STUDIES ON THE MODE OF ACTION OF DIPHTHERIA TOXIN*

VII. TOXIN-STIMULATED HYDROLYSIS OF NICOTINAMIDE ADENINE DINUCLEOTIDE IN MAMMALIAN CELL EXTRACTS

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In a preceding paper it was shown that low concentrations of diphtheria toxin, in the presence of NAD[1] as an essential cofactor, inhibit the incorporation of amino acids into TCA-precipitable peptides in mammalian cell extracts (1). Collier (2) has demonstrated that this inhibitory action of the toxin is due to the specific inactivation of transferase II, one of the highly labile soluble enzymes involved in transfer of amino acids from aminoacyl-tRNA to the growing peptide chain on the ribosome (3, 4). Goor and Pappenheimer (5) also concluded that transferase II was the site of toxin action and provided evidence that soluble transferase II was the *only* factor needed for protein synthesis that is lacking in extracts from intoxicated cells. It was further concluded from quantitative studies (6) that toxin and transferase II interacted stoicheiometrically in the presence of NAD to form an inactive toxin-transferase II complex.

Although the model involving toxin-transferase II complex formation proposed by Goor et al. (6) appeared to account for most of the quantitative relationships between toxin, NAD, and inhibition of peptide bond formation in cell-free extracts, it failed to explain how a mere 25–50 toxin molecules located in the outer cell membrane (7) could completely block protein synthesis and inactivate all of the soluble transferase II in the living cell within a relatively short period of time. Moreover, the model failed to provide a satisfactory explanation for the striking reactivation of previously intoxicated cell extracts upon addition of nicotinamide (6). Finally, despite repeated efforts, we have been unable to obtain any direct evidence for the postulated toxin-NAD-transferase II complex using either ^{125}I-labeled toxin or ^{14}C-labeled NAD.

[1] Abbreviations used: NAD, nicotinamide adenine dinucleotide; ADPR, adenosine diphosphate ribose; GTP, guanosine triphosphate; Phe-tRNA, phenylalanyl-transfer RNA; Tx, toxin; Tfase, transferase.

In the present paper we will show that in soluble fractions containing the aminoacyl transferases from rabbit reticulocytes, or from HeLa cells, toxin causes the reversible hydrolysis of NAD with liberation of free nicotinamide and formation of an enzymatically inactive derivative of transferase II. Evidence will be presented that in cultures of growing HeLa cells a few toxin molecules in the cell membrane rapidly catalyse the conversion of the entire cell content of soluble transferase II into its inactive ADP-ribose derivative. Soon after these observations had been made, we received from Dr. I. Kato of Tokyo the manuscript of the paper by Honjo et al. (8), showing that diphtheria toxin catalyses the splitting of NAD with release of nicotinamide and transfer of ADP ribose to form the inactive ADP riboside of transferase II purified from rat liver. Thus, the findings that we are reporting may be regarded as an independent confirmation and extension of the findings reported by the Japanese workers.

Materials and Methods

Diphtheria Toxin.—Diphtheria toxin, lot 18, partially purified by ammonium sulfate fractionation and dialysis, was obtained through the courtesy of Mr. Leo Levine of the Antitoxin and Vaccine Laboratory, Massachusetts Department of Health. The preparation which had been preserved with merthiolate 1:10,000, consisted of a mixture of monomeric and dimeric forms (Goor, [9]). It was further purified by DEAE-cellulose chromatography (5). The fraction eluting with 0.05 M phosphate at pH 6.9 showed two peaks at 6.6 and 4.3S in the ultracentrifuge corresponding to dimer and monomer respectively.[2] When the concentration of eluting buffer was raised to 0.1 M, the eluate contained the 6.6S component only. A partially purified preparation of toxin obtained some years ago from the Rijksinstitut voor de Volksgezondheit in Utrecht that had not been treated with merthiolate, was completely eluted from the DEAE column with 0.05 M phosphate and showed only a single peak at 4.3S. The absorbance of a 1% solution of toxin at OD 276 is 9.65. Both toxin preparations had about 2.6 μg protein per Lf and 30–60 MLD per Lf.

^{125}I-Labeled Toxin was prepared according to the method of Greenwood et al. (10) as described previously (7) except that unlabeled carrier iodine was added. The labeled toxin contained an average of 2 atoms iodine per molecule.

^{14}C (carbonyl)-NAD—(20.6 mc/mmole) was obtained from Nuclear-Chicago Corp., Chicago, Ill., and ^{14}C(carbonyl)nicotinamide (11 mc/mmole) from New England Nuclear Corp., Boston, Mass.

^{14}C-phenylalanyl-tRNA.—Mixed tRNA from *Escherichia coli* (11) was stripped of amino acids and charged with uniformly labeled ^{14}C-L-phenylalanine (316 mc/mmole) according to Bergman et al. (12). The product was stored frozen at a concentration of 5 mg/ml.

NADases.—Streptococcal NADase (13) was kindly given us by Dr. A. W. Bernheimer. The NADase preparations from *Neurospora*, from horse brain, and from pig brain were gifts from Dr. N. O. Kaplan.

Cells and Cell Extracts.—HeLa cells (S3 strain) were grown in spinner cultures on Eagle's medium supplemented with 5–8% antitoxin-free horse serum. The latter was kindly supplied by the Antitoxin and Vaccine Laboratories of the Massachusetts Department of Health.

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[2] We are indebted to Dr. Moshe Katz for determination of the sedimentation constants in the Model E ultracentrifuge.

Cells were collected by centrifugation at 800 g and washed several times by resuspending them to the original volume in Eagle's salt solution. In some experiments cells were disrupted in a French pressure cell as previously described (5). In most cases, however, packed, washed HeLa cells were suspended in 2 volumes ice-cold salt medium (10 mM KCl, 7.5 mM magnesium diacetate, 10 mM 2-mercaptoethanol, and 10 mM Tris-HCl at pH 7.4). After the cells had been allowed to swell for 10–15 min, they were broken by vigorous homogenization in a small hand-held homogenizer, brought to 100 mM KCl, and centrifuged for 10 min at 10,000 g to remove nuclei and cell debris. Before such "crude extracts" were assayed for their aminoacyl-transferase activity, they were first passed through a Sephadex G25 column equilibrated with 0.25 M sucrose, 80 mM KCl, 7.5 mM magnesium diacetate, 10 mM 2-mercaptoethanol, and 50 mM Tris-HCl pH 7.4.

Reticulocytes were collected from rabbits rendered anemic with phenylhydrazine as previously described (5).

