STUDIES ON THE MODE OF ACTION OF DIPHTHERIA TOXIN*

III. Site of Toxin Action in Cell-Free Extracts

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(Received for publication 5 June 1967)

The quantitative effect of diphtheria toxin on growth and metabolism of mammalian cells in tissue culture was first investigated by Strauss and Hendee (1). They showed that within 2 hr after addition of an excess of toxin, HeLa cells were no longer able to incorporate amino acids into protein, although aerobic glycolysis and oxygen uptake continued at normal rates for several hours thereafter. Later, it was shown by Kato and Pappenheimer (2) and by Strauss (3) that RNA synthesis is maintained and that intoxicated cells continue to take up and concentrate potassium ions from the external medium (2) for a considerable time after growth ceases. Moreover, the ATP and GTP contents of intoxicated HeLa cells remain at their normal levels for many hours after protein synthesis has been arrested (4) and the cells retain their normal morphological appearance. The effect of toxin on protein synthesis in vivo, therefore, seems to be highly specific. Additional confirmation of the specificity of toxin action comes from recent observations by Duncan and Groman (5) who have shown that toxin blocks synthesis of poliomyelitis viral proteins in infected HeLa cells.

Collier and Pappenheimer (6) found that incorporation of amino acids into polypeptides by cell-free systems extracted from HeLa cells and from rabbit reticulocytes could be inhibited up to 90% or more by low concentrations of toxin, provided that a specific cofactor, identified as nicotinamide adenine dinucleotide (NAD) was present. Since the toxin was found to be without effect on the activation of amino acids or on the formation of aminoacyl-sRNA, they concluded that inhibition of protein synthesis by toxin takes place at the level of transfer from aminoacyl-sRNA to the growing peptide chain on the ribosomes.

Mammalian cells are known to contain at least two soluble and highly labile enzymes that are required for binding of aminoacyl-sRNA to ribosomes and for catalysis of peptide bond formation (7, 8). Gasior and Moldave (8) have termed these

* 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 during tenure by Dr. Goor of a Predoctoral Fellowship of the National Science Foundation.

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enzymes transferases I and II and have shown that, after precipitation from ribosome-free extracts of calf liver cells by ammonium sulfate, they can be separated from one another by gel filtration. Recently, Collier (9), working with the rabbit reticulocyte cell-free system, has found that toxin has neither a direct nor an indirect effect either on ribosomes or polyribosomes; nor does it bring about release of nascent polypeptide chains from the latter. He has obtained convincing evidence, however, that in the presence of NAD, toxin specifically inactivates a reticulocyte factor corresponding to the transferase II found in liver by Gasior and Moldave. Transferase I is not affected by toxin.

In the first of the present series of papers, we are reporting our attempts to locate the exact site of action of diphtheria toxin in extracts from HeLa cells. Evidence will be presented showing that after removal of the ribosomes from HeLa cell extracts, toxin in the presence of NAD interacts *directly* with one of the remaining soluble transferases, and causes its complete inactivation. Ribosome-bound transferase, on the other hand, is no longer susceptible to inactivation by toxin. Our results point towards soluble, free transferase II as the specific site of toxin interaction in HeLa cell extracts and thus are in full agreement with the more direct evidence of Collier's work with the reticulocyte system (9).

In the two following papers, we shall report the results of studies on the specificity of NAD and its analogues (10), and on the quantitative relationships between NAD, toxin, and transferase II (11). It will be demonstrated that the inactivation by toxin in in vitro systems is reversible under certain conditions. The results have led to the formulation of a model to explain the mode of action of toxin in vitro (11).

Materials and Methods

Reagents.—Creatine phosphate, creatine phosphokinase, polyuridylic acid (poly U), ATP, GTP, NAD, and 2-mercaptoethanol (ME) were obtained from Calbiochem., Los Angeles, Calif. Glutathione (GSH), potassium deoxycholate (DOC), and protamine sulfate were purchased from Mann Research Labs., New York. General Biochemicals, Chagrin Falls, Ohio, supplied stripped sRNA prepared from rat liver.

Radiosotopes.—Uniformly labeled L-leucine-^{14}C and L-phenylalanine-^{14}C were obtained from New England Nuclear Corp., Boston, Mass. Each amino acid was diluted with the corresponding unlabeled amino acid to the desired specific activity.

