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

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(Received for publication 5 June 1967)

In the preceding paper (1), we reported on the relative ability of various nucleotides related to nicotinamide adenine dinucleotide (NAD) to serve as cofactors for inhibition by diphtheria toxin of protein synthesis in cell-free extracts. Those few analogues which could replace NAD as activators of diphtheria toxin all proved to be nucleotides of demonstrated coenzyme activity. The results suggested that NAD and certain related substances are capable of interaction with the toxin protein. That diphtheria toxin does, in fact, reversibly bind one mole of NAD per mole of toxin was demonstrated by equilibrium dialysis and by gel filtration.

In the present paper, we are reporting studies on the quantitative relationships between NAD, diphtheria toxin, and inhibition of peptide bond formation in cell-free extracts from various species. The data have led us to the conclusion that inhibition of protein synthesis, in vitro, results from reversible interaction between three components: toxin, NAD, and transferase II (2, 3). Reduction of NAD is not involved since it has been found that the inactivation of transferase II by toxin in mammalian cell extracts can be prevented and even *reversed* by relatively low concentrations of nicotinamide.

Materials and Methods

Reagents and Radioisotopes.—Materials used to determine amino acid incorporation in the cell-free systems were the same as in the preceding papers (1, 3). Nicotinamide, nicotinic acid, and pyridine-3-sulfonic acid were obtained from Nutritional Biochemical Corp., Cleveland,

* This investigation was aided by a grant from the National Science Foundation. The work was conducted in part under the sponsorship of the Commission on Immunization, Armed Forces Epidemiological Board, and supported in part by the Office of the Surgeon General, Department of the Army.

‡ This work was carried out by Dr. Goor of during tenure of a Predoctoral Fellowship of the National Science Foundation.

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Ohio. The Sigma Chemical Co., St. Louis, Mo., supplied isonicotinamide hydrazide. Thionicotinamide was obtained from Aldrich Chemical Co., Milwaukee, Wis. We are indebted to Dr. A. W. Bernheimer, of New York University School of Medicine, for a generous gift of purified streptococcal NADase.

Diphtheria Toxin and Antitoxin.—Purified toxin containing 2.6 μg protein per Lf and 60–70 MLD per Lf was prepared as previously described (1, 3). Horse antitoxin 5353AD was a pepsin-digested globulin fraction of high specificity.

Cells and Cell Extracts.—Both HeLa cells and mouse L cells were grown in spinner cultures as described earlier (3) except that L cells were grown in Eagle's medium containing 5% calf

TABLE I

Effect of Diphtheria Toxin on Poly U–Directed Phenylalanine Incorporation in Bacterial, Yeast, and Mammalian Cell-Free Systems

Toxin	NAD added	Phenylalanine-^{14}C incorporation					
		*E. coli** MRE-600		*Saccharomyces cerivisae*		Mouse L cells	
		cpm	Per cent inhibition	cpm	Per cent inhibition	cpm	Per cent inhibition
μg/ml							
0	0	656	—	1784	—	—	—
0	3×10^{-5} M	656	0	1613	9.5	434	—
10	0	675	0	—	—	49	89
10	3×10^{-5} M	658	0	528	71	39	91
120	3×10^{-5} M	630	4	—	7	—	—

* "S-30" dialyzed extract from *E. coli* MRE-600 (10) prepared by Dr. Sam Raeburn. The yeast and L-cell extracts were *not* dialyzed. Poly U–directed phenylalanine uptake was allowed to continue for 15 min in the case of the bacterial extract, before adding TCA. TCA was added after 40 min to reaction mixtures containing extracts of yeast and L cells.

serum (Grand Island Biological Co., Grand Island, N. Y.) instead of horse serum. Reticulocytes were prepared from rabbits treated with phenylhydrazine (3). Washed cell suspensions were extracted in a French pressure cell and fractionated as described in preceding papers.

Saccharomyces Cerivisae.—A strain of bakers' yeast was grown at 32°C with shaking in the following medium: Bacto yeast extract (Difco Laboratories, Detroit, Mich), 4 g; glucose, 10 g; KH_2PO_4, 2 g, all brought to 1 liter and pH 5.5. When growth reached an OD 700 mμ = 2–2.3, the cells were harvested by centrifugation in the cold and washed with 0.2% potassium phosphate containing 1% glucose at pH 5.5. The washed pellet was stored at −15°C until used. Extracts were prepared in the French pressure cell by exactly the same procedure used for extraction of mammalian cells.

NAD and NADH.—These were determined by the method of Glock and McLean (4), modified as described in the preceding paper (1).

RESULTS

Effect of Toxin in Cell-Free Systems Isolated from Certain Species.—There is considerable variation among cells from different species in their sensitivity

to diphtheria toxin. Living cell lines from rats and mice, for example, are highly resistant (5, 6). Cold-blooded vertebrates are sensitive to toxin above 20°C but not below this temperature (7). Insects are sensitive at certain stages in their life cycle but not at others (8). Unicellular forms such as bacteria, protozoa, and yeasts have always proved completely resistant.