Soluble Enzymes from Reticulocytes and from HeLa Cells.—Aminoacyl transferase–containing enzymes were prepared from crude extracts as described by Allen and Schweet (14). However, we replaced the dialysis step by passage of the final solutions through Sephadex G25 columns, eluting with 1 mM EDTA, 25 mM 2-mercaptoethanol, and 20 mM Tris-HCl pH 7.4, in the case of reticulocyte extracts, and with 1 mM EDTA, 25 mM 2-mercaptoethanol, 50 mM Tris-HCl pH 7.4; and 1 M sucrose in the case of extracts from HeLa cells. In order to reduce the hemoglobin content of the reticulocyte preparation somewhat, we collected the protein precipitating between 40 and 65% (AS65) saturation rather than between 40 and 70% (AS70) saturation with ammonium sulfate. The preparations contained about 20 mg protein/ml as determined by biuret assay.

Reticulocyte Ribosomes.—Ribosomes were prepared from lysates of rabbit reticulocytes and washed according to the method of Allen and Schweet (14). They were further washed by centrifugation through ammonium chloride as follows: 0.5 ml portions of ribosomes suspended in 0.25 M sucrose, 1 M NH_4Cl, 7.5 mM magnesium acetate, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol were layered over 3.5 ml portions of 1 M sucrose containing the same salt mixture supplemented with 0.1 M Tris pH 7.4 and centrifuged for 4 hr at 200,000 g. The pellets were resuspended in 0.25 M sucrose to OD260 mμ = 60 and stored frozen.

Aminoacyl Transfer Assay.—Reaction mixtures in a total volume of 0.1 ml of standard assay buffer (see below) contained 5 μl reticulocyte AS65 or 10 μl HeLa AS70, 0.6 O.D units of reticulocyte ribosomes, 0.04 μmoles GTP, 16 μg polyuridylic, and 10 μl (50 μg) ^{14}C-phenylalanyl-tRNA (ca. 1500 cpm). After 40 min at 37°C, hot TCA-insoluble material was collected on Millipore filters, glued to planchets, and counted in the gas flow counter.

Standard Assay Buffer.—Buffer used so as to contain after dilution concentrations of 50 mM Tris-HCl, pH 7.4, 80 mM KCl, 7.5 mM magnesium acetate and 10 mM 2-mercaptoethanol.

Polyacrylamide Gel Electrophoresis.—was carried out at pH 8.7 with 7.5% polyacrylamide gel according to the method of Ornstein (15) and Davis (16). Bromthymol blue was used as tracking dye. Gels were frozen and sliced so as to yield 50 1-mm sections, each of which was counted in a Picker β-γ Liquimat counter.

RESULTS

Aminoacyl Transferase Activity in Intoxicated HeLa Cells.—When one or more saturating doses of diphtheria toxin are added to a culture of HeLa cells, amino acid incorporation continues at its normal rate for 1.5–2 hr before coming to a complete halt (17–19). It was of interest to assay the aminoacyl transfer activity in preparations made during this latent period of apparent normal growth from cells treated with excess toxin. Fig. 1 shows that the poly-

U–directed phenylalanine incorporating activity, extracted from HeLa cells
that had been exposed for only 20 min to 3 Lf/ml (about ten saturating doses)
of toxin, had already dropped to about 10% of its initial value, whereas in the
presence of the same toxin concentration, the culture continued to incorporate
leucine into its cell protein for nearly 2 hr. In the experiment described by
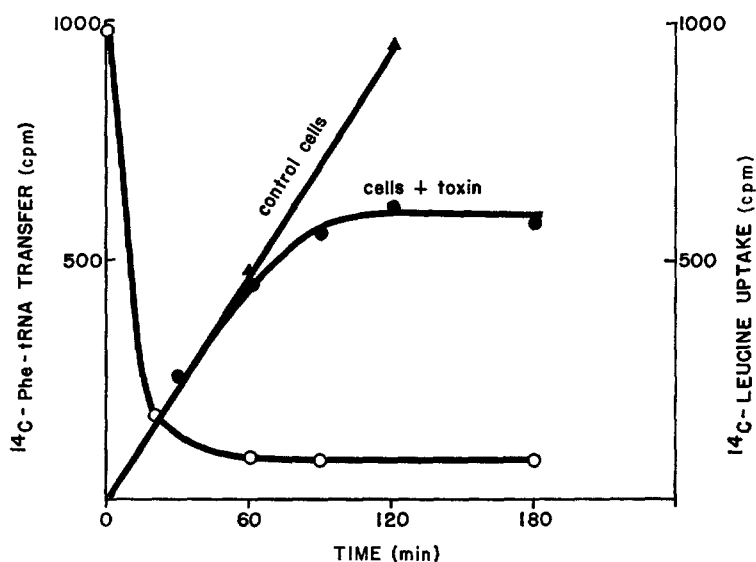

FIG. 1. Aminoacyl transfer activity in extracts from intoxicated cells. 2 liters of
HeLa cells in spinner culture (4 × 10⁵ cells/ml) were treated with 3 Lf/ml (ca. 10^{-7} M)
toxin. A 25 ml aliquot was removed to a small spinner flask containing 25 μc ¹⁴C-leu-
cine in order to follow protein synthesis. From the main culture, 300-ml samples were
taken just before addition of toxin and at 20, 60, 90, and 180 min thereafter (a 40
min sample was lost). The rapidly chilled cells were harvested, washed, and extracted
in a French pressure cell at 0°C as described in Materials and Methods. After passage
through Sephadex G25, ribosomes were removed by 30 min centrifugation at 405,000
g in the International Centrifuge. The supernates were assayed for poly-U–directed
¹⁴C-Phe-tRNA transfer in the usual manner.

Fig. 1, 300-ml samples were removed from the intoxicated culture at the times
specified and were chilled as rapidly as possible in an ice bath before cen-
trifuging and extracting their enzymes. In a subsequent experiment, the
further absorption of toxin after sampling was prevented by immediate addi-
tion of excess antitoxin to the sample when it was taken. Even with this pre-
caution, AS70 preparations containing the transferases from cells that had
been intoxicated for only 10 min showed less than 30% the aminoacyl transfer
activity of AS70 fractions from the control preparation extracted just before
addition of toxin.

