies of Infectious Centers.—In terms of cell population the reduction in virus yield from an intoxicated monolayer could be accounted for by one of the following hypotheses:

TABLE II

Determination of Infectious Centers in Intoxicated HeLa Cells

	Average plaque count	
	Toxin	Control
Experiment I	41 (16%)*	250
Experiment II	30 (14%)*	209

* Per cent of control.

1. All cells in the intoxicated monolayer produce only 10% of their normal virus yield.
2. 10% of the cells make a normal yield and 90% make no virus.
3. The cells make variable amounts of virus.

If the second hypothesis were correct it could be differentiated by a study of infectious centers.

Eight monolayer cultures were infected. Four bottles received toxin and the other four received control solution. After incubation for 1 hr the cells were washed with antitoxin, and the monolayers were dispersed with 0.05% trypsin. Cells from intoxicated and control cultures were each pooled and counted. Dilutions were made so that the intoxicated and control cultures contained the same number of cells. Samples of intact cells from each pool were plated on HeLa cell monolayers to determine the number of infectious centers.

The results of two such experiments shown in Table II indicate that only 15% of the intoxicated cells produce virus, suggesting that the second hypothesis is correct.

Effect of Ammonia on the Toxin-Virus System.—Kim and Groman (10) showed that ammonia prevented the killing action of diphtheria toxin on HeLa cells.

We have studied the effect of ammonia on the toxin-virus system using various ammonium salts and the aliphatic amines, glycamine and prolamine.

The cells were infected, and toxin or control medium and one of the ammonium salts or amines was added. After 1 hr the cells were washed with antitoxin. Mature virus was assayed 9 hr after infection.

TABLE III

*Effect of Ammonium Salts and Amines on the Inhibition of Poliovirus Replication by Toxin**

Addition to toxin	Amount	Virus yield
	μM/ml	*% of control*
Ammonium citrate $(NH_4)_2HC_6H_5O_7$	1.0	98
Ammonium sulfate $(NH_4)_2SO_4$	1.5	95
Ammonium chloride‡ NH_4Cl	3.7	96
Prolamine $CH_2(CH_2)_3NH_2$	3.0	99
Glycamine CH_3NH_2	3.0	99
None		8

* Infected cells were exposed to the various mixtures for 1 hr before the addition of antitoxin.

‡ The ammonium chloride-toxin mixture remained on the infected cells up to the time of virus assay (9 hr). No antitoxin was given.

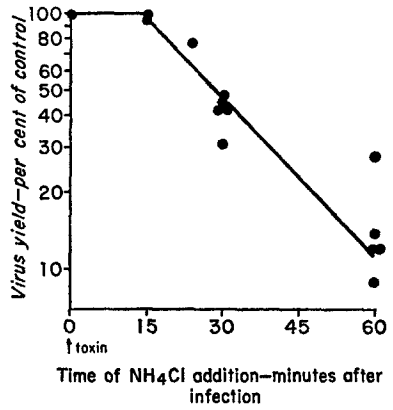

FIG. 4. Effect of NH_4Cl on poliovirus replication in HeLa cells incubated in toxin.

The results (Table III) show that ammonium salts and aliphatic amines prevented the inhibition of viral replication by toxin.

The inhibiting action of ammonium chloride was examined in more detail. The cells were infected and toxin or control solution added. Ammonium chloride (0.2 mg/ml) was added at intervals after infection, and mature virus production was assayed at the 9th hr of infection. Parallel uninfected control cultures were treated with toxin and NH_4Cl at corresponding times. After 9 hr they were washed with antitoxin and maintained for at least 5 days. The results are shown in Fig. 4.

The addition of NH_4Cl to infected cells 15 min after the addition of toxin completely prevents the action of toxin on viral replication. Uninfected control monolayers show no signs of intoxication. When NH_4Cl is added 30 min after the cells receive toxin the virus yield is reduced by at least 50%. Parallel uninfected control monolayers show variable intoxication. Generally the monolayer is intact but numerous dead cells are present in the medium and on the monolayer by the 5th day. The virus yield from cells receiving NH_4Cl 60 min after toxin is 85–90% less than the control yield, indicating that NH_4Cl provided essentially no protection when given at that time. This lack of protection is also reflected in uninfected control monolayers which are completely destroyed by this treatment.

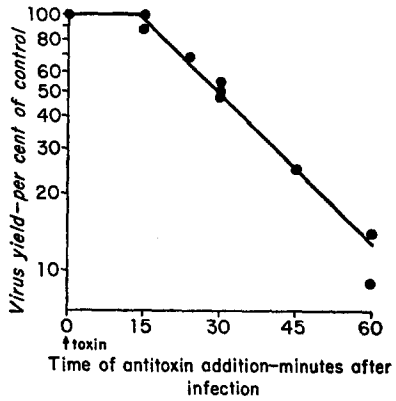

FIG. 5. Effect of antitoxin on poliovirus replication in HeLa cells exposed to toxin.