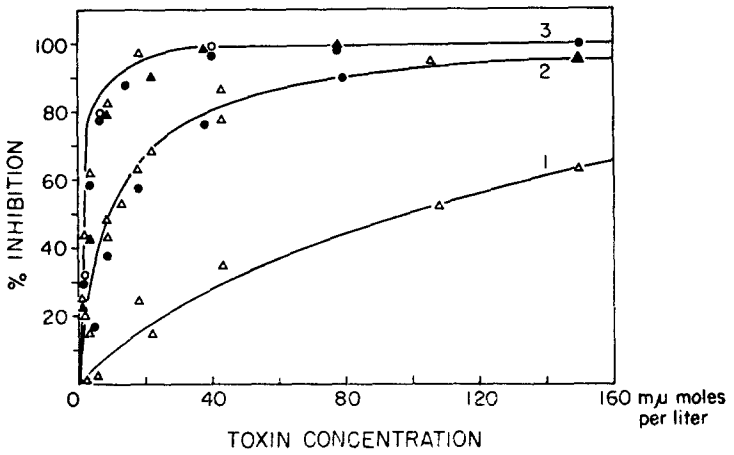

FIG. 1. Effect of diphtheria toxin on amino acid incorporation in cell-free extracts at constant NAD levels. The curves shown have been drawn as predicted from equation 3 (see Discussion) using the experimentally determined value for $K_1 = 2.4 \times 10^3$ liters/mole and assuming a value for $K_2 = 1.4 \times 10^{10}$ liters/mole. All of the experimental points have been normalized to a maximum inhibition of 100%. Since the experiments were carried out by different individuals on extracts from different species at different times, the actual values found for maximal inhibition varied between about 70 and 90%, depending on relative amounts of "free" and "bound" transferase II. The NAD concentrations were as follows: Curve 1, 0.3 μM; curve 2, 3 μM; curve 3, 30 μM. Poly U-directed phenylalanine-^{14}C incorporation was followed in all cases except for one set of points which have been recalculated from earlier published data (9) using leucine-^{14}C at 30 μM NAD. ●, HeLa cells; △, rabbit reticulocytes; ○, HeLa cells (leucine-^{14}C); ▲, mouse L-cells.

We have confirmed earlier observations (9) that the cell-free amino acid incorporating system extracted from *Escherichia coli* is not inhibited at all by diphtheria toxin. On the other hand, as can be seen from Table I, toxin at a concentration of 10 μg/ml inhibited poly U-directed phenylalanine uptake by at least 70% in a cell-free extract prepared from bakers' yeast, even though very much larger toxin concentrations have no effect whatsoever on living yeast cells. Similarly, as will be discussed in more detail below, an incorporating system extracted from mouse L cells proved to be just as sensitive to diphtheria toxin as systems extracted from cells of human or rabbit origin.

The fact that inhibition by toxin of phenylalanine uptake in *undialyzed*

mouse L cell extracts was almost 90%, even without addition of NAD, suggests that the resistance of living cells of this strain to toxin cannot be explained in terms of a low NAD content. A series of analyses by the method of Glock and McLean (4) on freshly prepared and frozen HeLa cells, whether normal or intoxicated, have indicated a total NAD + NADH content of 3.5 ± 0.7 mg/g cell protein. The NAD:NADH ratio varied between 4:1 and 10:1 depending on the method of extraction. The NAD + NADH content of mouse

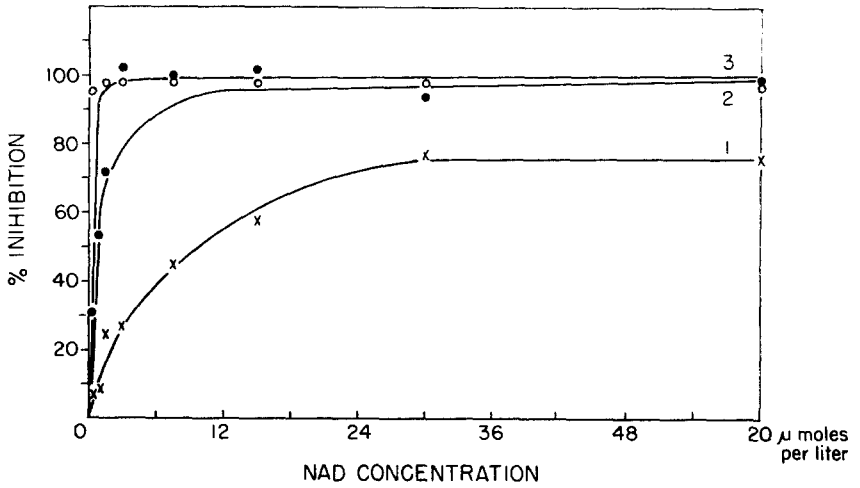

FIG. 2. Effect of NAD on poly U-directed phenylalanine-^{14}C incorporation in a crude HeLa extract at constant toxin levels. Curves 2 and 3 have been drawn as predicted from equation 3 (see Discussion) using values of 2.4×10^3 and 1.4×10^{10} liters/mole for K_1 and K_2 as in Fig. 1. The experimental points have been normalized to a maximum inhibition of 75% for curve 1 and to 100% for curves 2 and 3. The actual maximal inhibitions observed were 59, 78, and 78% respectively. In contrast to Fig. 1, all experimental points were obtained on a single preparation. Toxin concentrations used were: curve 1, 4×10^{-9} M; curve 2, 4×10^{-8} M; curve 3, 4×10^{-7} M.

L cells fell between the same limits. It would therefore appear that the resistance of living yeast cells and of rodent cells is due to failure of toxin to reach a target site, either within the cell or on its membrane, probably because of a permeability barrier.

Quantitative Studies.—The inhibition of amino acid incorporation by increasing concentrations of diphtheria toxin at three different NAD levels (0.3, 3, and 30 μM) was examined in systems extracted from cells of three different species: human HeLa, rabbit reticulocyte, and mouse L cells. The results, plotted in Fig. 1, have been recalculated on the basis of 100% maximal inhibition in each case. In the actual experiments, the observed maximal inhibition approached varied between 70 and 90%, depending on the proportion

of resistant ribosome-bound transferase II present in the given preparation. In a similar manner, we have plotted in Fig. 2, the effect on peptide bond formation of increasing concentrations of NAD at three different toxin levels (0.3, 3, and 30 μg/ml). The three experiments summarized in this figure were carried out at the same time using the same HeLa extract.

