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Mode of Inhibition of Diphtheria Toxin by Ammonium Chloride

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ABSTRACT

KIM, K. (University of Washington, Seattle), AND N. B. GROMAN. Mode of inhibition of diphtheria toxin by ammonium chloride. J. Bacteriol. **90**:1557-1562. 1965.—The inhibition of diphtheria toxin by ammonium salts was independent of toxin concentration over a 100-fold range of toxin. Inhibition by minimal concentrations of ammonium chloride was abolished by lowering the *p*H, indicating that free ammonia is the active form of inhibitor. A single addition of ammonium chloride inhibited toxin for a limited period of time, but periodic readdition of the ammonium salt was required to sustain inhibition indefinitely in the absence of antitoxin. Toxin was not destroyed and its adsorption occurred equally well in the presence or absence of ammonium chloride. Preadsorbed toxin was also effectively inhibited by the addition of ammonium chloride. Inhibited toxin remained accessible to antitoxin neutralization. Attempts to reverse ammonia inhibition by the addition of succinate or reduced nicotinamide adenine dinucleotide were unsuccessful. Attempts to inhibit toxin by interfering with active transport were also unsuccessful.

It was reported previously (Kim and Groman, 1965) that ammonium salts, glutamine, prolamine, or glycamine inhibit the action of diphtheria toxin on HeLa cell cultures. Two general explanations of inhibition are possible: first, that inhibition is due to the direct interaction of inhibitor with either free or bound toxin; second, that toxin action is indirectly affected because the inhibitor renders the cell insusceptible to toxin. Inhibition can also be viewed in terms of the adsorption and toxicity steps delineated by Strauss and Hendee (1959) in their study of toxin action. The present study is concerned with some details of the mode of inhibition of diphtheria toxin by ammonium chloride, and the findings bear on some of the proposed mechanisms of inhibition.

MATERIALS AND METHODS

All procedures and media relating to the culture and maintenance of HeLa cells, and the standard toxicity test, were previously described (Kim and Groman, 1965). As before, the criterion for toxicity was cell death, with a positive reading indicating that 80 to 100% of the cell sheet was destroyed within 5 days after exposure to toxin. Tests were scored as plus-minus when the cell sheet was partially destroyed. Irreversible toxicity is defined as the stage in intoxication beyond which antitoxin can no longer rescue the cells.

In most experiments, a partially purified toxin preparation kindly supplied by A. M. Pappenheimer, Jr., was employed. It was used at either a minimal level (0.004 Lf) or a saturating level (0.3 Lf). The former produced irreversible toxicity in 12 hr and the latter in 30 to 45 min. In some experiments, a crude toxin was employed at a concentration which produced irreversible toxicity in 6 to 8 hr. At this concentration, the crude inhibitor material present was diluted beyond the range of effectiveness. It should be noted that all agents were dissolved in Hanks' balanced salt solution containing 1.5 ml of 5% sodium bicarbonate per 100 ml (HBSS) and were added to monolayers in 1-ml amounts. Where employed, antitoxin was used in amounts sufficient to neutralize all added toxin.

RESULTS

Minimal effective concentration of ammonium chloride. A solution of ammonium chloride was diluted in HBSS, and various concentrations were tested for their ability to inhibit a minimal and a saturating level of partially purified toxin by the standard test procedure. The results in Table 1 show that the minimal effective concentration for both toxin levels was between 0.02 and 0.13 mg/ml. There was slight toxicity with saturating levels of toxin in the presence of 0.13 mg/ml of ammonium chloride; however, the cell sheet re-

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TABLE 1. *Effect of ammonium chloride concentration on a minimal and saturating level of toxin**

NH_4Cl/ml added to HBSS	Toxin (Lf/ml)	
	0.004	0.325
mg		
None	+	+
0.4	−	−
0.2	−	−
0.13	−	−
0.02	+	+
0.002	+	+

* Cells were exposed to the mixture for 13 hr at 37 C before the addition of antitoxin. Results indicate presence or absence of cytotoxicity.

mained uniformly intact. The experiment also shows that the cells were unaffected by exposure to ammonium chloride levels greater than those required for inhibition of toxin. The lack of correlation between toxin concentration and the concentration of ammonium chloride required for protection suggests that a direct interaction between ammonium ions and free toxin cannot explain inhibition, although interaction of toxin with a limited number of HeLa cell sites is not excluded.