These results suggest that toxicity is occurring in some cells 15–30 min after the addition of a saturating dose of toxin. By 30 min 50% of the cells are intoxicated in the sense that they can no longer produce a full yield of virus.

Effect of Antitoxin on the Toxin-Virus System.—

The cells were infected and incubated in toxin or control solution. At various intervals toxin was removed and the cells washed with a 10-fold excess of antitoxin. Mature virus production was assayed 9 hr after infection. Parallel uninfected cultures were treated similarly and observed for at least 5 days.

The results (Fig. 5) show that the kinetics of the protective action of antitoxin are very similar to those of NH_4Cl. There is a 50% reduction in virus yield when antitoxin is given to infected cells 30 min after the addition of toxin. Uninfected cultures again show variable intoxication under these conditions. The monolayers usually remain intact but numerous dead cells are present.

DISCUSSION

It has been shown that poliovirus replication is almost completely inhibited in diphtheria intoxicated HeLa cells. Toxin appears to act on certain biosynthetic steps in the virus replication cycle rather than through direct combination with either infectious viral RNA or mature virus particles.

The action or lack of action of toxin on various stages of the viral replication cycle gives some indication of its mode of action on the cell. Poliovirus adsorption and eclipse proceed normally for at least 90 min after a saturating dose of toxin is added to HeLa cells. This implies that the virus receptors, which are presumed to be surface lipoproteins (11), were not affected during this period. The normal eclipse of virus during this period also suggests that the host cell membrane is not affected by toxin. Eclipse probably involves a rearrangement of virus capsid just before or at the time of pinocytosis of virus by the cell (11). These results concur with the observations of Strauss (12) and Kato and Pappenheimer (13) in indicating that both membrane structure and function are unaffected during the first few hours following exposure to a saturating dose of toxin.

The data presented are compatible with the interpretation that toxin prevents poliovirus replication by inhibiting protein synthesis. If one assumes a 2-hr interval between the time of toxin addition and inhibition of protein synthesis [Strauss and Hendee (3)] a correlation between toxin action and the synthesis of infectious viral RNA (I-RNA) becomes apparent. Synthesis of I-RNA is dependent on the appearance of virus-induced RNA–dependent RNA polymerase. The enzyme first appears in polio-infected cells about 2 hr after infection and must be synthesized throughout the period of viral RNA replication because of its rapid turnover (14, 15). In the present study I-RNA synthesis was completely inhibited when toxin was added 2 hr before the appearance of virus (see Fig. 3) and progressively less inhibited with later additions. The simplest interpretation is that no functional RNA polymerase is made if toxin is added at least 2 hr before the scheduled time of appearance of this enzyme and that later additions inhibit synthesis of this enzyme which is normally required to compensate for its rapid turnover. The virus yield presumably reflects the I-RNA produced prior to the time toxin action is manifest. It appears that toxin also inhibits synthesis of capsid protein. As seen in Fig. 3 the yields of mature virus can be inhibited for a longer period of time than yields of I-RNA. While this is interpreted as due to inhibition of capsid synthesis, it is possible that toxin interferes with maturation rather than synthesis.

It has been observed by Strauss and Hendee (3) and ourselves that populations of cells exhibit some residual synthetic activity despite exposure to a saturating dose of toxin. The question is whether all the cells in a population are partially affected by toxin or whether a certain fraction of the cells are resistant.

Our data suggest that a certain fraction of the cells in an exposed population are resistant since about 15% of the intoxicated cell population produced infectious centers while the remainder did not.

The final point to be discussed is related to the speed with which irreversible toxicity is attained. Cells exposed to a saturating dose of toxin for 15–30 min eventually lose about 50% of their virus-producing capacity. In other studies in which cytotoxic effects (1) and protein synthesis (13) were used as criteria to judge irreversible toxicity, at least 30 min exposure to a saturating dose of toxin was required before any effect was eventually detected. Viral replication may be a slightly more sensitive method of detecting intoxication than other methods employed. This sensitivity could be due either to an intrinsically greater sensitivity of the process or a more rapid uptake of toxin by virus-infected cells.

SUMMARY

It has been demonstrated that a saturating dose of diphtheria toxin produced a 90% inhibition of poliovirus replication in HeLa cells. This inhibition was reflected in infectious viral RNA synthesis and in mature virus production. Toxin had no direct effect on virus particles or I-RNA, and poliovirus adsorption and eclipse appeared to be carried out normally in intoxicated cells.

When toxin was given at various time intervals after infection, the amount of inhibition depended on the time of toxin addition. Toxin given before or immediately after infection gave maximum inhibition, while toxin given several hours after infection had little effect. The data suggest that toxin inhibits viral replication through its effect on protein synthesis. It is likely that a critical step in the viral replication cycle, the production of poliovirus-induced RNA polymerase, is inhibited, and possibly the synthesis of capsid protein.

Ammonium salts and the aliphatic amines, glycamine and prolamine, prevented the inhibition of viral replication by toxin. The kinetics of the protective action of ammonium chloride and diphtheria antitoxin are remarkably similar.

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