Several conclusions may be drawn from the curves plotted in Figs. 1 and 2: (*a*) There is an inverse relationship between NAD and the toxin. The higher the NAD concentration, the less toxin is required for inhibition of peptide bond formation. (*b*) Despite the considerable differences in sensitivity to toxin between various cell lines already alluded to above, Fig. 1 shows that at any given NAD concentration, the toxin inhibition curves using cell extracts from three species (human, rabbit, and mouse) are virtually superposable. It will be noted, too, that in the HeLa system the same curve is obtained using poly U and phenylalanine-^{14}C as with leucine-^{14}C and natural messenger RNA. (*c*) When the effect of varying NAD concentrations on amino acid incorporation was studied, it was found that at the lowest toxin concentrations (curve 1 of Fig. 2) maximal inhibition of peptide bond formation could not be reached even at very high NAD concentrations. This suggests that toxin may interact directly, mole for mole, with transferase II rather than catalytically to cause its inactivation. This conclusion is in agreement with our earlier observation (3) that, at low toxin concentrations, inhibition in the cell-free system may be overcome provided that more than a certain critical amount of soluble supernate containing transferase II is added back. (*d*) The curves shown in Figs. 1 and 2 are best fitted by equations that describe rectangular hyperbolae. Their shape suggests that reversible equilibria may exist between toxin and NAD on the one hand, and transferase II on the other, and that the extent of interaction between the three components may be governed by mass law considerations.

Reversibility of Toxin Action—Effect of Nicotinamide.—The quantitative relationships between toxin and NAD that were discussed in the preceding section strongly suggested that inhibition of amino acid incorporation might result from *reversible* interactions with transferase II. Consistent with this hypothesis is our finding that incorporation of phenylalanine-^{14}C in extracts, whether derived from HeLa, reticulocyte, or mouse L cells, may be protected to a considerable extent by the addition of relatively low concentrations of nicotinamide. Table II shows that, in the presence of 10^{-7} M toxin and a nicotinamide concentration about 100 times that of NAD, inhibition of phenylalanine uptake was reduced from 80–90% to only 50% or less in extracts from each of the three species.

The results of an experiment to determine the effect of increasing concentrations of nicotinamide on inhibition of the poly U–directed incorporation of phenylalanine at different toxin and NAD levels in the HeLa cell-free system

are plotted in Fig. 3. It will be noted that nicotinamide is most effective either at a low toxin concentration (4×10^{-9} M as in curve 5) or at a low NAD concentration (6×10^{-6} M as in curve 4). Under these conditions, at a nicotinamide concentration of 5 mM, the observed phenylalanine uptake was at least 80% that of the control to which no toxin had been added. Conversely, by raising either toxin or NAD concentrations, the effectiveness of nicotinamide in relieving the inhibition is reduced.

TABLE II

Effect of Nicotinamide on Inhibition of Phenylalanine Incorporation by Toxin in Mammalian Cell Extracts

Additions	Phenylalanine-^{14}C-incorporated					
	HeLa cells*		Rabbit reticulocytes‡		Mouse L-cells‡§	
	cpm	Per cent inhibition	cpm	Per cent inhibition	cpm	Per cent inhibition
Control	990	0	5422	0	434	0
+ Nicotinamide	990	0	5309	2	381	12
+ Toxin + NAD	171	83	1171	78	39	91
+ Toxin + NAD + Nicotinamide	798	20	2763	48	185	51

* HeLa cell system: phenylalanine-^{14}C, 0.625 μc (12.5 μc/μmole); toxin, 15 μg; NAD, 5.4×10^{-5} M; nicotinamide, 1.5×10^{-2} M. Total volume, 1.4 ml. Incubation at 37°C for 40 min.

‡ Rabbit reticulocyte system: phenylalanine-^{14}C; 0.5 μc (125 μc/μmole); AS-70 fraction, 10 μl; reticulocyte deoxycholate-treated ribosomes, 240 μg; rat liver sRNA, 25 μg; toxin, 0.75 μg; NAD, 1.5×10^{-5} M; nicotinamide, 3.3×10^{-3} M. Total volume, 0.25 ml. Incubation at 37°C for 40 min.

§ Mouse L-cell system: phenylalanine-^{14}C, 0.5 μc (125 μc/mole); toxin, 1.25 μg; NAD, 3×10^{-5} M; nicotinamide, 8.25×10^{-3} M. Total volume, 0.25 ml. Incubation at 37°C for 40 min.

The action of nicotinamide is specific. Several compounds, structurally related to nicotinamide, have been tested for their ability to relieve the inhibition of amino acid incorporation caused by toxin and NAD. Thionicotinamide was found to be just as active as nicotinamide itself, but nicotinic acid, isonicotinic acid hydrazide, and pyridine-3-sulfonic acid each proved to be completely ineffective in protecting against the toxin.