Diptheria Toxin.—We are indebted to Mr. Leo Levine of the Antitoxin and Vaccine Laboratory, Massachusetts Department of Health, for supplying us with generous amounts of diphtheria toxin that had already been partially purified by ammonium sulfate precipitation followed by dialysis. It was further fractionated with ammonium sulfate, the fraction precipitating between 0.45 and 0.60 saturation being retained. After equilibration with 0.01 M phosphate buffer, pH 6.9, the toxin was applied to a DEAE-cellulose column equilibrated with the same buffer. It was eluted when the buffer concentration was raised to 0.05 M and the pH to 7.9. The eluate was precipitated with two-thirds saturated ammonium sulfate and the precipitate collected and dialyzed against 0.25 M tris buffer, pH 7, containing 0.2 M KCl and 0.025 M Mg^{++}. The purified toxin contained about 2.5 μg protein and 60–70 MLD per Lf unit. It was stored in the frozen state.

Cells—HeLa strain S-3 cells were collected from spinner cultures growing exponentially in Eagle's medium supplemented with 5–10% horse serum[1] as described previously (4, 6). Intoxicated HeLa cells were obtained by incubating spinner cultures for 5–6 hr in complete growth medium containing 1–2 Lf (2.5–5 μg) toxin per milliliter. Leucine-^{14}C incorporation in 15 ml aliquots was followed as a function of time in order to confirm the cessation of protein synthesis within 2–3 hr after adding toxin.

Normal and intoxicated cells were harvested by centrifugation of cultures containing about 5×10^5 cells per milliliter. The cells were washed twice by resuspending them in the original volume in chilled Eagle's salt solution followed by centrifugation. The final pellet was either extracted immediately or stored frozen at -15°C. It is possible to store the frozen cells for many months at this temperature and still obtain extracts of high activity from them.

Reticulocytes were prepared from phenylhydrazine-treated rabbits as described by Allen and Schweet (12).

Cell Extracts.—Both normal and intoxicated cells were suspended in 5 ml chilled tris-sucrose buffer[2] for each milliliter of packed cells. The ice-cold suspensions were lysed by passing them through a French pressure cell at 2000 psi. Cellular debris and nuclei were removed by centrifugation at 2000 *g* in the International. The supernate was then centrifuged at 12,000 *g* in the Sorvall SS2 to remove mitochondria and smaller cell fragments. The final crude supernate was equilibrated with 0.25 M tris buffer[3] either by dialysis or by passage through Sephadex G25.

Preparation of Cell Fractions—Ribosomes.—The crude cell extract (from either HeLa cells or from rabbit reticulocytes) was centrifuged at 105,000 *g* in the Spinco ultracentrifuge, model L, for 90 min. The supernate was again centrifuged at high speed and the top one-half carefully withdrawn and saved for the preparation of sRNA and soluble enzymes. The pellet from the first centrifugation was rinsed several times by careful overlay with and decantation of medium B[4] (Allen and Schweet, reference 12). It was resuspended in the same medium and again centrifuged for 90 min at 105,000 *g*. The ribosomes were suspended in 0.25 M sucrose and centrifuged at low speed to remove a small amount of debris. They were either used at at once, purified further, or stored in the frozen state. Further purification was accomplished by treatment with potassium deoxycholate (DOC) (13). One-fourth volume of neutralized 5% DOC was added to the ribosome suspension and incubated at 37°C for 3–5 min. Ten volumes of medium B were added and the ribosomes collected by centrifugation for 90 min at 105,000 *g*. The pellet was rinsed several times, resuspended in 0.25 M sucrose and stored frozen. The concentration of the suspension was estimated from its optical density at 260 mμ, assuming an OD of 1 $\simeq$ 50 μg/ml.

sRNA.—The ribosome-free supernate was treated with protamine sulfate, and the precipitated sRNA was further purified by phenol extraction and ethanol precipitation according to the method of Allen et al. (14). The supernate from the protamine precipitation was saved for preparation of soluble enzymes (see below). The sRNA was dissolved in distilled water, dialyzed against 0.02 M Tris at pH 7.5, and stored frozen. Its concentration was estimated assuming OD 260 mμ $\simeq$ 40 μg sRNA/ml.

