STUDIES ON THE MODE OF ACTION OF DIPHTHERIA TOXIN*

IV. Specificity of the Cofactor (NAD) Requirement for Toxin Action in Cell-Free Systems

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It was shown by Collier and Pappenheimer (1) that inhibition, by diphtheria toxin, of amino acid incorporation into polypeptides in cell-free systems isolated from mammalian cells requires the presence of nicotinamide adenine dinucleotide (NAD) as an essential cofactor. In the absence of NAD, there is no inhibition of amino acid incorporation, even in the presence of high toxin concentrations. More recently, Collier (2) and ourselves (3) have demonstrated that toxin causes inhibition of peptide bond formation (in vitro) by inactivation of a soluble enzyme, transferase II. However, the mechanism by which NAD helps to bring about this inactivation has not been elucidated. The early studies (1) had already suggested that the NAD requirement is highly specific since NAD cannot be replaced by nicotinamide adenine dinucleotide phosphate (NADP).

In the present paper, we are reporting further experiments on the specificity of the NAD requirement for inhibition of amino acid incorporation by diphtheria toxin in cell-free extracts. A series of NAD analogues have been tested for their ability to replace NAD as a cofactor for inactivation of amino acid transfer by the toxin. The results have been interpreted as indicating that interaction takes place between toxin and certain of the analogues. In preliminary experiments, such interaction has been demonstrated directly, using the method of equilibrium dialysis.

Materials and Methods

Diphtheria Toxin.—The purified toxin used contained 2.6 μg protein per Lf unit and 60–70 guinea pig MLD/Lf. The method of preparation was similar to that already described (3).

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Toxin used for equilibrium dialysis contained 11,000 Lf/ml. Its OD at 276 mμ was 26.6.

Diphtheria Toxoid.—Purified toxoid NZ157P containing 1600 Lf/ml and 1.51 mg N/ml was kindly supplied by Mr. Leo Levine of the Massachusetts Department of Health. It was further fractionated with amnonium sulfate and the fraction precipitating between 0.35 and 0.6 saturation was collected and dissolved in a small volume of distilled water. It was then filtered through Sephadex G50. The toxoid used for equilibrium dialysis contained 8500 Lf/ml. Its OD at 278 mμ was 34.7.

Cell-Free Extracts.—These were prepared from HeLa cells and rabbit reticulocytes. Incorporation of phenylalanine-^{14}C using the synthetic poly U messenger was followed in the cell-free systems as described in detail in the preceding paper (3).

NAD and Its Analogues.—Nicotinamide was purchased from Nutritional Biochemicals, Cleveland, Ohio. Cytochrome *c* (horse heart, type II), crystalline yeast alcohol dehydrogenase (ADH), reduced glutathione (GSH), NAD, reduced nicotinamide adenine dinucleotide (NADH), nicotinamide mononucleotide (NMN), deamino-NAD, thionicotinamide-AD, 3-acetylpyridine-AD, 3-acetylpyridine–deamino-AD, 3-carbonylpyridine-AD and 3-carbonylpyridine–deamino-AD were all obtained from Sigma Chemical Co., St. Louis, Mo. We are indebted to Dr. Norbert Swislocki, of Brandeis University, who kindly supplied generous samples of α-NAD, ethylnicotinate-AD, 3-nicotinylhydroxamic acid-AD, 4-methylpyridine-AD, nicotinamide(1, 2-hydroxyethyl)–AD, and 4-amino-5-imidazolecarboxamide-AD. We also wish to thank Dr. A. W. Bernheimer, of New York University, who generously supplied the NADase used in this work.

Determination of NAD and NADH.—The method of extraction and assay was similar to that described by Glock and McLean (4) except that the cytochrome b_5–mediated NADH–cytochrome *c* reductase system used was prepared from guinea pig liver microsomes according to Chance and Williams (5). NAD was extracted by treating 0.5 ml HeLa extract in 0.1 N HCl for 30 sec at 100°C followed by homogenizing for 1.4 min in a Potter-Elvejhem type homogenizer. The extract was then quickly chilled and neutralized. NADH was extracted with 0.1 N NaOH for 30 sec at 100°C, homogenized, chilled, and neutralized as above. The neutralized extracts were centrifuged for 30 min at 20,000 *g* and the supernate used immediately for assay of NAD or NADH.