Effect of depletion and renewal of ammonium chloride on toxicity. An experiment was done to determine how long HeLa cells were protected against intoxication by a single addition of ammonium chloride. The cells were exposed to a low level of crude toxin in the presence of 0.2 mg of ammonium chloride per ml in HBSS containing 0.1% yeast extract (HYE) and were incubated at 37 C. At successive time intervals, the ammonium chloride mixture was replaced with HYE medium containing antitoxin and was incubated at 37 C for an additional 5 days. HYE medium was used for exposure to toxin and ammonium chloride because HBSS alone could not sustain the cells for more than 24 hr at 37 C. The results showed that 0.2 mg of ammonium chloride could protect HeLa cells for approximately 24 hr.

On the assumption that loss of protection was due to depletion of ammonium ions, the effect of renewal of ammonium chloride on intoxication was tested next. HeLa cells were exposed to a low level of crude toxin in the presence of 0.2 mg of ammonium chloride per ml of HYE. At 24, 48, and 72 hr, ammonium chloride was renewed by adding 0.2 mg of the salt in 0.1 ml of HYE to the test cultures. The cultures were incubated at 37 C throughout the experiment and were observed microscopically for toxicity 5 days after the initial exposure to toxin. Toxin remained on the cultures for the entire 5-day period. None of the cultures showed evidence of toxicity; hence, renewal of the salt assured continued protection even in the absence of antitoxin. Toxin remained active under these conditions for at least 2 to 3 days, for if the toxin-ammonium chloride mixture was replaced with HYE medium at this time the cells were destroyed. The survival of cultures for at least 48 hr after the last addition of NH_4Cl, in contrast to the 24-hr period noted above, was probably due to thermoinactivation of the toxin. At the level employed above, crude toxin loses its activity in about 3 days when incubated at 37 C in HYE medium.

Taken together, the results of these experiments show that the protection afforded by a given level of ammonium chloride is only temporary and in all probability is lost owing to its depletion. Equally important, they show that toxin is not irreversibly inactivated by a protective level of ammonium chloride.

Effect of ammonium chloride on adsorption of toxin. The effect of ammonium ions on the adsorption of toxin to HeLa cells was tested next. HeLa cells were exposed to a saturating level of toxin in HBSS containing 0.2 mg of ammonium chloride per ml. At successive intervals, the toxin-ammonium chloride mixture was poured off and the cultures were washed once with 1.5 ml of HBSS. A 1-ml amount of HBSS was then added to each sample tube, and the cultures were incubated for an additional 30 min to allow any toxin which remained adsorbed to act. This latter time was selected because irreversible toxicity occurs within 30 min when a saturating level of toxin is present. The medium was then replaced with 1 ml of HYE medium containing 2% calf serum (HYE-2) and antitoxin. As a control on residual free toxin, toxin in HBSS was added to a blank screw-cap tube in 1-ml amounts, incubated, poured off, and the tubes were washed once. The tubes were then rinsed once with 1 ml of HBSS and the 1-ml rinse was added to HeLa cells for 30 min, followed by treatment with antitoxin.