Phenylalanyl-^{14}C-sRNA.—2 μc phenylalanine-^{14}C (125 μc/μmole) were added to 1.4 ml of a cell-free system containing rabbit reticulocyte extract and lacking only GTP, ribosomes,

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[1] Commercial horse sera frequently contain appreciable titers of diphtheria antitoxin. The Antitoxin and Vaccine Laboratory has kindly supplied us with sterile horse serum shown to contain less than 0.001 unit of antitoxin per milliliter.

[2] Tris buffer pH 7.5, 0.01 M; sucrose, 0.25 M; KCl, 0.06 M; $MgCl_2 \cdot 6H_2O$, 0.025 M; GSH or ME, 0.01 M.

[3] Tris buffer pH 7.5, 0.25 M; KCl, 0.2 M; $MgCl_2 \cdot 6H_2O$, 0.025 M; GSH or ME, 0.01 M.

[4] Sucrose, 0.25 M; $KHCO_3$, 0.0175 M; $MgCl_2 \cdot 6H_2O$, 0.002 M.

and poly U. After 20 min at 37°C, the mixture was extracted twice with equal volumes of 90% phenol and then precipitated several times with two volumes of ethanol (15). The final precipitate of phenylalanyl-^{14}C-sRNA was dialyzed against 1 mM acetate, pH 6.5, and stored frozen. The final preparation contained 34 μc/mg.

Soluble Enzymes.—The supernate from sRNA precipitation by protamine sulfate was fractionated with ammonium sulfate, the fraction precipitating between 40 and 70% saturation being retained. This AS70 fraction (12) was dialyzed against 0.02 M Tris at pH 7.5 containing 1 mM GSH and 0.1 mM EDTA. Preparations were stored frozen in the presence of 25 mM GSH and 2.5 mM EDTA. The fraction contained both soluble activating and transfer enzymes (12).

Incorporation of Amino Acids.—Phenylalanine-^{14}C incorporation into trichloroacetic acid (TCA)–precipitable peptides was carried out in 12 ml conical tubes as previously described (6), except that in most of the experiments the total volume was reduced from 1.4 to 0.25 ml. The reaction mixture contained 9 μmoles Tris, pH 7.5, 9 μmoles KCl, 0.9 μmoles $MgCl_2 \cdot 6H_2O$, 2.6 μmoles GSH or ME, 1.8 μmoles creatine phosphate, 0.18 μmoles ATP, 0.045 μmoles GTP, 22 μg creatine phosphokinase, 18 μg poly U, and 0.004 μmoles phenylalanine-^{14}C (125 μc/μmole). Crude cell extract, toxin, and NAD were added to appropriate tubes. The concentrations of AS70 fraction, sRNA, and ribosomes used in the reconstituted system from rabbit reticulocytes are given in the experimental section. The final volume was brought to 0.25 ml with distilled water and incorporation allowed to proceed at 37°C for 40 min. The reaction was then stopped by addition of 0.25 ml 10% TCA. The precipitates were washed three times with 5 ml changes of 5% TCA, suspended in 5 ml 5% TCA, and heated for 15 min at 90°C. After cooling and two further washings with TCA, the precipitates were suspended in 5% TCA collected on Millipore filters, and again washed twice on the filter with TCA. The filters were glued to planchets, dried and counted in a thin-window gas flow counter.

Characteristics of the Incorporating Systems.—Both HeLa and the reconstituted reticulocyte systems require ATP and an ATP-generating system. There was no incorporation in the presence of puromycin or in the absence of added sulfhydryl (GSH or ME) or of poly U. There was no measurable uptake of phenylalanine-^{14}C into TCA-precipitable material at 0°C even after prolonged periods of time.

EXPERIMENTAL

Phenylalanine Uptake in Extracts from Intoxicated Cells.—Within 2 hr after treatment with a saturating dose of diphtheria toxin, HeLa cells in culture at 37°C stop synthesizing protein. Nevertheless, extracts made from cells exposed to excess toxin for 5–6 hr, are still capable of incorporating leucine into TCA-precipitable peptides, although at a rate that may be less than 20% of that found in extracts from an equivalent number of normal cells (6). Likewise, we have observed that phenylalanine-^{14}C uptake in crude extracts from thoroughly washed intoxicated HeLa cells is usually only 30–40% that of equivalent extracts from normal cells,[5] even without further supplement of NAD (see Table I).