The assay system contained the following components in a total volume of 2.5 ml: glycylglycine buffer, pH 8.7, 0.05 M; ethanol, 0.04 M; antimycin, 1 μg/ml; nicotinamide, 0.03 mM; yeast alcohol dehydrogenase, 40 mg/ml; cytochrome *c* reductase preparation, 0.05 ml; and 1.5 ml containing the sample to be analyzed. The reaction was initiated by addition of 0.1 ml of 1 mM cytochrome *c*. Its rate of reduction was measured at 550 mμ and was proportional to the NAD or NADH concentration.

Assay of NADase Activity.—The effect of streptococcal NADase on NAD and its analogues was examined by the cyanide method of Kaplan (6). NAD or one of its analogues was incubated at 37°C and pH 7.5 with 2100 units NADase for 8 min in a volume of 0.6 ml. A control was incubated in the absence of NADase. 3 ml 1 M KCN were then added to stop the reaction, and the mixutres were allowed to stand at room temperature for 1 hr before reading the absorbance in the region 325–340 mμ. The exact wavelength used depended on the particular NAD analogue. Only those analogues that are capable of being reduced with dithionite form cyanide complexes (7).

Equilibrium Dialysis—Binding of NAD by purified toxin and toxoid was measured by equilibrium dialysis using leucite microchambers made by Future Plastics, Inc., Cambridge, Mass., according to a design suggested by Professor H. N. Eisen, Washington University, St. Louis, Mo. Either 60 or 80 μl purified toxin or toxoid ($\approx 3 \times 10^{-4}$ M) was placed in one chamber, and the same volume of NAD added to the other chamber which was separated from the first by a small sheet of cellophane. At each equilibrium concentration of NAD

tested, ranging from 1.25×10^{-4} M to 2×10^{-8} M, a control was run with buffer (20 mM Tris, 60 mM KCl, 5mM $MgCl_2 \cdot 6\ H_2O$, pH 7.5) instead of toxin. After 16–18 hr equilibration, optical densities were measured at 260 mμ and 276 mμ on samples from each compartment. Calculations were made assuming molar extinctions of 1.6×10^4 for NAD at 260 mμ and 6.4×10^5 for toxin at 276 mμ.

RESULTS

Failure of NADH to Replace NAD.—In the experiment summarized in Table I, a crude extract from HeLa cells was freed from NAD by passage through Sephadex G25. Then NADH was added to a concentration of 0.3 mM and the extract was treated for 1 hr at 0°C with 17,000 units of streptococcal NADase. The mixture was added to the otherwise complete phenylalanine-^{14}C–incorporating system containing 6 μg/ml diphtheria toxin. Table I shows that phenylalanine-^{14}C incorporation, in the control that contained 0.03 mM NAD and had *not* been pretreated with NADase, was inhibited 80% by toxin. Within the limits of experimental error, the same amount of toxin caused no inhibition

TABLE I

*Failure of NADH to Replace NAD as Cofactor for Toxin Action**

Additions before pretreatment with NADase	Additions after NADase	Phenylalanine-^{14}C-incorporated	Inhibition
		cpm	%
No NADase	Crude HeLa extract‡	3680	0
No NADase	Crude HeLa extract + toxin (6 μg/ml) + NAD (3×10^{-5} M)‡	731	80
Crude HeLa extract§ + NADH (3×10^{-4} M)§	Toxin (6 μg/ml)	3867	0
	Toxin (6 μg/ml)	3409	7

* The incorporating system was the same as described previously (3). Each tube contained crude extract from normal HeLa cells, 72 μg poly U and phenylalanine-^{14}C, 2 μc/ml (125 μc/μmole) in a final volume of 0.25 ml. After 40 min at 37°C, the reaction was stopped with TCA; the precipitate was collected and assayed in the usual manner.

‡ No NADase added to these tubes.

§ Pretreated for 1 hr at 0°C with 17,000 units streptococcal NADase before addition of toxin and phenylalanine. After 40 min at 37°C, NADH concentration (by analysis) had fallen to 5×10^{-5} M.

of amino acid uptake in the presence of 10 times as much NADH. Since streptococcal NADase does not split NADH (8), its concentration at the end of the experiment could be determined. There was some disappearance of NADH, doubtless because of its oxidation by small amounts of microsomal cytochrome b_5 that were present in the cell extract. Nevertheless, the final NADH concentration was still nearly 0.05 mM, which is well above the concentration of NAD needed for maximal inhibition.