The results in Table 2 demonstrate that ammonium chloride does not inhibit the adsorption of toxin. Enough toxin adsorbed in 15 min in its presence so that after the removal of toxin and the ammonium salt the stage of irreversible toxicity was reached in an additional 30 min. Thus, the toxin which remained adsorbed after washing produced irreversible toxicity in the time normally required for a saturating dose. The fact that the control washings were not toxic for the cells indicates that residual toxin could not have produced this rapid development of

TABLE 2. *Adsorption of toxin in the presence of ammonium chloride*

Additions to HBSS	Toxin (0.325 Lf)	Time exposed to toxin	Time antitoxin added*	Cytotoxicity
		min	*min*	
None	−	−	−	−
	+	15	15	−
	+	30	30	+
	+	45	45	+
NH_4Cl, 0.2 mg/ml (present only during period of exposure to toxin)	−	−	−	−
	+	15	15	−
	+	15	45	+
	+	30	30	−
	+	30	60	+
	+	45	45	−
	+	45	75	+
None (residual toxin control)	+	−	30	−

* Measured from the beginning of exposure to toxin.

TABLE 3. *Effect of ammonium chloride on preadsorbed toxin*

Toxin (0.325 Lf/ml)	Time of addition*		Cytotoxicity
	NH_4Cl	Antitoxin	
+	None	0	−
	None	15 min	−
	None	30 min	±†
	None	45 min	±
	None	60 min	+
+	0	5.5 hr	−
	15 min	5.5 hr	−
	30 min	5.5 hr	±
	45 min	5.5 hr	±
	60 min	5.5 hr	+
−	—	—	−
	0	—	−
	60 min	—	−
	120 min	—	−

* Elapsed time beginning with incubation at 37 C.

† Partial toxicity.

irreversible toxicity. Furthermore, it is estimated that the removal of toxin and the subsequent washing reduced the level of residual toxin at least 100-fold, and at this reduced level irreversible toxicity would not have been reached for at least 12 hr. Thus, the data show that ammonium chloride does not prevent the adsorption of toxin but protects the cells even though a lethal dose of toxin has been adsorbed.

Effect of ammonium chloride on preadsorbed toxin. Strauss and Hendee (1959) reported that HeLa cells exposed to a saturating level of toxin for 1.5 hr at 10 C did not proceed to the stage of irreversible toxicity at this temperature. This observation provided a method of testing whether ammonium chloride could inhibit preadsorbed toxin. HeLa cells were exposed to a saturating level of toxin in HBSS for 1 hr at 4 C. The toxin was poured off and the cultures were washed once with 1.5 ml of chilled HBSS. A 1-ml amount of HBSS was added to each tube and the temperature was raised to 37 C. At successive intervals, one set of tubes received antitoxin and a second set received ammonium chloride. Ammonium chloride was removed from the latter set 5.5 hr after incubation at 37 C had begun.

The results in Table 3 show that ammonium chloride prevented intoxication even when added after the adsorption of a lethal dose of toxin. The fact that the control without ammonium chloride reached the stage of irreversible toxicity 30 to 60 min after incubation at 37 C had begun shows that a saturating dose of toxin had indeed adsorbed during the 1 hr in the cold. As shown in the previous section, toxin remaining after the wash could not have produced this rapid transition to irreversible toxicity. The data also show that the protective effect of ammonium chloride is instituted rapidly, at least as rapidly as that of antitoxin. The addition of either antitoxin or ammonium chloride at identical times to toxin-treated cultures conferred the same degree of protection or lack of it at each time of addition.

Effect of pH on inhibition. Warren (1962) reported that the penetration of free ammonia (NH_3) into a variety of plant and animal cells occurred rapidly, whereas ammonium ions penetrated poorly. Ammonia in solution exists as an equilibrium between free ammonia (NH_3) and ammonium ions (NH_4^+), and the percentage of each molecular species in the equilibrium is *p*H-dependent. The effect of *p*H on ammonium inhibition was determined to provide some indication of the molecular species responsible for inhibition of toxin. Ammonium chloride was dissolved in HBSS and the *p*H was adjusted with 5% sodium bicarbonate or 1 N HCl. A saturating level of partially purified toxin was then added, and the solutions were tested for toxicity in the usual manner. The cells were exposed to toxin at 37 C for 1.5 hr before the addition of antitoxin.