[5] There was a good deal of variation in the capacity of extracts from different batches of intoxicated cells to incorporate phenylalanine. Recent observations suggest that this variation may be determined by cultural conditions during the period of exposure to toxin. In order to reduce both the latent period and the amino acid-incorporating capacity in extracts from intoxicated cells to a minimum, it is essential to use cells growing exponentially in fresh medium.

Table I also shows that extracts from washed, intoxicated cells contain little if any free toxin, since in the presence of excess NAD there was no inhibition of amino acid uptake on mixing intoxicated with normal extract.[6] On the contrary, the incorporation observed was additive. From experiments with methionine-^{3}H-labeled toxin, Pappenheimer et al. (16) had also concluded that only a small fraction of the added toxin is actually taken up by the cells, as they were unable to detect significant uptake of label even after 6 hr continuous exposure to several saturating doses of toxin.

TABLE I

Phenylalanine Incorporation in Crude Extracts from Normal and Intoxicated HeLa Cells

Additions	Phenylalanine-^{14}C incorporated	Inhibition
	cpm	%
Crude normal HeLa extract (0.5 ml)	1530	—
Crude normal HeLa extract (0.5 ml) + toxin + NAD	294	81
Crude normal HeLa extract (0.25 ml)	1010	—
Crude intoxicated HeLa extract (0.5 ml)	500	67
Crude intoxicated HeLa extract (0.5 ml) + NAD	473	69
Normal (0.25 ml) + intoxicated (0.25 ml) HeLa extract + NAD	1290	—

Extracts from both normal and intoxicated cells were adjusted to the same protein content. Concentrations used were: phenylalanine-^{14}C, 0.625 μc/tube (12.5 μc/μmole); toxin, 10 μg/ml; NAD, 5.4×10^{-5} M. The volume was 1.4 ml/tube. Incubation at 37°C for 40 min.

As an initial step towards identification of the toxin target, it was decided to attempt to restore activity to normal levels in extracts from intoxicated cells by addition of fractions isolated from normal cell extracts.

Aminoacyl-sRNA.—Toxin does not interfere with the initial steps in protein synthesis that involve amino acid activation (6). Neither the yield nor the rate of formation of aminoacyl-sRNA are affected by relatively high concentrations of toxin. The earlier studies also showed that purified diphtheria toxin not only lacks RNase activity but also fails to increase RNase activity when added to HeLa cell extracts. Moreover, as can be seen from Table II, comparatively large amounts of sRNA from rabbit reticulocytes or from rat liver cause very little stimulation of poly U–directed incorporation of phenylalanine in intoxicated HeLa cell extracts.

Despite the above findings, the possibility remained that toxin might in some

[6] The absence of free toxin was confirmed by guinea pig test. We have injected the extract from 10^{8} intoxicated HeLa cells (collected from a 700 ml culture) into a single guinea pig without observing even a local reaction at the site of injection.

way inactivate aminoacyl-sRNA. The results of an experiment, plotted in Fig. 1, make this appear unlikely. In this experiment, increasing amounts of phenylalanyl-^{14}C-sRNA were added to reconstituted systems prepared from NAD-free extracts of normal and intoxicated cells. Ribosomes were removed by centrifugation and RNA by precipitation with protamine sulfate. The final supernates from both normal and intoxicated cells were supplemented with NAD and with sufficient amounts of washed DOC-treated reticulocyte ribosomes and of phenylalanine-^{14}C to insure that neither would limit the phenyl-

TABLE II

Effect of sRNA and of Washed and DOC-Treated Ribosomes on Phenylalanine Incorporation in Normal and Intoxicated HeLa Cell Extracts

Additions	Phenylalanine-^{14}C uptake	Net increase
	cpm	*cpm*
Crude normal HeLa extract (0.5 ml)	1395	—
+ rat liver sRNA	2080	+685
+ washed rabbit reticulocyte ribosomes	2940	+1545
+ washed DOC-treated ribosomes	2200	+805
Intoxicated crude HeLa extract (0.5 ml)	614	—
+ rat liver sRNA	795	+185
+ rabbit reticulocyte sRNA	670	+56
+ washed reticulocyte ribosomes	1770	+1156
+ washed DOC-treated ribosomes	620	+6

Normal and intoxicated cell extracts adjusted to the same protein concentration.