We have earlier pointed out (5) that ribosomally bound transferase II is resistant to inactivation by toxin. We tentatively conclude from the above experiments, therefore, that in the living cell exposed to a large dose of toxin, the excess free transferase II becomes rapidly depleted, and cell protein syn-
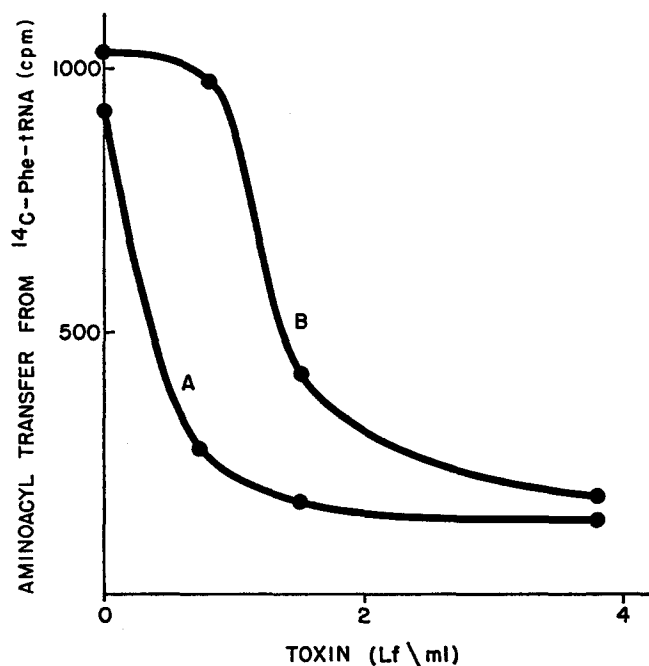

Fig. 2. Effect of preincubation with toxin on inhibition of aminoacyl transfer. Aliquots of the reaction mixture for aminoacyl-transfer using reticulocyte AS65 fraction and lacking only ^{14}C-Phe-tRNA, were incubated for 10 min at 37°C with 20 μM NAD. The tubes were quickly cooled to 0°C, ^{14}C-Phe-tRNA was added, and incubation at 37°C continued for a further 40 min. Incorporation of the label into TCA-insoluble peptides was then measured in the usual manner. *Curve A*, toxin added *before* preincubation; *curve B*, toxin added after cooling to 0°C.

thesis is maintained for the duration of the latent period by transferase bound to the ribosomes.

Effect of Preincubation of Toxin and NAD with the Aminoacyl-Transferase System.—In previous work, the effects of toxin and NAD concentrations on the degree of inhibition of protein synthesis in vitro were examined without any attempt to follow the rate of transferase II inactivation as a function of time. In fact, the system was shown to behave as if the final extent of the inactivation was reached almost instantaneously at 37°C (1, 6). In the earlier studies, toxin and NAD were mixed with the complete amino acid incorporating system at 0°C, quickly brought to 37°C and incubated for 40 min (5).

It is well known that with incorporating systems in vitro, the uptake of labeled amino acids slows down rapidly after the first 10 min and usually stops within 20 min. Therefore, any toxin-specific inactivation would have to take place within a few minutes of the start of the incubation in order to be detected.
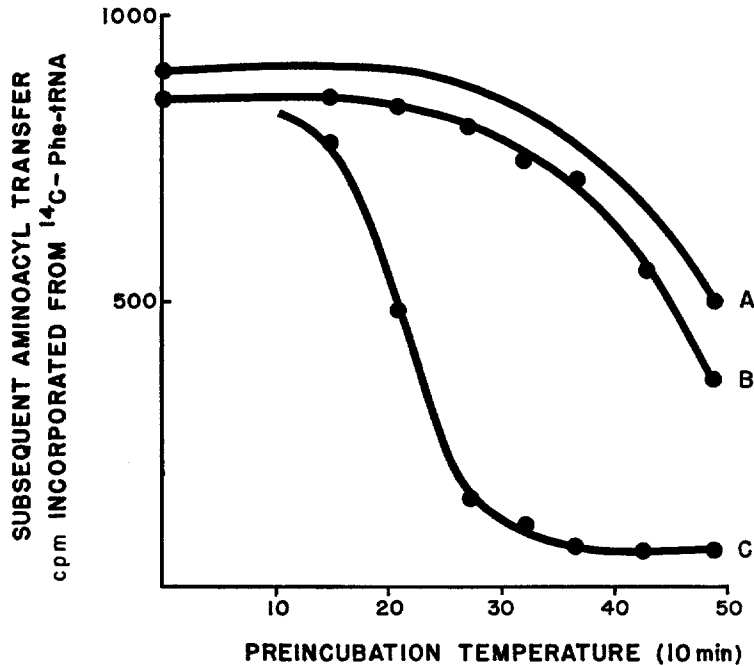

FIG. 3. Effect of temperature on inactivation of reticulocyte transferase by toxin. Tubes with complete reaction mixtures containing reticulocyte AS65 fraction and lacking only ^{14}C-Phe-tRNA, were incubated with 20 μM NAD for 10 min at the temperatures indicated. The tubes were then quickly cooled to 0°C, ^{14}C-Phe-tRNA was added to each and the incubation continued for 40 min at 37°C. Curve A, no toxin added; curve B, preincubated with 0.75 Lf/ml toxin; curve C, 0.75 Lf/ml toxin added after preincubation.

It is possible to separate the intoxication reaction from assay of peptide bond formation simply by delaying addition of one or more of the components required for amino acid incorporation. In this way we have now found that the degree of inhibition may be considerably increased by a few minutes preincubation at a suitable temperature. Fig. 2 demonstrates the increased inhibition of the poly-U-directed formation of polyphenylalanine following preincubation of constant amounts of NAD and of an AS65 fraction from reticulocytes together with varying concentrations of toxin. With excess toxin, the

preincubation has little effect, since maximal inhibition is obtained anyway; but with limiting amounts of toxin (in this case in the neighborhood of 4×10^{-8} M) preincubation may increase the extent of inhibition from very little to 70% or more. Even greater effects may be obtained by lengthening the preincubation period, but the instability of transferase II at 37°C makes prolonged preincubation undesirable.

Fig. 3 summarizes an experiment in which the concentrations of NAD and of toxin were chosen so as to give very little inhibition unless preincubated with the AS65 fraction. The 10 min preincubation was then conducted

TABLE I

Aminoacyl Transfer Activity after Incubation of Certain Components of the Assay System with Toxin and NAD

Additions to preincubation mixture	Aminoacyl transfer activity
	cpm
No preincubation	784
AS65 (toxin added *after* preincubation)	714
AS65	78
AS65 + ^{14}C-Phe-tRNA	76
AS65 + poly-U + GTP	84
AS65 + poly-U + GTP + ribosomes	241
^{14}C-Phe-tRNA + poly-U + GTP + ribosomes	677

In each case the mixtures containing the additions mentioned above were incubated with 30 μM NAD and 0.75 Lf/ml toxin in standard assay buffer for 10 min at 37°C and then cooled to 0°C. All other components needed for poly-U-directed incorporation of ^{14}C-Phe-tRNA were then added and the reaction was allowed to proceed for 40 min at 37°C.

at several temperatures. Below 15°C no significant effect of the toxin could be detected, but the rate of inactivation by toxin increased very rapidly above this temperature. In a subsequent experiment we measured the preincubation times required at different temperatures to give a 50% reduction in counts incorporated, thus avoiding complications arising from nonlinearity of the assay and continued intoxication during the course of the assay. From the latter experiment we obtained a value of $Q_{10} = 2.1$ for the inactivation of transferase II by toxin.