Reactivation of Inhibited Extracts by Nicotinamide.—If the inactivation of transferase II by toxin is truly reversible, it should be possible to reactivate an intoxicated system by the addition of nicotinamide. Two experiments, summarized in Table III, show that such is indeed the case for intoxicated cell-free systems isolated from HeLa cells and from rabbit reticulocytes. The

table shows, as expected (2, 3), that when active extracts from either cell type were treated with NADase at 25°C for 5 min *before* the addition of toxin no inhibition by toxin of incorporation of phenylalanine into trichloroacetic acid (TCA)-precipitable peptides was observed during the subsequent incubation at 37°C. On the other hand, when NADase was added *after* a 5 min treatment at 25°C with both toxin and NAD, activity was not restored to the system and

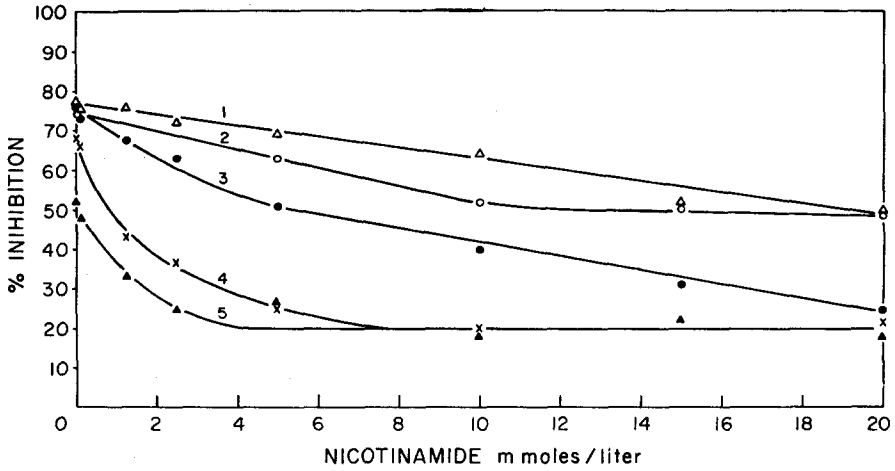

FIG. 3. Effect of increasing nicotinamide concentrations on inhibition of phenylalanine-^{14}C incorporation in a crude HeLa extract at different levels of toxin and of NAD.

Curve No.	Toxin concentration × 10^7 M	NAD concentration × 10^5 M
1	4.0	3
2	0.4	6
3	0.4	3
4	0.4	0.6
5	0.04	3

incorporation of label was only about 50% that of the control extracts.[1] Removal of excess NAD thus does not result in rapid reactivation of an already intoxicated system. Nevertheless, as clearly shown in Table III, addition of nicotinamide to the NADase-treated extract resulted in immediate restoration of amino acid incorporation almost to the control level. This reversal of intoxication brought about by nicotinamide is almost certainly due to the

[1]Presumably, a considerable proportion of the highly labile soluble transferase II irreversibly lost activity during the brief incubation at 25°C. This would account for the relatively low per cent inhibition by toxin observed in experiments in which 0.25 M sucrose is not used as a stabilizing medium (11).

liberation of free active transferase II from an inactive complex, and it demonstrates clearly that the role of NAD as an essential cofactor for the inactivation of this enzyme by toxin is not its usual one of acting as a coenzyme for a dehydrogenation reaction.

The reactivation of an intoxicated amino acid–incorporating system by nicotinamide is strikingly demonstrated by the experiment illustrated in Fig. 4. In this experiment, the poly U–directed incorporation of phenylalanine in a crude HeLa extract was followed as a function of time in four tubes, all of

TABLE III

Reversal of Inhibition by Nicotinamide in HeLa and Reticulocyte Extracts

Line	Additions before NADase	Additions after NADase	Phenylalanine-^{14}C-incorporated			
			Reticulocyte extract		HeLa extract	
			cpm	Per cent inhibition	cpm	Per cent inhibition
	(1)	(2)	(3)	(4)	(5)	(6)
1	Control ± nicotinamide	—	1156	0	1002	0
2	+ NAD	Toxin	1105	4	1000	0
3	+ toxin + NAD	—	542	53	497	50
4	+ toxin + NAD	Nicotinamide	926	20	990	1

Extracts from rabbit reticulocytes and from HeLa cells were preincubated for 5 min at 25°C with the additions listed in column 1: toxin, 15 μg; NAD, 3×10^{-5} M; nicotinamide, 3×10^{-2} M. The reaction mixtures were then rapidly cooled to 0°C and 15,000 units streptococcal NADase were added. After 1 hr at 0°C, the mixtures were supplemented with phenylalanine-^{14}C, 0.625 μc (12.5 μc/mole) and the additions listed in column 2. Total volume was 1.4 ml. Incubation was at 37°C for 40 min. The results listed in line 4 represent averages from duplicate tubes in each case. The mixtures were aliquots of those used in line 3 to which nicotinamide was added.

them containing NAD at a concentration of 3×10^{-5} M. Toxin, 6 μg/ml, was added to tubes 2, 3, and 4. At 0 time, the labeled amino acid was added and the tubes were brought rapidly to 37°C. 10 min later, NADase was added to tubes 3 and 4 to destroy excess NAD, and at the same time, nicotinamide (8.25×10^{-3} M) was added to tube 4. At suitable intervals, 0.25 ml aliquots were removed from each tube, immediately precipitated with cold TCA to stop the reaction, washed and processed for counting as described under Materials and Methods. Fig. 4 shows that, from the very outset, toxin caused maximal inhibition of amino acid incorporation in tubes 2, 3, and 4. Addition of excess NADase to tube 3 at 10 min, when amino acid uptake had almost stopped, failed to alter the kinetics of the reaction in the intoxicated extract. In the presence of nicotinamide, however, there was immediate recovery and

renewed rapid uptake of the labeled amino acid which continued for at least 20 min after incorporation had completely stopped in the control tubes.