Concentrations: phenylalanine-^{14}C, 0.625 μc/tube (12.5 μc/μmole); rat liver sRNA, 78 μg/ml; rabbit reticulocyte sRNA, 93 μg/ml; washed reticulocyte ribosomes, 210 μg/ml; washed DOC-treated reticulocyte ribosomes, 205 μg/ml. Total volume per tube, 1.4 ml. Reaction at 37°C for 40 min.

alanine uptake. Fig. 1 shows that incorporation was stimulated far less in the intoxicated than in the normal system by phenylalanyl-sRNA. As will become clear below, the relatively small linear increase observed in the intoxicated extract can be attributed to small amounts of a ribosome-bound transfer enzyme that is insensitive to toxin. We conclude from these observations that aminoacyl-sRNA is not altered by toxin and is not limiting in intoxicated extracts.

Ribosomes.—Transfer of amino acids from aminoacyl-sRNA to the growing peptide chain takes place on ribosomes as directed by mRNA. When washed ribosomes from rabbit reticulocytes were added to crude extracts from intoxicated HeLa cells, the poly U–directed incorporation of phenylalanine into TCA-precipitable peptides was restored to its normal level or even higher. However, this stimulation was not due to the ribosomes themselves, since treatment

with deoxycholate always greatly reduced the effect and, occasionally, as in the experiment summarized in Table II, almost abolished it entirely. Tables II and III show that, whereas DOC-treated ribosomes may double the already high incorporation of phenylalanine in extracts from normal cells, the treated ribosomes are very much less stimulatory in extracts from intoxicated cells.

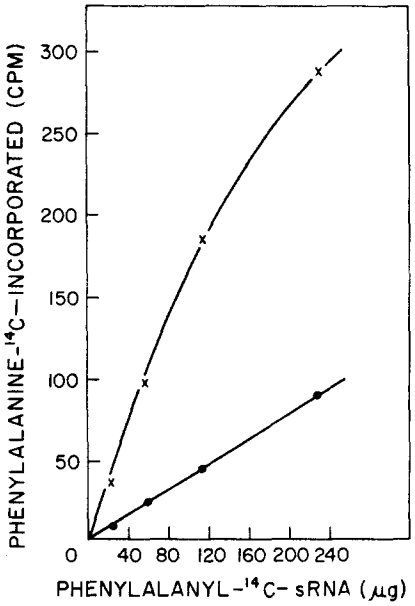

FIG. 1. Effect of phenylalanyl-^{14}C-sRNA on phenylalanine incorporation in normal and intoxicated HeLa extracts. The incorporating system contained 0.5 ml HeLa extract from which ribosomes and sRNA had been removed (see Materials and Methods). It was supplemented with 350 μg DOC-treated reticulocyte ribosomes, 0.625 μc (0.02 μmoles) phenylalanine-^{14}C, and increasing amounts of phenylalanine-^{14}C-sRNA (34 μc/mg). Extracts were brought to 1.4 ml and incubated 40 min at 37°C. ×, control; ●, intoxicated extract.

Because sRNA was not completely removed by the protamine precipitation, both normal and intoxicated extracts showed a small ^{14}C uptake in the absence of any added aminoacyl-^{14}C-sRNA. The curves have been corrected for this endogenous incorporation.

The different patterns of response exhibited by extracts from normal and intoxicated cells to additions of the various components (Tables II and III) demonstrate that the two systems are not limited by the same factor(s). In the normal system, it is the ribosomes that appear to be the principal factor that is limiting. On the other hand, intoxicated extracts seem to be saturated with respect to ribosomes but do respond to a soluble factor present in normal HeLa supernate (Table III, last column).

Further evidence that it is not the ribosomes themselves that are attacked

by the toxin is given in Fig. 2. This figure shows that stimulation of phenylalanine incorporation by addition of increasing amounts of DOC-treated reticulocyte ribosomes to ribosome-free extracts from normal HeLa cells was strongly inhibited by excess toxin in the presence of NAD. Nevertheless, the extracts to which excess toxin was added did show a small but important linear

TABLE III

Effect of Addition of Normal HeLa Supernate Enzymes on Incorporation in Normal and Intoxicated HeLa Cell-Free Systems