We have used a similar technique to study other factors that might influence the intoxication reaction, choosing toxin and NAD concentrations that give minimal inhibition without preincubation. In agreement with our previous study, intoxication was prevented by addition of suitable concentrations of nicotinamide (6). The only other component that we have found to be essential, is the transferase II–containing AS65 fraction itself; preincubation of ribosomes with toxin had little or no effect on their subsequent activity. In-
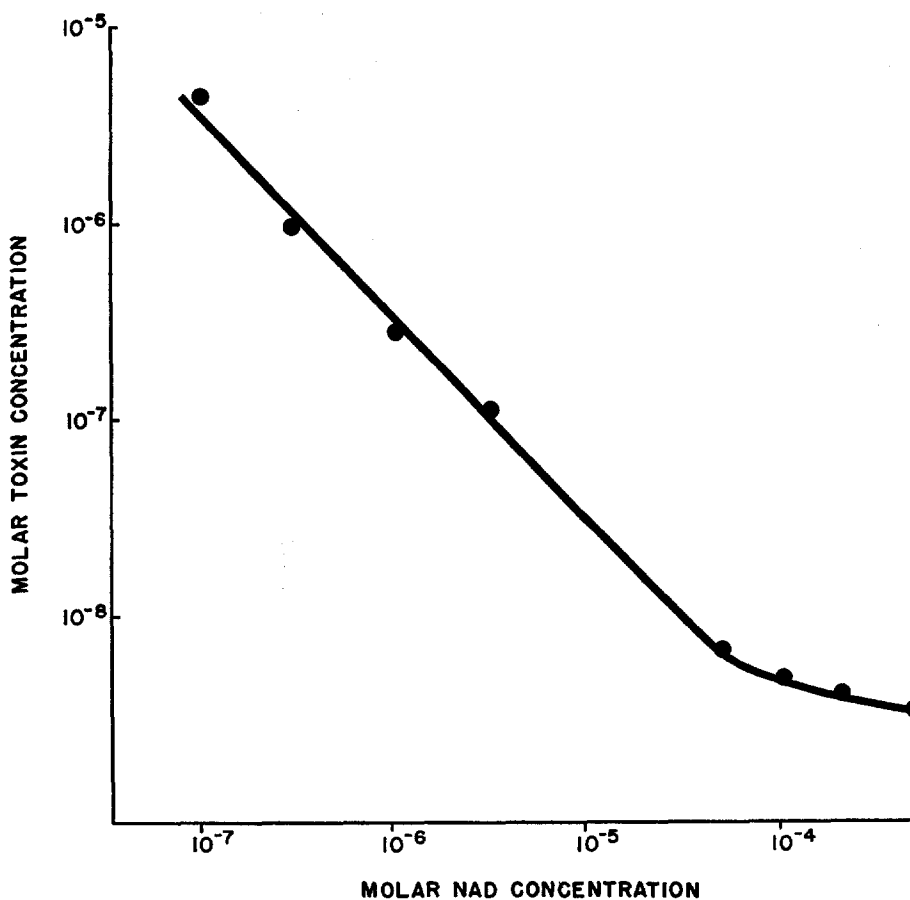
FIG. 4. Toxin and NAD concentrations required for 50% inhibition of aminoacyl transfer (Poly U, ^{14}C-Phe-tRNA). Each point on the graph was obtained by a separate set of measurements. For each set a number of 5 μl aliquots of reticulocyte AS65 fraction were incubated for 10 min at 37°C with a fixed amount of NAD and different amounts of toxin. The aminoacyl transfer activity remaining was then assayed in the usual manner. The results were plotted against toxin concentration and from this curve was read the amount of toxin necessary to reduce the number of counts incorporated by 50%.

deed inclusion of ribosomes actually reduces the extent of inactivation of the AS65 fraction as shown in Table I. The table shows that no other component of the aminoacyl transferase system has any noticeable effect on inactivation of transferase II by toxin.

We therefore conclude that inactivation of transferase II in vitro requires only toxin, NAD, and the specific transferase II–containing enzyme fraction.

It is not, as previously supposed, a rapidly attained equilibrium, but is a progressive reaction with a temperature coefficient characteristic of most enzyme reactions.

Quantitative Relationships.—It will be recalled that Goor et al. (6), assuming the formation of an inactive toxin-NAD-transferase II complex, derived a simple equation to describe the degree of inhibition of amino acid incorporation in cell-free systems at any given toxin and NAD concentration. The model
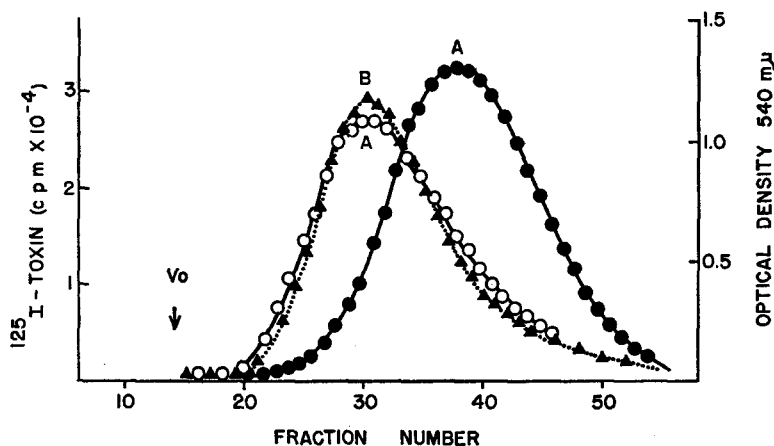

FIG. 5. Filtration of $^{125}I_2$-toxin through Sephadex G150 before and after incubation with NAD and reticulocyte AS65 fraction. A G150 column, 1.5 × 30 cm was equilibrated with 0.05 M tris at pH 7.5 containing 0.1 M KCl, 1 mM EDTA, 10^{-5} M dithiothreitol, and 10^{-5} M NAD. Curve *A*, 0.5 ml oxyhemoglobin (OD540 mμ = 12) incubated for 20 min at 37°C with 10^{-5} M NAD and 2 Lf $^{125}I_2$-toxin in standard assay buffer. —O——O— toxin (counts per min). —●——●— hemoglobin (OD540 mμ). Curve *B*, 0.5 ml reticulocyte AS65 fraction incubated for 20 min at 37°C with 10^{-5} M NAD and 2 Lf/ml $^{125}I_2$-toxin in same buffer. ...▲....▲. toxin (counts per min).

that they proposed assumed that inactivation of the enzyme was instantaneous and did not consider any possible effects due to natural decay of the highly unstable transferase II during the course of the assay. It was of interest to determine whether preincubation of toxin and NAD with enzyme fractions would influence the quantitative relationships previously reported, other than to increase the extent of inhibition, as would be expected from the results reported in the preceding section. As shown in Fig. 4, the inverse relationship between toxin and NAD described by Goor et al. (6) was fully confirmed and the product of NAD and toxin concentrations required for 50% reduction in aminoacyl transfer after 10 min preincubation at 37°C proved to be remark-

ably constant over a wide range of concentrations. Only at very low toxin concentrations does the curve deviate from linearity. The results differ in one respect from those reported previously; even with preincubation, the reticulocyte AS65 fraction appears to be less sensitive to toxin than fractions isolated from HeLa cells.