Effect of Toxin on Stability of Soluble Transferase II.—Free, unbound transferase II is an extremely labile enzyme (11, 12). In crude HeLa extracts in the

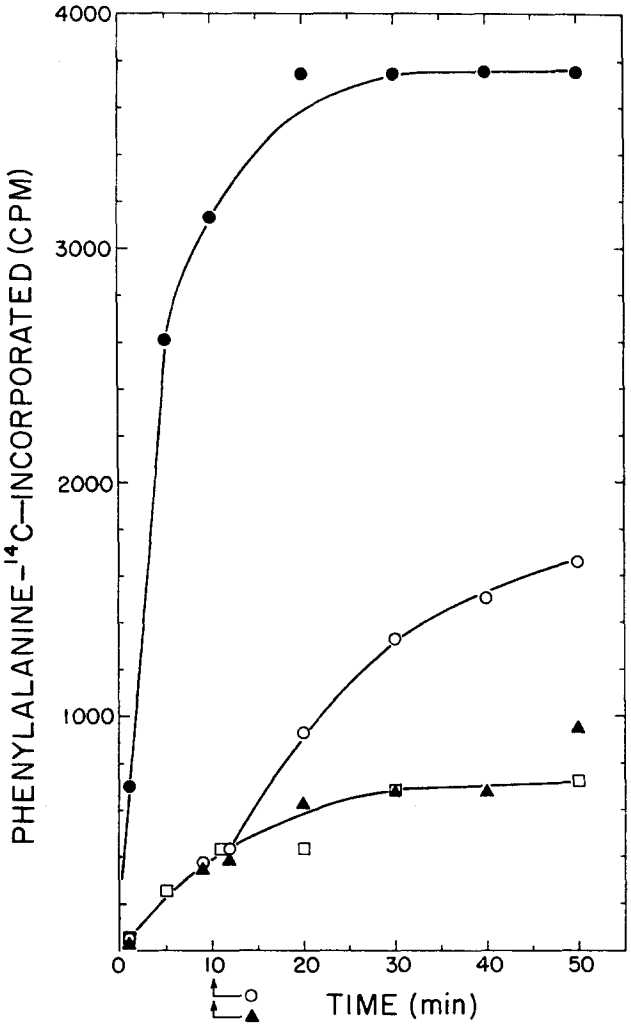

FIG. 4. Kinetics of inhibition of poly U-directed phenylalanine-^{14}C incorporation at 37°C in a cell-free HeLa extract by toxin and reversal of inhibition by nicotinamide. ●, control; normal HeLa extract, no additions; □, toxin (6 μg/ml) + NAD (30 μM) at 0 time; ▲, toxin (6 μg/ml) + NAD (30 μM) at 0 time; streptococcal NADase (8500 units/ml) added after 10 min; ○, toxin (6 μg/ml) + NAD (30 μM) at 0 time; NADase and nicotinamide (0.03 M) were added after 10 min.

absence of sucrose, the soluble enzyme may become completely and irreversibly inactivated within a few minutes at 37°C. When bound to ribosomes, however, the enzyme becomes more stable and remains active after all activity in the supernate has been lost. In the presence of NAD, toxin also seems to stabilize transferase II by interaction to form a relatively stable complex. We have already seen from Fig. 4 that addition of nicotinamide can restore

TABLE IV

Stabilization of HeLa Transferase II by Toxin and NAD

Line	Additions before NADase	Additions after NADase	Phenylalanine-^{14}C-incorporated	
			cpm	Per cent inhibition
	(1)	(2)	(3)	(4)
1	—	Control ± nicotinamide	3508	0
2	—	Control + toxin + NAD	988	72
3	—	Control + toxin + NAD + nicotinamide	2997	15
4	Control ± nicotinamide	—	1000	—
5	Control + toxin + NAD	—	1006	—
6	Control + toxin + NAD	Nicotinamide	2005	—

Normal crude HeLa extracts used in lines 1–3 were *not* preincubated or otherwise treated with NADase. Reaction mixtures contained: toxin, 1.5 μg; NAD, 3×10^{-5} M; nicotinamide, 8.25×10^{-3} M; streptococcal NADase, 2500 units; phenylalanine-^{14}C, 0.5 μc (125 μc/mole). Final volume, 0.25 ml. The extracts used in lines 4–6 were preincubated for 7 min at 37°C with the additions listed in column 1. The tubes were then rapidly cooled to 0°C and NADase was added. After 1 hr at 0°C, mixtures were supplemented with phenylalanine-^{14}C and other additions as listed in column 2. Incorporation of label into TCA-precipitable material was measured after 40 min at 37°C. The reaction given in line 6 was the average of triplicate determinations using aliquots removed after NADase treatment from the tube used in line 5.

transferase activity to an intoxicated system at a time when all incorporation had stopped in control extracts. The stabilizing effect of toxin is shown even more clearly by the experiment summarized in Table IV. In this experiment, addition of toxin to the crude HeLa extract in the usual manner resulted in 72% inhibition of phenylalanine incorporation. The 28% of toxin-resistant activity that still remained may be accounted for by transferase II that was bound in a more stable form to the ribosomes (13). As can be seen from line 4 of the table, preincubation of the extract with nicotinamide at 37°C for only 7 min, in the absence of toxin and before initiation of incorporation by addition of labeled phenylalanine, reduced its uptake to the intoxicated level. On the other hand, when preincubated under the same conditions together

with toxin and NAD, incorporation could be doubled by addition of nicotinamide. This result is most readily explained by assuming that nicotinamide caused liberation of free transferase II by dissociation of a relatively stable toxin-enzyme complex.