Additions	Phenylalanine-^{14}C incorporated	Increased incorporation above control	Soluble enzyme-dependent increased incorporation above control saturated with DOC-ribosomes and sSNA
	cpm	*cpm*	*cpm*
Crude normal HeLa extract (0.4 ml)	1160	0	—
+ reticulocyte DOC-ribosomes	2400	1240	—
+ rat liver sRNA	1530	370	—
+ DOC-ribosomes + sRNA	3110	1950	0
+ DOC-ribosomes + sRNA + normal HeLa supernate (0.2 ml)	3580	2420	470
+ DOC-ribosomes + sRNA + normal HeLa supernate (0.4 ml)	3700	2540	590
Crude intoxicated HeLa extract (0.4 ml)	725	0	—
+ DOC-ribosomes	944	219	—
+ sRNA	1010	285	—
+ DOC-ribosomes + sRNA	1120	395	0
+ DOC-ribosomes + sRNA + normal HeLa supernate (0.2 ml)	3700	2975	2580
+DOC-ribosomes + sRNA + normal HeLa supernate (0.4 ml)	4560	3835	3440

Normal and intoxicated HeLa extracts were adjusted to the same protein concentration. Concentrations: phenylalanine-^{14}C, 0.625 μc (12.5 μc/μmole); reticulocyte DOC-ribosomes, 273 μg/ml; rat liver sRNA, 67 μg/ml. Total volume, 1.4 ml/tube. Incubation at 37°C for /0 min.

response to the addition of treated ribosomes. Gasior and Moldave (8, 13) have found that although treatment with DOC readily removes all of the transferase I from ribosomes, transferase II is usually only partially removed. That at least one of the transfer enzymes had been completely removed by DOC treatment of the HeLa ribosomes is proved by the fact that DOC ribosomes stimulated no incorporation whatsoever from phenylalanine-^{14}C-sRNA unless HeLa supernate was added.

It thus appears that it is the particular transfer enzyme that *cannot* be readily

removed from ribosomes with DOC that is the toxin target. Although resistant to inactivation by toxin when bound to ribosomes, the free soluble enzyme is completely inactivated by toxin provided NAD is present. In any case, ribo-

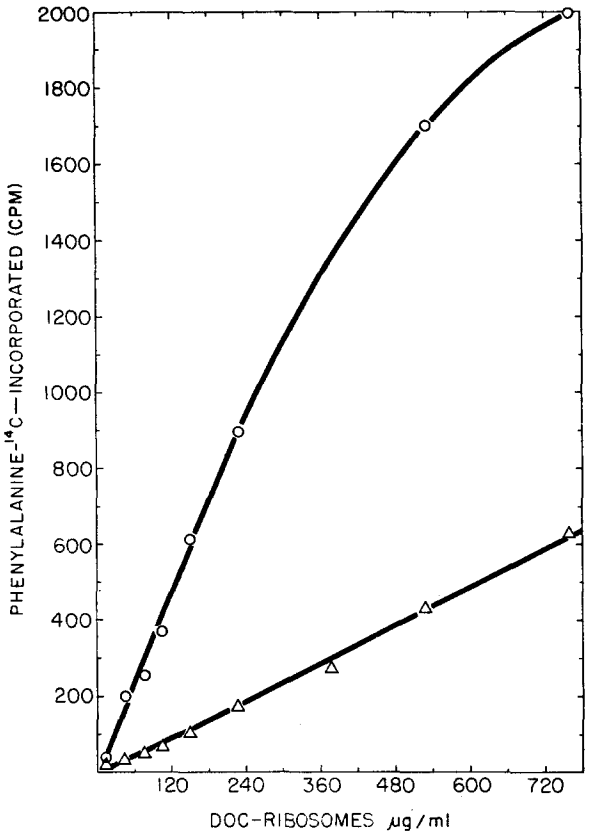

FIG. 2. Effect of ribosomes on phenylalanine-^{14}C incorporation in presence and absence of toxin. Increasing amounts of DOC-treated HeLa ribosomes were added to a ribosome-free supernate from normal HeLa cell extract containing 3×10^{-5} M NAD. Total volume, 0.25 ml. Incubation was for 40 min at 37°C. ○, control; △, 6 µg/ml toxin.

somes themselves are probably never limiting in crude extracts from intoxicated cells.

Transfer Enzymes.—The results summarized in the preceding paragraphs point towards one of the soluble transfer enzymes as the most probable site of toxin action. We have seen from Table III that, although the amino acid incorporating system extracted from normal cells seems to be limited mainly by the ribosomes and to a lesser extent by sRNA, the intoxicated system shows

marked stimulation only when the soluble fraction from normal extracts, freed from ribosomes and sRNA, is added.