The data in Table 4 demonstrate that inhibition of toxin was *p*H-sensitive when a minimal

TABLE 4. *Effect of pH on toxin inhibition by ammonium chloride**

Additions to HBSS	*p*H	Toxin (0.325 Lf)	Cyto-toxicity
None	7.8–8.9	−	−
		+	+
	6.1–6.3	−	−
		+	+
NH_4Cl, 0.13 mg/ml	7.7–7.8	−	−
		+	−
	6.9–7.0	−	−
		+	−
	6.3–6.5	−	−
		+	+
NH_4Cl, 0.2 mg/ml	8.4	−	−
		+	−
	7.0–7.4	−	−
		+	−
	6.3–6.5	−	−
		+	−

* Cells were exposed to the mixtures for 1.5 hr at 37 C before the addition of antitoxin.

level of ammonium chloride (0.13 mg/ml) was present but not when a slightly higher concentration (0.2 mg/ml) was used. These concentration effects were reproducible. The controls show that intoxication of HeLa cells can occur over the *p*H range tested, indicating that the *p*H effect was dependent on a change in the NH_4^+—NH_3 equilibrium and not on the toxin molecules or the cells. Since the concentration of ammonium ions was not appreciably affected by the shift in *p*H whereas the concentration of free ammonia was affected over a 100-fold range, the results indicate that free ammonia is the molecular species responsible for inhibition.

Attempts to reverse ammonia inhibition. Since the interaction of ammonia and unadsorbed toxin is not required for inhibition, two alternative mechanisms for ammonia action are suggested: (i) that ammonia combines directly with toxin after its adsorption to the cell, or (ii) that ammonia interferes with the intoxication process through its effect on the cell or some cell process. Consideration was given to the latter hypothesis.

Very little is known about the effect of ammonia on cells. There are several reports (McKhann and Tower, 1961; Worcel and Erecinska, 1962; Erecinska and Worcel, 1963) that ammonium salts interfere with pyruvate and α-ketoglutarate metabolism, probably by virtue of reduced levels of nicotinamide adenine dinucleotide (NAD) resulting from reductive amination of these compounds to alanine and glutamate, respectively. The addition of succinate restored the NAD levels but did not affect the reductive amination of these compounds. To test the possibility that ammonia inhibited toxin by virtue of its effect on NAD levels, the effect of succinate, pyruvate, and reduced NAD ($NADH_2$) on ammonia inhibition was tested. Pyruvate was used in an attempt to overload the system, on the assumption that some would be oxidized via the Krebs cycle. Pyruvate or succinate was dissolved in HBSS and the *p*H was adjusted to 7.6 to 7.8 with 5% sodium bicarbonate before the addition of ammonium chloride and a minimal level of partially purified toxin. The final concentrations were: succinate, 12 μmoles/ml, or pyruvate, 10 μmoles/ml, plus ammonium chloride, 3 μmoles/ml. The solutions plus toxin were tested in the usual manner. Toxin was present at a minimal level which produced irreversible toxicity in 12 hr. Antitoxin was added at 13 to 15 hr. $NADH_2$ at a final concentration of 10 μmoles/ml was tested differently because of its instability in solution. It was dissolved in HBSS containing ammonium chloride at a *p*H between 7.6 and 7.8 to insure maximal stability. A saturating level of toxin was used to shorten the time required for irreversible toxicity. HeLa cells were exposed to the NH_4Cl—NAD-toxin solution for 2 to 6 hr before the addition of antitoxin. The exposure time was far beyond the time necessary for irreversible toxicity to occur with a saturating level of toxin. All test cultures survived, so that in no case was ammonia inhibition reversed by these agents. There was no assurance that the compounds had entered the cells. The effect of $NADH_2$ was also tested by adding it to cells pre-exposed to NH_4Cl-toxin mixtures for 6 hr to allow toxin adsorption to take place. Again, ammonium inhibition was not reversed.