Failure to Demonstrate a Stable Toxin–NAD–transferase II Complex.— If toxin and transferase were to interact in the presence of NAD to form a stable complex as earlier postulated (6) we would expect such a complex to differ in both size and charge from either protein alone. However, our numerous attempts to demonstrate directly the existence of such a complex have all met with failure. The experiments shown in Figs. 5 and 6 are typical. Using $^{125}I_2$-toxin (6.6S dimeric form) the elution pattern of the iodine label from a Sephadex G150 column was almost superposable on that of the same amount of toxin which had been incubated with a highly active AS65 preparation from reticulocytes in the presence of 10^{-5} M NAD (Fig. 5). A polyacrylamide gel electrophoresis pattern of $^{125}I_2$-toxin (4.3S monomeric form) after incubation with NAD and the AS65 fraction is shown in Fig. 6. Here again the distribution of radioactivity was not appreciably different from that observed with labeled toxin alone. It is not known if the small secondary peaks observed in both gels represent an impurity in this particular preparation or a small amount of toxic dimer.

Attempts to demonstrate a stable protein complex containing NAD were also negative. In a typical experiment, 400 μg AS65 protein was incubated for 10 min at 37°C in 2 ml tris buffer containing 10^{-6} M, ^{14}C-labeled NAD, and 3×10^{-8} M toxin (76 Lf/ml) and then precipitated with an equivalent amount of antitoxin. The floccules were washed once with buffer and then suspended in buffer containing 0.01 M nicotinamide for 10 min at 37°C. The total radioactivity released by the nicotinamide was less than observed in control floccules of toxin-antitoxin prepared in the absence of AS65, and in any case, was equivalent on a molar basis to less than 0.05% of the toxin added.

It was concluded from the foregoing experiments that a stable NAD-containing toxin-transferase complex of the type previously postulated, probably does not exist. We therefore decided to investigate the possibility that toxin was bringing about a splitting of the NAD molecule in the presence of the aminoacyl transferase system.

Toxin-Stimulated Hydrolysis of NAD.—That toxin, concomitant with the inactivation of transferase II, does indeed stimulate the hydrolysis of NAD with release of free nicotinamide is shown by the experiments illustrated in Fig. 7. In these experiments, a high concentration of toxin was allowed to react with AS65 fraction from reticulocytes in the presence of NAD labeled in the carbonyl position with ^{14}C. After incubation at 37°C and pH 7.5 for
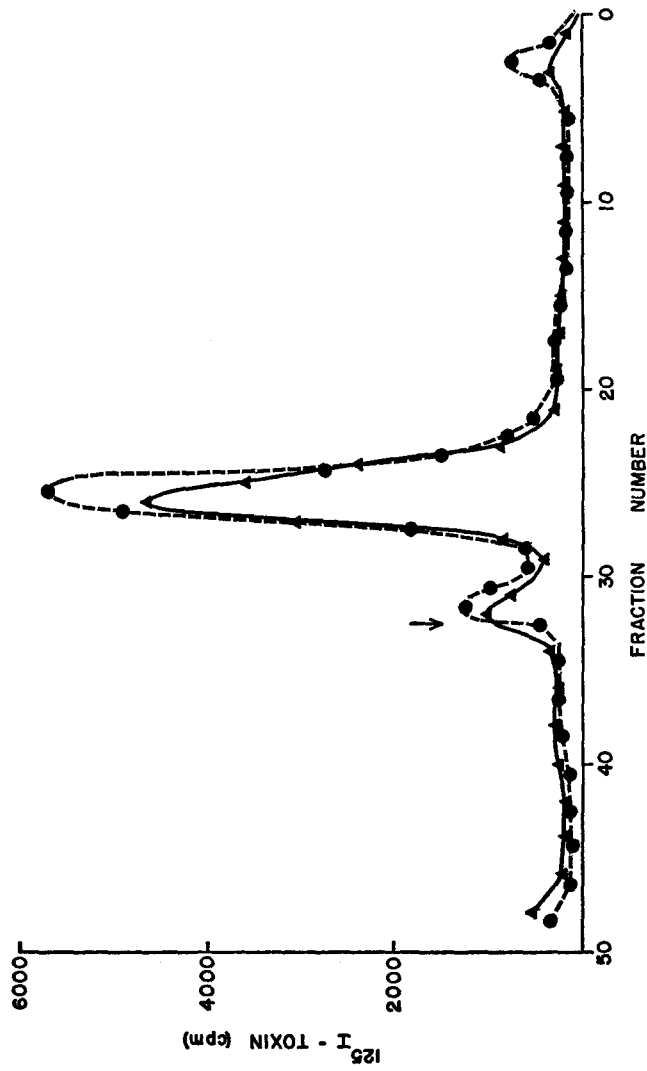
FIG. 6. Polyacrylamide gel electrophoresis of $^{125}I_2$-toxin before and after incubation with NAD and reticulocyte AS65 fraction. 0.1 Lf $^{125}I_2$-toxin incubated for 10 min with 3×10^{-5} M NAD in standard assay buffer. --●--●-- with 10 μl reticulocyte AS65 --▲--▲-- no. AS65. The bromthymol blue marker dye emerged with fraction 3. Position of hemoglobin band marked with arrow. The Tris-glycine buffer at pH 8.7 contained 3×10^{-5} M NAD and 10^{-4} M dithiothreitol.
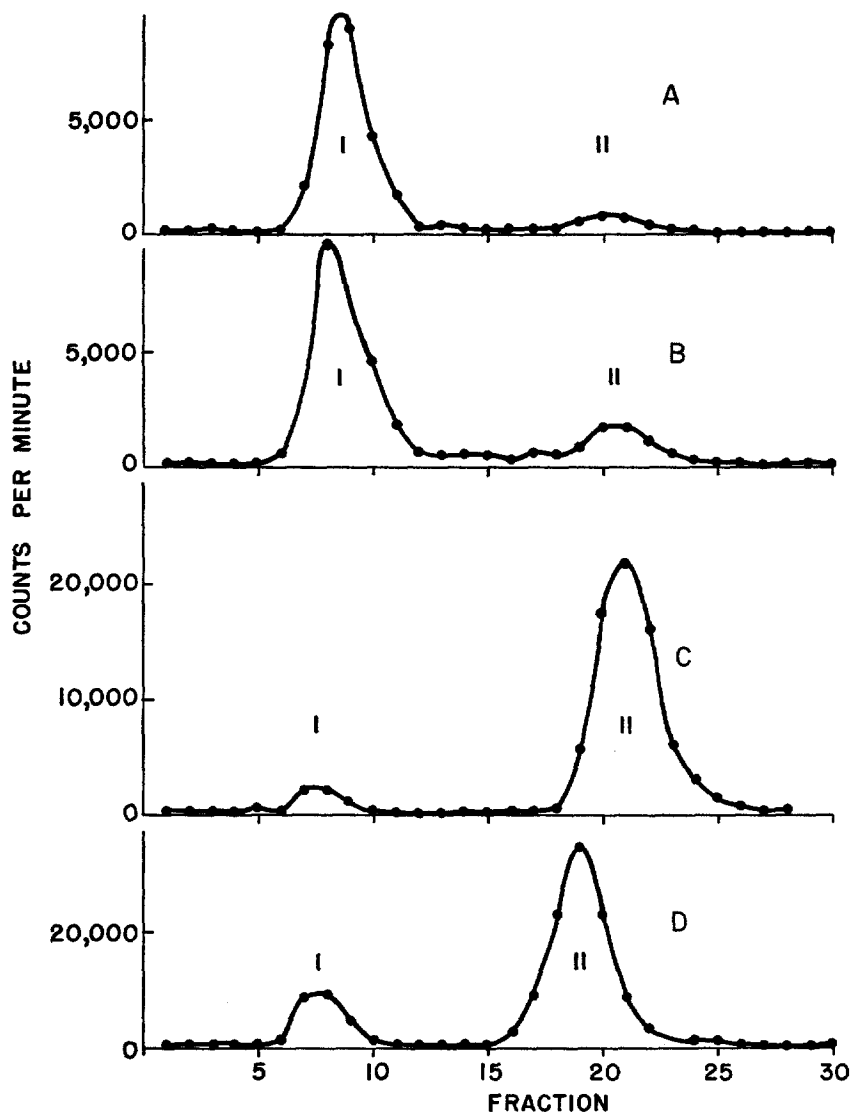
FIG. 7. Toxin-stimulated hydrolysis of NAD ^{14}C-(carbonyl) NAD. was incubated at 37°C for 10 min in 2 ml standard assay buffer, either (A) with 3 × 10^{-6} M diphtheria toxin alone or (B) with 2 mg/ml reticulocyte AS65 fraction alone or (C, D) with both together. An equal volume of 10% TCA containing 10^{-4} M NAD, and 10^{-4} M nicotinamide was added and the precipitate removed by centrifugation. The supernate was extracted three times with 8 ml changes of ether and the aqueous layers pervaporated in dialysis tubing to a volume of 0.5 ml. Each sample was then applied to a small Sephadex G10 column (V_0 = 3.5 ml). Elution of NAD (I) and nicotinamide (II) was monitored at 260 mμ and 0.5 ml fractions were collected on glass fiber pads, dried, and counted by liquid scintillation (20). The ^{14}C-NAD concentrations were 2 × 10^{-7} M in (A) and (B), 4 × 10^{-7} M in (C), and 8 × 10^{-4} M in (D).