Analogues of NAD as Cofactors for Activation of Diphtheria Toxin.—The analogues that have been tested for their ability to replace NAD in supporting the inhibition of protein synthesis by toxin were all compounds that have been examined previously for their ability to replace NAD as coenzymes for a variety of dehydrogenases (for review, see reference 9). They included analogues of the following types: (*a*) with substituents replacing the 3-carboxamide group in the pyridine ring; (*b*) replacement of the pyridine ring; (*c*) alterations in the purine ring, and (*d*) simultaneous alterations in both rings. In order to remove traces of NAD that might have remained in the HeLa extracts, even after filtration through Sephadex G25, and in order to prevent accumulation of any

TABLE II

*Inhibition of Phenylalanine Incorporation by Diphtheria Toxin in the Presence of NAD and Related Nucleotides**

Additions to crude HeLa extract	Phenylalanine-^{14}C-incorporated	Inhibition	Coenzyme activity for dehydrogenases
1	2	3	4
	cpm	%	
None‡	4202	—	—
Toxin‡	3910	7	—
Toxin + NAD‡	1175	72	—
Pretreated NADase + toxin + NAD	4009	5 (72)§	Yes
Nicotinamide	3804	10	No
NMN	4016	5 (36)	Yes
3-Thiocarboxamide pyridine-AD	1099	74 (70)	Yes
3-Acetylpyridine-AD	2046	51 (50)	Yes
Deamino-NAD	4116	2 (29)	Yes
3-Acetylpyridine deamino-AD	4028	5 (6)	Yes
3-Carbonylpyridine-AD	3904	7 (32)	Yes
3-Carbonylpyridine deamino-AD	3737	11 (4)	Yes
3-Nicotinylhydroxamic acid-AD	3986	5	Yes
Ethylnicotinate-AD	3916	7	No
α-NAD	4093	2	No
Nicotinamide(1,2-hydroxyethyl)-AD	3998	5	No
4-Amino-5-imidazolecarboxamide-AD	3424	19	No
4-Methylpyridine-AD	3913	7	No

* The phenylalanine-^{14}C incorporating system was as described in Table I. All tubes except the control (line 1) contained 3 μg/ml diphtheria toxin. Unless otherwise indicated (lines 1–3), each nucleotide was pretreated in the HeLa extract with 1300 units/ml streptococcal NADase for 1 hr at 0°C before adding toxin and phenylalanine-^{14}C and bringing to 37°C. All nucleotides were added so as to give a final concentration of 6×10^{-5} M. The final concentration of nicotinamide was 8.35×10^{-3} M.

‡ No pretreatment with NADase.

§ Numbers in parentheses give per cent inhibition *without* pretreatment with NADase.

NAD that might have been resynthesized from an analogue due to enzymes present in the crude cell extracts, an excess of streptococcal NADase was added to the system before adding toxin and phenylalanine-^{14}C.

Table II summarizes the ability of a number of compounds related to NAD to support inhibition by toxin of phenylalanine incorporation into trichloroacetic acid (TCA)–precipitable peptides. Table III summarizes the sensitivity of the same analogues to attack by streptococcal NADase. Column 3 of Table II

TABLE III

Cleavage of Nucleotides Related to NAD by Streptococcal NADase

Analogue	Concentration $\times 10^4$	Absorption maximum	Cleavage
		mμ	%
NAD	8.34	325	74.0
NMN	4.25	325	9.5
Deamino-NAD	8.34	325	68.0
3-Thiocarboxamide pyridine–AD	8.34	335	3.4
3-Acetylpyridine-AD	8.34	340	5.8
3-Acetylpyridine-AD	4.17	340	2.0
3-Carbonylpyridine-AD	4.17	330	5.9
3-Carbonylpyridine deamino-AD	4.17	330	1.0
α-NAD	8.34	333	0.5
Nicotinamide(1,2-hydroxyethyl)–AD	8.34	325	15.4
Nicotinylhydroxamic acid–AD	8.34	325	11.4
Ethylnicotinate-AD	8.34	325	0