DISCUSSION

Although ammonium ion has been referred to as the inhibitor of diphtheria toxin, the effect of *p*H on inhibitory activity indicates that ammonia is the effective component. Although this conclusion is tentative, ammonia will be so designated for this discussion.

It is evident that ammonia does not prevent the adsorption of toxin to susceptible cells; rather, it inhibits toxin after it has adsorbed but before the stage of irreversible toxicity is reached. Inhibition is reversible since cell death follows if ammonia is removed after a lethal dose of toxin has been adsorbed in its presence. This reversibility clearly shows that toxin is not destroyed during the inhibitory reaction. The data also indicate that ammonia acts to keep toxin at the

cell surface during inhibition. Thus, inhibited cultures can be permanently rescued by antitoxin at a time when uninhibited control cultures have passed the stage of irreversible toxicity (Table 2).

It is interesting to note that almost parallel effects of ammonium salts have been observed on influenza virus infections in tissue culture (Eaton and Scala, 1961; Eaton et al., 1962; Jensen, Force, and Unger, 1961). Ammonium salts are not virucidal and do not prevent the adsorption of virus. Inhibitory concentrations of ammonium salts appear to be grossly independent of virus concentrations, and a similar independence is observed with toxin concentrations. The significance of the similarities remains to be determined. One difference appears to exist. Whereas ammonium salts apparently prevent toxin penetration, they do not appear to inhibit virus penetration, although they do inhibit virus reproduction at a very early stage in infection.

Assuming that ammonia inhibits toxin after it has adsorbed, can any more specific mechanism of inhibition be visualized? One possibility is that a direct but reversible combination of toxin and ammonia occurs after adsorption. One might speculate that such a combination could only take place after adsorption had produced some accommodating change in the toxin molecule. A more likely alternative is that ammonia acts on the HeLa cells rather than the toxin and renders them temporarily resistant to intoxication. An ammonia-induced change might prevent a critical step in the intoxication process, such as the transport of toxin into the cells. Since ammonia cannot act once the stage of irreversible toxicity is reached, it is unlikely that it acts on the intracellular phase of intoxication suggested by the recent work of Kato (1962) and Collier and Pappenheimer (1964). These workers showed that diphtheria toxin inhibits amino acid incorporation into protein in vitro, and the inference is clear that this same mechanism operates in vivo.

A number of efforts were made to test the theory that some transport mechanism was involved in moving toxin into the cell. However, cell susceptibility to toxin was unaffected by ouabain (10^{-7} M), an agent reported as inhibitory to the sodium-potassium pump (Post and Albright, 1960), or by D- or L-histidine, agents that affect bacterial membranes (Grossowicz and Ariel, 1963). Furthermore, starved HeLa cells could still be intoxicated even in the presence of 2,4-dinitrophenol (3×10^{-3} M) or potassium cyanide (10^{-3} M) in the absence of glucose. Lowering the temperature to 4 C was the only condition which mimicked ammonia action, indicating that inhibition of a transport mechanism must still be considered as a possible mode of action of ammonia.

Present knowledge of toxin action indicates that adsorption, penetration, and interference with protein synthesis constitute the sequence of intoxication. It appears that ammonia prevents toxin penetration. One reservation to this description must be noted. Although toxin can inhibit protein synthesis in an in vitro system, there is yet no direct evidence that toxin actually penetrates into the interior of the cell. The primary locus of toxin action might still be the outer cell membrane. If selected functions of the membranes were disrupted, such as, for example, loss of ability to transport an essential amino acid or metabolite, then one could still account for all the observed effects of toxin and all the observations relative to ammonia inhibition. However, since cells undergoing intoxication exhibit no early changes in permeability (Strauss and Hendee, 1959; Kato and Pappenheimer, 1960) as might be expected with this hypothesis, the formulation as given seems the most reasonable.

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