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10 min, the mixture was deproteinized and the supernate filtered through Sephadex G10. Fig. 7a shows that when toxin and ^{14}C(carbonyl)-NAD were incubated together in the absence of AS65 fraction, only about 5% of the label eluted with the nicotinamide fraction[3]. That the AS65 fraction from rabbit reticulocytes apparently still contained a trace of NADase activity (Hofmann, [21]) is shown in Fig. 7b. When both toxin and aminoacyl transferase-containing AS65 fraction were present, however, (Figs. 7c and 7d) most of the NAD was hydrolyzed and 90–95% of the label appeared in the nicotinamide fraction. These results suggest that in the presence of transferase

TABLE II

Toxin Requirement for Reversal of In Vitro Intoxication

Reaction mixture	Aminoacyl transfer activity
	cpm
A. Normal AS65 control	562
B. Intoxicated AS65 supernate (toxin removed)	50
C. Intoxicated supernate + nicotinamide (5 mM)	130
D. Same as (C) + 50Lf/ml fresh toxin	539
E. Same as (C) + Tx-antiTx floccules	423
F. Same as (C) + fresh washed floccules	405

Except for the control (A) from which toxin was omitted, 50 μl reticulocyte AS65 fraction per ml of standard assay buffer containing 0.5 μM NAD and 100 Lf/ml diphtheria toxin were incubated at 37°C for 10 min. An equal volume of antitoxin (100 units/ml) was then added. After a further 20 min at 25°C, the specific floccules were removed by centrifugation. Assay of the supernates for aminoacyl transfer activity, using 50 μl aliquots, was carried out in the presence of 5 mM nicotinamide except in the case of (B). In (C) there were no further additions; in (D) free toxin was added; in (E) the toxin-antitoxin floccules previously removed were added back and in (F) freshly prepared washed toxin-antitoxin floccules were added in the same concentration as free toxin.

II, diphtheria toxin stimulates the hydrolysis of NAD with release of free nicotinamide under the identical conditions that result in the inactivation of transferase II.

Reversibility of the Inactivation of Transferase II by Toxin.—In the experiments to be described below, it will be shown that diphtheria toxin can not only stimulate the hydrolysis of NAD with release of nicotinamide and concomitant inactivation of transferase II, but also, in the presence of excess nicotinamide will catalyze the reverse reaction in which transferase II inhibition is relieved. Reactivation of inactive transferase II by nicotinamide and toxin has been demonstrated in preparations from normal HeLa cells, in AS65 fractions from

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[3] The labeled NAD alone showed about 5% of an impurity with the same V_e on G10 and the same R_f on paper chromatography as nicotinamide.

reticulocytes intoxicated in vitro and in AS70 fractions prepared from cells grown for several hours in the presence of excess toxin.

In the experiments of Goor et al. (6), who first showed reversal of intoxication in vitro by addition of a relatively high nicotinamide concentration, the inhibitory toxin was still present when the nicotinamide was added so that its importance for reversal was not recognized. Table II shows that nicotin-

TABLE III

Inhibition of Aminoacyl Transfer and Its Release by Free and Antitoxin-Bound Diphtheria Toxin

Experiment	[NAD]	Inhibition Reaction		Reversal Reaction	
		Toxin	Aminoacyl Tfase activity	Toxin	Aminoacyl Tfase activity after addition of nicotinamide
	μM		*cpm*		*cpm*
A	2.5	None	1225	None	1058
B	0.5	Free	99	Free	1143
	2.5	Free	82	Free	1097
C	0.5	Free	62	Floccules	870
	2.5	Free	53	Floccules	781
D	0.5	Floccules	403	Floccules	801
	2.5	Floccules	56	Floccules	677

For inhibition, 5 μl aliquots of reticulocyte AS65 fraction were incubated with 10 Lf free toxin and NAD at the concentration indicated in standard assay buffer for 10 min at 37°C. The total volume was 0.1 ml in each case. In (D) 10 Lf toxin-antitoxin floccules were added instead of free toxin. In (C), 10 units of antitoxin were added and the incubation was continued a further 2 min. For reversal, duplicate mixtures were incubated an additional 5 min at 37°C with 2 μ moles nicotinamide. Aminoacyl transfer activity was then assayed as described under Materials and Methods.

amide is far less effective in reactivating the aminoacyl transferase activity in an intoxicated reticulocyte AS65 preparation when the toxin is first removed by specific precipitation with diphtheria antitoxin. Appreciable transferase activity is only restored to the system by readdition of free toxin or the toxin-antitoxin floccules themselves. The small stimulation of incorporation with nicotinamide alone is doubtless due to catalysis by a trace of soluble toxin-antitoxin complex that failed to precipitate within 20 min at 25°C.