Conditions: Each analogue at the concentration indicated was incubated in the absence or presence of 2130 units of streptococcal NADase. Total volume of the incubation mixture was 0.6 ml and the pH was 7.5. The reaction was allowed to proceed at 37°C for 8 min and then stopped by adding 3 ml of 1 M KCN. The mixtures stood at room temperature for 1 hr before reading the absorbance at the indicated wavelength.

gives the per cent inhibition for each analogue in extracts that had been pretreated with excess NADase for 1 hr at 0°C before addition of toxin and initiation of peptide bond formation by adding phenylalanine-^{14}C. The numbers shown in parenthesis give the per cent inhibition observed when the system was *not* pretreated with NADase, although in each case free NAD had been removed by gel filtration. Finally, column 4 indicates whether or not the analogue has been found capable of replacing NAD in dehydrogenase reactions at least to some extent. Many of the analogues have been shown to possess variable activity as coenzymes, depending on the particular dehydrogenase system in question (Colowick et al., reference 9).

Inspection of Tables II and III shows that among those NAD analogues that cannot serve as substrates for streptococcal NADase, only 3-thiocarboxamide pyridine–AD is fully active in replacing NAD as a cofactor for diph-

theria toxin. 3-acetylpyridine-AD appears to be about 70% as active. Three other nucleotides related to NAD supported the toxic inhibition of phenylalanine incorporation in the absence of NADase but not in its presence. These included nicotinamide mononucleotide (NMN), 3-carbonylpyridine-AD, and deamino-NAD. Deamino-NAD, which appears to be about 40% as effective

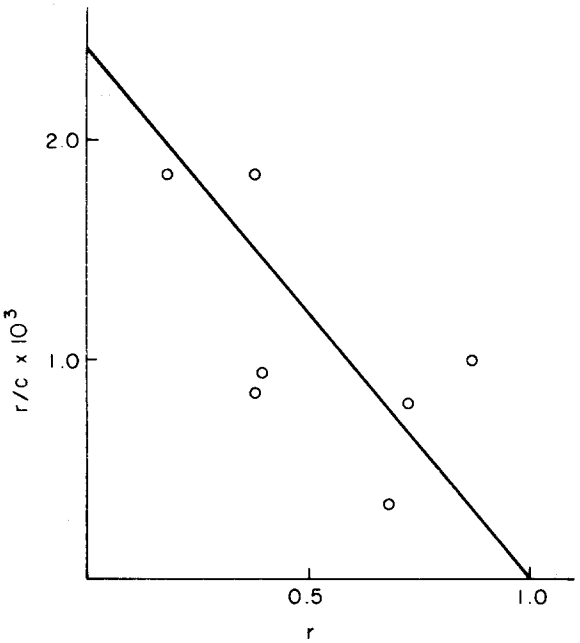

FIG. 1. Equilibrium dialysis of diphtheria toxin (3×10^{-4} M) against increasing concentrations of NAD in 0.02 M Tris, pH 7.5, containing 0.06 M KCl and 0.005 M $MgCl_2$. The equation for the straight line is $r/c = (1 - r)K$ where r = bound NAD, c = free NAD in moles per liter, and $K = 2.4 \times 10^3$ liters/mole. The molecular weight of toxin is taken at 65,000 (Raynaud et al., reference 11).

as NAD in the absence of NADase, is shown in Table III to be an excellent substrate for the streptococcal enzyme. It would thus appear that its failure to activate toxin in the presence of NADase was due to its cleavage by this enzyme. On the other hand, both NMN and 3-carbonylpyridine-AD, which appeared to activate toxin in the absence of NADase, showed no activity in its presence despite the fact that neither analogue can serve as a substrate for the enzyme. It seems probable, therefore, that these last two nucleotides were converted to NAD by enzymes present in the crude HeLa extract. In the presence of NADase, any resynthesized NAD would, of course, be destroyed as fast as it was formed.