The stimulation of phenylalanine incorporation by soluble enzymes present in the AS70 fraction from rabbit reticulocytes and its inhibition by diphtheria

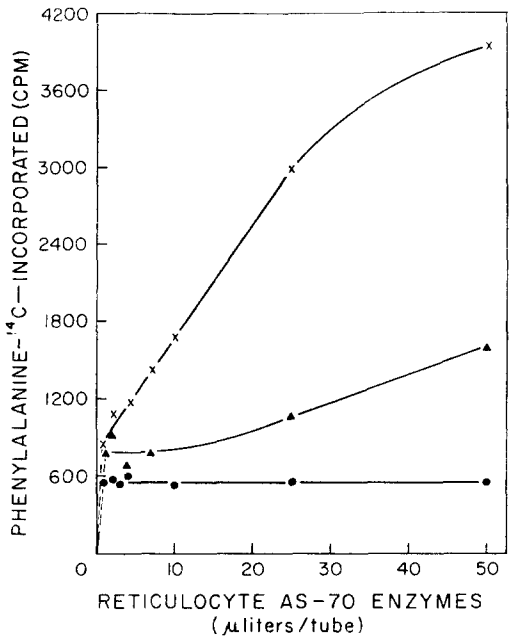

FIG. 3. Effect of soluble AS70 fraction from reticulocytes on phenylalanine-^{14}C incorporation. Increasing amounts of reticulocyte AS70 fraction were added to an incorporating system supplemented with 3×10^{-5} M NAD, 750 μg DOC-treated reticulocyte ribosomes, and 25 μg sRNA from rat liver. Volume 0.25 ml, incubation for 40 min at 37°C. ×, control; ●, 1.5 μg toxin; ▲, 0.0625 μg toxin.

Note that all three curves are discontinuous in the region near the origin. Incorporation was nil unless a small amount of AS70 fraction was added. However, only 1 μl of this soluble fraction sufficed to raise incorporation to 590 cpm or above.

toxin is strikingly illustrated in Fig. 3. The basal system in this case contained rat liver sRNA, DOC-treated reticulocyte ribosomes, and 3×10^{-5} M NAD and gave no incorporation until a small amount of AS70 had been added. Thereafter, phenylalanine-^{14}C uptake in the control tubes was directly proportional to the amount of AS70 fraction added until the level of incorporated amino acid had increased 5–6-fold. In the presence of 6 μg/ml toxin, however, even the maximal amount of AS70 fraction added failed to increase incorporation above the initial level of 590 cpm. At low toxin concentration (0.25 μg/ml), on the other hand, the inhibition of phenylalanine uptake could be overcome by addition of a sufficiently large amount of AS70 fraction.

We conclude from the foregoing experiment that a transfer enzyme present in soluble form in the AS70 fraction may be completely inactivated by excess toxin and partially inactivated by smaller amounts of toxin. When the transfer enzyme is bound to the ribosome, however, it becomes resistant to toxin. Thus the per cent inhibition observed in any given cell-free system in the presence of excess toxin is a measure of the per cent of the total transfer enzyme that remains free in solution.

Inactivation, by toxin, of a soluble transfer enzyme does not require the concomitant presence of either sRNA or of ribosomes. An extract from normal HeLa cells was centrifuged at 105,000 *g* to remove ribosomes and the supernate

TABLE IV

Effect of Toxin + NAD on Ribosome- and sRNA-Free Supernates from HeLa Cell Extracts

Additions	Phenylalanine-^{14}C dincorporated
	cpm
Supernate A* (0.5 ml)	6
Rat liver sRNA + rabbit reticulocyte DOC-ribosomes	5
Supernate A (0.5 ml) + rat liver sRNA + reticulocyte DOC-ribosomes	440
. + toxin + NAD	98
Toxin-treated supernate B* (0.5 ml) + rat liver sRNA + DOC-ribosomes	88
+ 0.1 ml supernate A	179
+ 0.2 ml supernate A	214

* Both supernates A and B were incubated for 10 min at 37°C, chilled, and dialyzed to remove excess NAD as described in the text.