In Table III, the relative efficiency of free toxin at two different NAD concentrations is compared with that of toxin-antitoxin floccules both in bringing about the inhibition of transferase II and in reactivating the inhibited

enzyme in the presence of nicotinamide. It is evident that antitoxin-bound toxin is somewhat less effective than free toxin in catalyzing both the inhibition reaction and its reversal.

Our finding that the toxin-stimulated release of nicotinamide from NAD is closely linked to inhibition of transferase II and that the inactivated enzyme can be reactivated again, provided *both* toxin and nicotinamide are present, can best be explained by assuming that toxin catalyzes the reversible

TABLE IV

Reactivation of Transferase II in AS70 Fraction Prepared from Intoxicated HeLa Cells

Additions	Aminoacyl transfer activity in AS70 preparation	
	From intoxicated cells	From normal cells
	cpm	*cpm*
None	103	568
Toxin, 5 Lf/ml	88	536
Nicotinamide (5mM)	106	565
Toxin (5Lf/ml) + Nicotinamide (5 mM)	612	577
Toxin (5Lf/ml) + NAD (10 μM)	78	142
Streptococcal NADase (20 unit/ml) + nicotinamide	98	—
Neurospora NADase (10 unit/ml) + nicotinamide	78	—
Pig brain NADase (10 unit/ml) + nicotinamide	79	—
Horse brain NADase (10 unit/ml) + nicotinamide (5 mM)	30	—
No. AS70 fraction	15	—

AS70 fractions were prepared from normal HeLa cells and from cells harvested from a parallel culture grown for 4 hr in the presence of 1 Lf/ml toxin. Amino acid incorporation stopped within 2 hr in the intoxicated culture. Aminoacyl transfer activity was assayed without preincubation.

transfer of ADP-ribose from NAD to transferase II. The reaction probably involves the formation of an unstable toxin-NAD-enzyme intermediate according to the following scheme:

1. Tx + NAD ⇌ Tx-NAD

2. Tx-NAD + Tfase II ⇌ [NAD-Tfase-Tx]

3. [NAD-Tfase-Tx] ⇌ ADPR-Tfase II + Tx + nicotinamide

Proof that the ADP-ribose moiety is firmly linked to the transferase has been provided by Honjo et al. (8) using NAD labeled in the adenine ring and in the phosphate groups.

It is noteworthy that ADP-ribosyl-transferase II is formed and accumulates

in living cultures of HeLa cells exposed to diphtheria toxin. In the experiments summarized in Table IV, a culture of HeLa cells was treated with several saturating doses of toxin for 4 hr. ^{14}C-leucine incorporation into cell protein ceased altogether within 2 hr. The AS70 fraction prepared from the thoroughly washed intoxicated cells contained no detectable toxin and showed very low aminoacyl transfer activity. The table shows that although either toxin or nicotinamide alone was without effect, a mixture of the two restored transferase activity to a level that was even higher than found in the control preparation from normal cells. Table IV shows also that NADases from other sources such as *Streptococcus*, *Neurospora*, and animal brain, fail to cause inhibition of aminoacyl transfer activity in the presence of NAD.

Even when stabilizing agents, such as sucrose, glycerol, and 2-mercaptoethanol are present, free transferase II rapidly loses activity at moderate temperatures. As previously noted, the toxin-inactivated enzyme appears to be considerably more stable (6). The relative stability of ADP-ribosyl transferase II, probably explains the fact that AS70 fractions prepared from intoxicated HeLa cells usually show somewhat higher aminoacyl transfer activity after reactivation with toxin and nicotinamide, than do similar fractions prepared from normal cells.

DISCUSSION

When soluble fractions containing the aminoacyl transferring enzymes from rabbit reticulocytes or from HeLa cells are incubated with diphtheria toxin and NAD, the dinucleotide is rapidly hydrolyzed to yield free nicotinamide and simultaneously the aminoacyl transferase activity is lost. As previously shown (2, 5) this loss in activity is due to the specific inactivation of transferase II. The reaction is reversible and active transferase II may be regenerated in the presence of toxin by addition of nicotinamide to the system. We therefore conclude that inhibition of the amino acid transfer by toxin is due to the formation of an inactive adenine diphosphate ribose (ADPR) derivative of transferase II and that diphtheria toxin catalyzes the reversible reaction:

$$\text{NAD} + \text{Tfase II} \rightleftharpoons \text{ADPR-Tfase II} + \text{nicotinamide}$$

This conclusion is in complete accord with the recent experiments of Honjo et al. (8). By using NAD isotopically-labeled in various positions, they have clearly shown that the entire ADPR moiety of NAD becomes firmly bound to transferase II, purified from rat liver, following incubation with toxin. They showed that ADPR was linked covalently to transferase II, presumably at or near the active site, through the ribose-5'-phosphate end.[4] The Japanese work-

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[4] The binding of NAD by diphtheria toxin has been demonstrated by Montanaro and Sperti (22) and by ourselves (23). We have recently shown in this laboratory, both by equilibrium dialysis and by binding to toxin-antitoxin floccules, that nicotinamide is almost as good a ligand as is NAD itself.[5] Sperti and Montanaro (24)

ers found that during a 40 min incubation at 37°C, each toxin molecule could catalyze the transfer of up to 50 ADPR equivalents from NAD to transferase II.

In the present studies, we have demonstrated that the action of diphtheria toxin on living cells likewise results in the intracellular formation and accumulation of the inactive ADPR derivative of transferase II. Extracts made from intoxicated cells freed from membranes and ribosomes were found to have greatly reduced aminoacyl transferase activity when prepared from cells exposed for only a few minutes to a saturating dose of the toxin. Similar extracts, even when made from cells exposed for several hours to a saturating dose, could be restored to near normal activity by addition of nicotinamide together with toxin—neither agent alone being sufficient.

In a recent paper, Moehring and Moehring (25) report the partial restoration of aminoacyl transfer activity by addition of nicotinamide *alone* to crude extracts from washed intoxicated KB cells. It would appear that in their experiments the reactivation of the incorporating system was a slow continuing process that required at least 90 min at 37°C. It seems likely that their extracts still contained traces of membrane-bound toxin sufficient to catalyze the reversal in the presence of a high nicotinamide concentration.