Failure of Nicotinamide to Restore Activity to Intoxicated Cells.—The reactivation of intoxicated cell-free extracts by nicotinamide raised the possibility that it might be possible to reverse the action of toxin on living cells. However, our attempts to demonstrate an effect of nicotinamide on intact intoxicated cells have thus far proved unsuccessful. For example, in the experiment summarized in Table V, HeLa cells growing exponentially in spinner

TABLE V

Effect of Nicotinamide and Diphtheria Toxin on Leucine-^{14}C Uptake by Normal and Intoxicated HeLa Cells

Spinner No.	Cell suspension	Additions	Fluorescent cells	Leucine-^{14}C
			counts/ml	*cpm/ml*
1	Normal	None	1.5×10^5	1353
2	"	Nicotinamide + antitoxin	1.7×10^5	1260
3	Intoxicated	None	0.75×10^5	37
4	"	Nicotinamide + antitoxin	0.8×10^5	38

* Washed cells, both intoxicated and controls, were suspended to 25 ml volumes and about 10^5 cells per ml in Eagle's medium containing 5% calf serum and leucine-^{14}C (0.4 μmoles per ml, 2.5 μc per μmole). Nicotinamide to a final concentration of 2×10^{-3} M and antitoxin, 5 units/ml were added as indicated. After 17 hr spinner culture at 37°C, amino acid incorporation was measured in the usual manner and cell counts were carried out in the fluorescent microscope on aliquots treated with fluorescein diacetate (14).

culture were treated with 0.5 μg/ml (0.2 Lf/ml) of diphtheria toxin. After 6 hr at 37°C, intoxicated and control cells were centrifuged and washed three times by resuspension to their original volume in buffered salt solution, followed by centrifugation in order to remove all traces of free toxin. After resuspension in growth medium containing 5% calf serum and leucine-^{14}C to a count of 10^5 cells per milliliter, control and intoxicated cultures were each further divided into two equal portions. To one of them was added nicotinamide (2×10^{-3} M) plus antitoxin (5 units/ml). After incubation in spinners for 17 hr, cells from 1 ml aliquots were collected on Millipore filters and counted in the usual manner. Table V shows that even in the presence of both nicotinamide and antitoxin, leucine uptake was negligible in comparison with the controls.

It is of interest to add that even after more than 20 hr, during which the intoxicated cells synthesized virtually no protein, their morphological appearance remained normal, and the cells became highly fluorescent upon treatment

with fluorescein diacetate[2] indicating that their cell membranes were still undamaged (14). Cultures in which protein synthesis had been blocked by cycloheximide for the same length of time also retained fluorescein, but were able to resume normal exponential growth after the antibiotic had been removed by washing.

DISCUSSION

A good many antibiotics are now known that inhibit protein synthesis by interfering with transfer of amino acids from sRNA to the growing peptide chain (Schweet and Heintz, reference 15). The various tetracyclines, for example, appear to act by specifically inhibiting the binding of aminoacyl-sRNA to the ribosomes (16, 17). Puromycin, on the other hand, functions by replacing aminoacyl-sRNA and reacting with the growing peptide chain to form a C terminus (18). This results in release of nascent polypeptide chains from the ribosomes and breakdown of polysomes. Other antibiotics, among them cycloheximide (Actidione), appear to block the final step in protein synthesis that involves formation of the peptide bond itself (19). Cycloheximide is of particular interest to us because in many ways it parallels diphtheria toxin in its mode of action. Like toxin, cycloheximide probably inhibits, reversibly, the action of transferase II in extracts from yeast as well as from mammalian cells. Like toxin, cycloheximide is completely without effect when added to the *E. coli* system (20). Neither toxin nor cycloheximide influence polysome breakdown or cause release of nascent peptide chains as is the case with puromycin (2, 19). Finally, inhibition of amino acid incorporation in living cells by either toxin or cycloheximide is complete and is brought about by lower concentrations than are required in cell-free systems.

The finding, that the inhibition by diphtheria toxin of peptide bond formation in cell-free systems can be prevented and even reversed by nicotinamide, permits us to draw important conclusions regarding the mechanism of intoxication. In the first place, it demonstrates clearly that transferase II is not inactivated by oxidation and therefore the requirement for NAD as a cofactor is not its usual one involving a dehydrogenation reaction with resulting reduction to NADH. Moreover, the ease of reversibility together with the fact that nicotinamide is a relatively unreactive compound indicates that inhibition of amino acid transfer probably involves an interaction in which neither toxin nor transfer enzyme is chemically altered. In the preceding paper, we showed that toxin (Tx) can interact reversibly with NAD:

$$1.\ \mathrm{Tx} + \mathrm{NAD} \rightleftharpoons \mathrm{Tx(NAD)}; \qquad K_1 = 2.4 \times 10^3 \text{ liters/mole.}$$

We now suggest that the product of the above reaction may react further with

[2]We are indebted to Dr. B. Papermaster for a generous gift of fluorescein diacetate.

transferase II (Tf II) to yield an inactive product:

$$2.\ \text{Tx(NAD)} + \text{Tf II} \rightleftharpoons \text{Tx(NAD)Tf II}.^{3}$$

Reaction 2 can be reversed by nicotinamide with the liberation of free, active transferase II once more. If the equilibrium constant for reaction 2 is K_2, then, provided that the law of mass action is applicable, it can be shown that the inhibition of soluble transferase II by toxin and NAD should be determined by the following equation:

$$3.\ \text{Per cent inhibition} = 100\,\frac{(\text{Tx})\,(\text{NAD})\,K_1K_2}{1 + (\text{NAD})\,K_1 + (\text{Tx})\,(\text{NAD})\,K_1K_2}$$

in which (Tx) equals the total toxin concentration added to the system and it is assumed that the transferase concentration is small in relation to the toxin added.

All of the curves shown in Figs. 1 and 2 were drawn assuming equation 3 to be correct with a value of $K_2 = 1.4 \times 10^{10}$ liters/mole and the experimentally determined value, $K_1 = 2.4 \times 10^{3}$ liters/mole.[4] It will be noted that the experimental points fall closely on the predicted curves irrespective of the cell species from which the extracts were derived or of whether or not leucine or phenylalanine was the amino acid whose incorporation was inhibited. This suggests that the high affinity of the transferase II molecule for the toxin-NAD complex varies relatively little from one species to another.