Concentrations used were: phenylalanine-^{14}C, 0.625 μc/tube (12.5 μc/μmole); toxin, 9 μg/ml; NAD, 5.4 $\times$ 10^{-3} M; rat liver sRNA, 67 μg/ml, reticulocyte DOC-ribosomes, 273 μg/ml. The volume was 1.4 ml. Incubation for 40 min at 37°C.

treated with protamine sulfate to precipitate RNA. The final clear supernate was divided into two equal portions, A and B. Toxin (4 μg/ml) and NAD (2.5 $\times$ 10^{-5} M) were added to the latter. Both A and B were then incubated for 10 min at 37°C in the tris 0.25 M sucrose buffer used for cell extraction, rapidly chilled, and dialyzed against two 2-liter changes of cold buffer containing 10 mM GSH to remove all excess NAD. Table IV shows that in the presence of rat liver sRNA and DOC-treated ribosomes, there was good incorporation of phenylalanine-^{14}C (440 cpm) in supernate A which was 80% inhibited by subsequent addition of excess toxin and NAD. On the other hand, the intoxicated supernate B was only 20% as effective as A when mixed with sRNA and ribosomes. Table IV shows that amino acid uptake in the presence of supernate B was stimulated by addition of the normal supernate A. The experiment also shows that while inhibition of protein synthesis requires NAD as a cofactor, removal of excess NAD from the system *after* the toxin has been allowed to

act, does not bring about any rapid reactivation of the transfer enzyme. (See also references 9 and 11.)

DISCUSSION

It has been demonstrated that extracts made from HeLa cells that have been continuously exposed for 6 hr in culture to a saturating dose of diphtheria toxin are still capable of stimulating the poly U–directed incorporation of phenylalanine into TCA-precipitable polypeptides. Even though the intoxicated cells from which the extracts had been made had synthesized no protein for a considerable period, amino acid incorporation in the cell-free system was often 30–40% or even more of that found in extracts from the same number of normal cells. In fact, despite the complete cessation of protein synthesis brought about by exposure of growing cells to toxin, most of the complex protein-synthesizing machinery of the cell remains unimpaired, since amino acid incorporation in the intoxicated extracts can be restored to its normal level or even higher by addition of soluble enzymes from normal HeLa cells or small amounts of the AS70 fraction prepared from rabbit reticulocytes.

The experiments that have been described leave no doubt that diphtheria toxin, in the presence of NAD, inactivates one of the soluble enzymes required for transfer of amino acids from aminoacyl-sRNA to the growing polypeptide chain. The target enzyme, when free in solution, is completely inactivated by the toxin. Ribosome-bound transfer enzyme, however, is completely resistant to toxin action. Of the two enzymes known to be involved in the transfer reaction in mammalian cell extracts, Gasior and Moldave (8, 13) have shown that only transferase I is completely removed from ribosomes by treatment with deoxycholate, whereas appreciable amounts of transferase II usually remain firmly bound. When DOC-treated ribosomes are added to otherwise complete systems containing excess toxin and NAD, poly U–mediated phenylalanine incorporation is directly proportional to the added ribosomes. We have therefore concluded that transferase II is the enzyme which, in its soluble form, is acted upon by toxin and which becomes limiting in intoxicated cells. Recently, Collier (9) has separated the two transferases from reticulocyte extracts by Sephadex chromatography and has shown directly that transferase II is specifically inactived by the toxin whereas transferase I is not affected.

Since only the free enzyme is sensitive, any incorporating activity that remains after treatment of an extract with excess toxin and NAD will provide a measure of that fraction of transferase II that is ribosomally bound. Finally, since transferase II is far less stable when free in solution than when it is bound to the ribosomes, the per cent inhibition by toxin of amino acid incorporation will always be greatest in freshly prepared extracts.

SUMMARY

Extracts from HeLa cells treated with excess diphtheria toxin for several hours, until all protein synthesis has been arrested, are still able to stimulate the poly U–directed incorporation of phenylalanine into polypeptides at a moderate rate. Activity may be restored to normal levels or above by addition of a soluble enzyme fraction containing transferase II. Our results are in agreement with those of Collier who has recently shown that toxin inactivates transferase II in extracts from rabbit reticulocytes. We have further demonstrated that amino acid incorporation in extracts from intoxicated HeLa cells is limited by their transferase II content whereas, in extracts from normal cells, it is the ribosomes and to a lesser extent sRNA that are limiting. We have found that only *soluble* transferase II is inactivated by toxin; the ribosome-bound enzyme is resistant.

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