Let us now consider what may take place in the living HeLa cell exposed to a saturating dose of diphtheria toxin. We know that, at most, only 25–50 molecules can become fixed in the cell membrane where they can no longer interact with agents added from without, such as specific antitoxin or trypsin (7). Nevertheless, membrane-bound toxin must be in contact with cytoplasmic components, since even this small number of toxin molecules suffices to bring about the reduction of the intracellular transferase II to a very low level within a few minutes. Since we suspect that there must be at least as many transferase II molecules in each HeLa cell as there are ribosomes, the number of which has been estimated to be about 10^7, each toxin molecule must cause inactivation of more than 10^5 molecules of transferase II per minute in the living cell. Can we account for such a high turnover number in vivo in terms of the observations on NADase activity of toxin in cell extracts?

Since the volume of 2×10^8 packed HeLa cells is about one ml, it follows that the maximum cell-bound concentration of toxin will be $1–2 \times 10^{-11}$ M. Taking the intracellular concentration of NAD to be 5×10^{-4} M (6), then the product, [NAD] $\times$ [toxin], for intoxicated cells must lie between 0.5 and 1 $\times$

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have found that adenine also binds to toxin. Presumably, when the toxin-NAD complex interacts with transferase II and the nicotinamide-ribose linkage is split, the nicotinamide moiety remains bound to toxin from which it then dissociates and the ADP ribose becomes covalently linked to the transferase through its ribose 5′-phosphate end (8).

10^{-14} M^2. From our previously published data, we know that the transferase II activity of HeLa cell extracts is 50% inhibited when the product, [NAD] $\times$ [toxin], is about 3 $\times$ 10^{-14} M^2, irrespective of the precise toxin concentration. We feel that the agreement between these two figures is quite remarkable, particularly in view of the several assumptions that have been made. Thus, we do not know what fraction of the intracellular NAD is available for interaction with the toxin; nor do we know how best to compare reaction rates in vitro with those observed in vivo. Nevertheless, on the basis of the above calculations, it does not appear necessary to postulate the enhancement of toxin activity in vivo by some unknown membrane component although, of course, such a possibility cannot be rigorously excluded at this time.

The importance of the inverse relationship between toxin and NAD concentrations required for a given degree of inhibition in vitro, first stressed by Goor et al. (6), has been confirmed and has now been shown to extend over a 1000-fold range of concentrations, even when toxin and NAD are preincubated with the soluble enzyme system. The extent of transferase II inactivation thus depends on the concentration of toxin-NAD complex present. The interpretation of these relationships, however, is not as simple as originally supposed (6). Thus it is not immediately clear why the concentration of toxin-NAD complex should so predictably govern the overall *extent*, as well as the *initial rate* of aminoacyl transfer. Transferase II is a highly unstable enzyme and quickly decays while incorporation of amino acids is being followed in extracts from normal cells. We would expect, therefore, from our preincubation experiments, that the loss of transferase II activity in extracts to which toxin has been added would be determined by the sum of the natural decay, on the one hand, and formation of inactive ADPR derivative by continued action of the toxin, on the other. Yet we have confirmed many times, and earlier studies have shown (1, 6), that in the presence of toxin and NAD, amino acid incorporation in vitro appears to start at a slower rate than in control systems, and thereafter intoxicated and control systems proceed in parallel until incorporation stops. In other words, toxin-stimulated inactivation of transferase II seems to be complete shortly after the start of the incubation and the remaining enzyme then decays at its usual rate. This explains the proportionality between the initial rate and over-all extent of inhibition, but itself requires explanation.

It was concluded in our previous study (5, see also reference 25) that ribosomally bound transferase II is not inactivated by toxin in vitro and as we have seen from Table I, preincubation of toxin and NAD with AS65 fractions leads to less inactivation of their transferase in the presence of washed ribosomes than in their absence. We suggest therefore, that when ribosomes are present they may compete with the toxin-NAD complex for transferase II. Either free transferase II quickly becomes converted by toxin to its inactive ADPR derivative or it becomes bound to ribosomes, active in peptide bond forma-

tion but no longer susceptible to rapid inactivation by toxin. The amount of inactivation during the initial short period will thus depend entirely on how much toxin-NAD complex is present.

A similar explanation may account for the curious finding that most of the transferase II becomes inactive in intoxicated HeLa cells well before any appreciable decrease can be detected in the over-all rate of protein synthesis by the cells. Perhaps this may be explained if we assume that transferase II can only influence the rate of protein synthesis in living cells when it is bound to ribosomes. If release of the enzyme from the ribosomes is a relatively slow process, then even after nearly all the *free* transferase II has been converted to its inactive ADP riboside, the level of *ribosome-bound* transferase will remain substantially unchanged and therefore, incorporation of amino acids by the whole cell will, at first, be relatively uneffected. The enzyme which is then released from the ribosomes will be inactivated at an increasingly slower rate, since it is more likely to encounter another ribosome than one of the few toxin molecules in the cell membrane.

Some mention should be made regarding NADases produced by other pathogenic microorganisms. Bernheimer et al. (13) have demonstrated a striking correlation between the capacity of certaio strains of hemolytic streptococci to form NADase and their leukotoxicity. In this case, toxic activity is probably due directly to destruction of NAD by NADase released within the cell by phagocyted streptococci. Purified plague toxin from *Pasteurella pestis* has also been shown to possess associated NADase activity and to inhibit NAD-linked mitochondrial respiration in tissue homogenates derived from susceptible species (Ajl and Rust, 26). Neither in the case of the streptococcus nor of the plague bacillus, however, can the action of the toxin in vivo be related with certainty to its NADase activity nor has a substrate other than the nucleotide itself been identified. In the case of diphtheria toxin, nonbacterial transferase II appears to be a specific acceptor for the ADPR-moiety of NAD. At any rate, there is no evidence that toxin catalyzes the transfer of ADPR to any other protein.

SUMMARY

When diphtheria toxin and NAD are added to soluble fractions containing aminoacyl transfer enzymes isolated from rabbit reticulocytes or from HeLa cells, free nicotinamide is released and, simultaneously, an inactive ADP ribose derivative of transferase II is formed. The reaction is reversible, and in the presence of excess nicotinamide, toxin catalyzes the restoration of aminoacyl transfer activity in intoxicated preparations. In living cultures of HeLa cells, the internal NAD concentration is sufficiently high to account for the rapid conversion, catalyzed by a few toxin molecules located in the cell membrane, of the entire cell content of free transferase II to its inactive ADP

ribose derivative. Completely inactive ammonium sulfate fractions containing soluble proteins isolated from cells that have been exposed for several hours to excess toxin, can be reactivated to full aminoacyl transfer activity by addition of nicotinamide together with diphtheria toxin. Transferase II appears to be a highly specific substrate for the toxin-stimulated splitting of NAD and thus far no other protein acceptor for the ADP ribose moiety has been found.

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