Interaction between Toxin and NAD.—The fact that the only analogues found capable of replacing NAD as cofactors for toxin were nucleotides already known to possess coenzyme activity for NAD-linked dehydrogenases suggested a possible interaction between toxin and NAD. Preliminary experiments using a technic similar to that described by Hummel and Dreyer (10), in which toxin was filtered through Sephadex G25 equilibrated with different concentrations of NAD, provided direct evidence for such interaction. Reversible binding of 1 mole of NAD per mole of toxin with an association constant of approximately 2.4×10^3 liters/mole, was also demonstrated by equilibrium dialysis. In Fig. 1, we have plotted the binding of NAD by 3×10^{-4} M toxin over a range of total NAD concentrations varying between 1.25×10^{-4} M and 2×10^{-3} M. Although the actual K value calculated must still be regarded as tentative, since the points show considerable scatter, it is significant that, over the same range of NAD concentrations, purified diphtheria toxoid showed no detectable binding whatsoever.

DISCUSSION

The studies of Collier (2) and of ourselves (3) have shown that diphtheria toxin in the presence of NAD inhibits protein synthesis in cell-free extracts by inactivation of transferase II, one of the soluble enzymes required for peptide bond formation from aminoacyl-sRNA. The experiments of Sutter and Moldave (12) suggest that this enzyme contains labile —SH groups and is exceedingly sensitive to inactivation by oxidation. The possibilities that first present themselves are, therefore, (*a*) that toxin may act directly as a NAD-linked dehydrogenase to inactivate transferase II or (*b*) that toxin may activate a "latent" dehydrogenase present in inactive form in cell extracts that in turn oxidizes transferase II. The latter possibility seems unlikely because of experiments, reported in the preceding paper (3), in which it was shown that, when extracts from normal and intoxicated cells are mixed together, the incorporation is additive. In either case, inactivation of transferase II should result in reduction of NAD and increased absorption at 340 mμ. We have been unable to detect any change in absorption resulting from the action of toxin, even in cell free systems reconstituted from purified components. If NAD is indeed reduced as a result of toxin inactivation of transferase II, then either the yield of NADH is too small to be detected by the means available to us, or it is reoxidized as rapidly as it is formed. The latter possibility seems improbable, since the better preparations were free from mitochondria and therefore contained little or no cytochrome oxidase.

The chemical and biological properties of a large series of nucleotides related to NAD have been extensively studied by Kaplan and his coworkers (7). Their work and that of others on the effectiveness of various analogues, including all those used in the present study, in replacing NAD as coenzymes

for dehydrogenases, has recently been reviewed by Colowick et al. (9). In no case where an analogue had been found to be totally inactive as a coenzyme could it replace NAD as a cofactor for inhibition of protein synthesis by diphtheria toxin. On the other hand, of those nucleotides found capable of activating various dehydrogenases to a greater or a lesser extent, several are capable of supporting inhibition, by diphtheria toxin, of phenylalanine incorporation into polyphenylalanine. The only analogue that we have found to be just as effective as NAD, is 3-thiocarboxamide pyridine–AD. However, 3-acetylpyridine-AD was found to be about 70%, and deamino-NAD about 40%, as active as NAD.

While the results with analogues still do not resolve the question whether or not inhibition of protein synthesis in cell-free extracts by toxin and NAD actually involves the direct oxidative inactivation of transferase II, they nevertheless strongly suggest that some sort of interaction between toxin and NAD and certain related nucleotides, takes place. Direct evidence for reversible binding of one NAD per molecule of toxin has been demonstrated by gel filtration and by equilibrium dialysis. From the preliminary experiments that we have reported, a tentative value of $K = 2.4 \times 10^3$ liters per mole has been calculated. The significance of this interaction and its relation to the mode of action of diphtheria toxin will be discussed in the paper that follows.

SUMMARY

The ability of a number of nucleotides related to NAD to replace NAD as cofactors for inhibition by diphtheria toxin of peptide bond formation has been examined. Neither NADH nor NADP are active. Of some 14 analogues closely related structurally to NAD that have been tested, only 3-thiocarboxamide pyridine–AD is as active as NAD itself. Replacement of the 3-carboxamide group on the pyridine ring by an acetyl group, or deamination of the purine ring, resulted in derivatives with reduced activity. The results were interpreted as suggesting that NAD and certain related nucleotides are capable of specific interaction with diphtheria toxin. Using the method of equilibrium dialysis, reversible binding of 1 mole of NAD per mole of toxin has been demonstrated. Toxoid does not interact with NAD.

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