Although the above model accurately predicts the quantitative inhibition of soluble transferase II as a function of NAD and toxin concentrations, it fails to explain why nicotinamide brings about the rapid dissociation of the toxin-transferase complex despite the fact that removal of excess NAD by treatment with NADase does not result in reactivation of the system. The failure of NADase to reverse the inactivation of transferase II suggests that NAD and nicotinamide may not be competing for the same site on the toxin molecule. It must be admitted that we have, as yet, obtained no evidence that NAD is actually present in the toxin-transferase complex. It is thus quite possible that, although NAD may so alter the tertiary conformation of toxin as to permit its interaction with the transfer enzyme, the nucleotide itself may be eliminated in the process. Whether or not NAD is present, the evidence that has been presented clearly indicates that a toxin-transferase complex is formed.[5]

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[3] A possible alternative to reaction 2 might be the following:

$$\text{Tx(NAD)} + \text{Tf II} \rightleftharpoons \text{Tx-Tf II} + \text{NAD}$$

[4] Curve 3 of Fig. 2 was not calculated according to equation 3, since it was assumed that insufficient toxin was present to react with all the transferase II.

[5] The existence of a toxin-transferase complex is proved by unpublished experiments in which insoluble toxin-antitoxin floccules in the presence of NAD were used to remove transferase II from cell free extracts. Treatment of the washed floccules with nicotinamide resulted in the release of active transferase.

The experiments of Collier (2) and those discussed above leave no doubt that the mode of action of toxin in cell-free systems involves its specific interaction with soluble transferase II to form an inactive complex that can be dissociated by nicotinamide. We may now ask the question: Can this same mechanism be used to explain the inhibition by toxin of amino acid incorporation by living cells in tissue culture, and can it eventually be extended so as to explain the lethal action of toxin when injected into susceptible animals? With regard to the latter question, the recent results reported by Bonventre and Imhoff (21) suggest that the answer may be exceedingly complex. Using radioautograms and incorporation of isotopes into tissue proteins, only the heart and the pancreas showed marked inhibition in intoxicated guinea pigs. Other tissues, such as adrenals, kidney, liver, etc., known to be severely damaged by toxin, showed no reduction in the quantity of leucine incorporated into protein.

As has already been discussed (3), inhibition of amino acid uptake by mammalian cells in tissue culture by diphtheria toxin is both highly specific and complete. Nevertheless, cell-free extracts from intoxicated cells are still capable of incorporating phenylalanine into TCA-precipitable peptides at a moderate rate that seems to be determined by their ribosome-bound transferase II contents and seems to be resistant to further inhibition by toxin. The model that we have proposed to explain the action of toxin in the cell-free system postulates the mole for mole interaction between toxin and soluble transfer enzyme. As was seen in Fig. 2, in the presence of excess NAD, toxin may become limiting at very low concentrations insufficient to interact with all the soluble transferase present. Yet, very much lower concentrations of toxin than this are effective in blocking the growth of mammalian cell cultures. Moreover, the effect of toxin on living cells is *irreversible*; the cells do not recover even when thoroughly washed and treated with both excess nicotinamide and antitoxin. Finally, it should be stressed that mere inhibition of protein synthesis is not sufficient to kill the cells. Amino acid incorporation in HeLa cultures may be blocked for many hours with either puromycin (22) or cycloheximide without significantly affecting the ability of the cells to resume normal growth and protein synthesis, once the antibiotic has been washed out. A further difficulty arises when we consider the amounts of toxin required for inhibition of living cells when compared with the systems extracted from them. It is easily calculated (22) from the experiments of Lennox and Kaplan (23) and of Gabliks and Solotorofsky (6), that at an added concentration of only 200–400 molecules per cell (ca. 10^{-17} M!) all growth of sensitive cells ceases within 3 days. Experiments with toxin, internally labeled with methionine-^{3}H (22) or trace-labeled with carrier-free ^{125}I, have shown that even when a saturating dose of toxin is added to growing HeLa cells, only a few molecules (not more than 100) become fixed to the cells.[6]

[6] Pappenheimer, A. M., Jr., and R. Brown. Unpublished observations.

Since transferase II is required for synthesis, on ribosomes, of *all* cellular proteins, it seems probable that there are at least as many molecules of this enzyme in a cell as there are ribosomes. The number of ribosomes per HeLa cell has been estimated to be in the order of $5–10 \times 10^6$. Obviously, the model we have postulated above to explain the mechanism by which toxin inhibits amino acid incorporation in cell extracts must be modified before it can be used to explain how toxin prevents protein synthesis in living cells.

At present we propose the following tentative hypothesis to explain how relatively few toxin molecules reacting per cell might bring protein synthesis to a standstill in living cultures. We suggest that the large toxin molecule (24) does not penetrate the interior of the cell, but rather interacts with transferase II located on the cell membrane itself. Let us suppose that on mammalian cell membranes, there exists a limited number of strategically located sites where peptide bonds are formed that are necessary for membrane function. If, for example, such sites were needed for transport of an essential amino acid across the cell membrane, then inactivation by toxin would be expected to arrest protein synthesis completely after a short lag during which the amino acid pool was becoming depleted. Cells intoxicated by such a mechanism would still contain all of their protein-synthesizing machinery undamaged except, perhaps, for soluble transferase II which, because of its extreme lability, would in all likelihood become inactivated. Extracts from intoxicated cells, when supplied with amino acids and a suitable messenger, would be expected to synthesize polypeptides in amounts determined by their ribosomally bound transferase II contents as was indeed shown to be the case in the third paper of this series (3). It is hoped that experiments, now in progress, will help to clarify the apparent discrepancies existing between the effects of toxin in cell-free extracts and in the living cell.

SUMMARY

Inhibition of soluble transferase II activity in cell-free systems by diphtheria toxin and NAD can be prevented or reversed in the presence of a sufficient concentration of nicotinamide. Quantitative studies on inhibition of peptide bond formation in cell-free extracts by toxin and NAD have indicated that two successive reversible reactions are involved. First, toxin and NAD interact mole for mole to form a relatively dissociable complex. This toxin-NAD complex then reacts with transferase II to form an enzymatically inactive product that is but slightly dissociated. In the presence of sufficient nicotinamide, however, the latter complex can be broken down to yield active transferase II once more.

Based on the above model, an equation has been derived that accurately predicts the per cent inhibition of amino acid incorporation in cell-free systems at any given toxin and NAD level. The observed inhibition appears to be in-

dependent of the sensitivity to toxin of the cell species from which the extracts were derived, and depends only on the toxin and NAD concentrations.

Although the model satisfactorily explains inhibition of peptide bond formation by toxin in cell-free systems, further assumptions are needed to explain how still lower concentrations of toxin are able to arrest protein synthesis completely in the living cell.

BIBLIOGRAPHY

1. Goor, R. S., and A. M. Pappenheimer, Jr. 1967. Studies on the mode of action of diphtheria toxin. IV. Specificity of the cofactor (NAD) requirement for toxin action in cellfree systems. *J. Exptl. Med.* **126**:913.
2. Collier, R. J. 1967. Effect of diphtheria toxin on protein synthesis: Inactivation of one of the transfer factors. *J. Mol. Biol.* **25**:83.
3. 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.
4. Glock, G. E., and P. McLean. 1955. Levels of oxidized and reduced diphosphopyridine nucleotide and triphosphopyridine nucleotide in animal tissues. *Biochem. J.* **61**:388.
5. Kato, I., and A. M. Pappenheimer, Jr. 1960. An early effect of diphtheria toxin on the metabolism of mammalian cells growing in culture. *J. Exptl. Med.* **112**:329.
6. Gabliks, J., and M. Solotorovsky. 1962. Cell culture reactivity to diphtheria, staphylococcus, tetanus and *Escherichia coli* toxins. *J. Immunol.* **88**:505.
7. Grasset, E., and A. Zontendyk. 1931. Immunological studies in reptiles and their relation to aspects of immunity in higher animals. *Publ. South African Inst. Med. Res.* **4**:377.
8. Pappenheimer, A. M., Jr., and C. M. Williams. 1952. Effects of diphtheria toxin on the cecropia silkworm. *J. Gen. Physiol.* **35**:727.
9. 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. Exptl. Med.* **120**:1019.
10. Nirenberg, M. W. 1963. Cell-free protein synthesis directed by messenger RNA. *In* Methods of Enzymology. S. P. Colowick and N. O. Kaplan, editors. Academic Press Inc., New York. **6**:17.
11. Grossi, L. G., and K. Moldave. 1960. The effect of soluble factors on the transfer of amino acids from soluble ribonucleic acid to microsomal proteins. *J. Biol. Chem.* **235**:2370.
12. Sutter, R. P., and K. Moldave. 1966. The interaction of aminoacyl transferase II and ribosomes. *J. Biol. Chem.* **241**:1698.
13. Gasior, E., and K. Moldave. 1965. Resolution of aminoacyl-transferring enzymes from rat liver by molecular sieve chromatography. *J. Biol. Chem.* **240**:3346.
14. Rotman, B., and B. W. Papermaster. 1966. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. *Proc. Natl. Acad. Sci. U.S.* **55**:134.

15. Schweet, R., and R. Heintz. 1966. Protein synthesis. *Ann. Rev. Biochem.* **35**:723.
16. Hierowski, M. 1965. Inhibition of protein synthesis by chlortetracycline in the *E. coli in vitro* system. *Proc. Natl. Acad. Sci. U.S.* **53**:594.
17. Clark, J. M., and A. Y. Chang. 1965. Inhibitors of the transfer of amino acids from amino acyl soluble ribonucleic acid to proteins. *J. Biol. Chem.* **240**:4734.
18. Nathans, D. 1964. Inhibition of protein synthesis by puromycin. *Federation Proc.* **23**:984.
19. Colombo, B., L. Felicetti, and C. Baglioni. 1966. Inhibition of protein synthesis in reticulocytes by antibiotics. I. Effects on polysomes. *Biochim. Biophys. Acta.* **119**:109.
20. Ennis, L. H., and M. Lubin. 1964. Cycloheximide: Aspects of inhibition of protein synthesis in mammalian cells. *Science.* **146**:1474.
21. Bonventre, P. F., and J. G. Imhoff. 1966. Studies on the mode of action of diphtheria toxin. I. Protein synthesis in guinea pig tissues. *J. Exptl. Med.* **124**:1107.
22. 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 cultures. *In* Cell Culture in the Study of Bacterial Disease. M. Solotorovsky, editor. Rutgers University Press, New Brunswick. 21.
23. Lennox, E. S., and A. S. Kaplan. 1957. Action of diphtheria toxin on cells cultivated *in vitro*. *Proc. Soc. Exptl. Biol. Med.* **95**:700.
24. Raynaud, M., B. Bizzini, and E. Relyveld. 1965. Composition en amino-acides de la toxine diphtherique purifee. *Bull. Soc. Chim. Biol.* **47**:261.