STUDIES ON THE MODE OF ACTION OF DIPHTHERIA TOXIN*

II. Effect of Toxin on Amino Acid Incorporation in Cell-Free Systems

By R. J. COLLIER,‡, § Ph.D., and A. M. PAPPENHEIMER, Jr., Ph.D.

(*From the Biological Laboratories, Harvard University, Cambridge, Massachusetts*)

(Received for publication, July 20, 1964)

In the preceding paper (1), it was shown that the cellular levels of ATP, GTP, and hexose phosphates remain unchanged for at least 5 to 6 hours after addition of a saturating dose of diphtheria toxin to growing cultures of HeLa cells. These observations appear to eliminate the possibility that the complete inhibition of protein synthesis in HeLa cells which occurs within 2 to 3 hours after addition of a saturating dose of toxin (2) is caused by a primary effect of toxin on the energy metabolism of the cells. Since there is evidence that the toxin does not alter cell permeability or interfere drastically with RNA or DNA synthesis for several hours after its addition to cultures of mammalian cells (3, 4), it seemed worthwhile to investigate its effect on polypeptide synthesis from amino acids *in vitro*.

In recent years, extensive studies on amino acid incorporation into polypeptides have been carried out using cell-free systems. These investigations have resulted in a reasonably clear understanding of the major steps involved in protein biosynthesis and in the identification of various enzymes and cofactors that are required. We have therefore studied the effect of purified diphtheria toxin on amino acid incorporation in cell-free extracts from HeLa cells and from rabbit reticulocytes. The results have demonstrated that low concentrations of purified toxin specifically inhibit the uptake of amino acids into protein by interfering with a step involved in transfer of amino acids from soluble ribonucleic acid to the growing polypeptide chain. A cofactor, which has been identified as nicotinamide adenine dinucleotide (NAD), *must* be present in order for toxin to exert its inhibitory effect in cell-free systems.

* 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. Collier of a Graduate Fellowship of the National Science Foundation.

§ Present address: Institute of Molecular Biology, University of Geneva, Geneva, Switzerland.

Materials and Methods

Diphtheria Toxin and Antitoxin.—The toxin used in these studies contained 2.6 μg protein per Lf and about 60 M.L.D. per Lf. The method of preparation was described in detail in the preceding paper. The antitoxin used was preparation 1520–5353AD from Lederle Laboratories, Pearl River, New York; this antitoxin was also described in the preceding paper.

Diphtheria Toxoid.—A preparation of diphtheria toxoid, partially purified by ammonium sulfate fractionation, was kindly supplied by Dr. Leo Levine of the Massachusetts Department of Public Health. It was further purified by fractionation on a column of G-200 sephadex. The final product was estimated to be about 65 per cent pure.

Reagents.—ATP (dipotassium salt) and nicotinamide adenine dinucleotide (NAD) were supplied by Pabst Laboratories, Milwaukee, Wisconsin. GTP and creatine phosphate were products of Calbiochem Company, Los Angeles. Reduced glutathione (GSH), creatine phosphokinase, and bovine pancreatic ribonuclease were obtained from Calbiochem Company. Sigma Chemical Company, St. Louis supplied nicotinamide adenine dinucleotide phosphate (NADP) and bovine pancreatic deoxyribonuclease (DN-C), and Nutritional Biochemicals Corporation, Cleveland supplied puromycin. Polyuridylic acid (poly U) was purchased from Miles Chemical Company, Clifton, New Jersey.

Isotopically Labeled Compounds.—Uniformly labeled C^{14}-L-leucine and C^{14}-L-phenylalanine were obtained from either Schwarz BioResearch, Orangeburg, New York or New England Nuclear Corporation, Boston. They were usually diluted with the corresponding non-radioactive amino acid to a specific activity of 6 to 12 μc per μmole before use. C^{14}-algal protein hydrolysate was purchased from New England Nuclear Corporation and was used without dilution.

Cells.—HeLa cells were cultivated in spinner culture as described in the preceding paper (1). Cultures were harvested by centrifugation after reaching a concentration of 3.3×10^5 to 4.0×10^5 cells per ml. The cells were washed twice by centrifugation with Eagle's salt solution containing glucose (5) and were either used immediately for preparing extracts or were stored frozen as a packed cell pellet. Active extracts could be obtained from cells which had been stored frozen as long as 2 to 3 months.

Rabbit reticulocytes were prepared according to the method of Allen and Schweet (6). Rabbits of 5 to 6 pounds were injected daily for 4 consecutive days with 1 ml of a solution of 2.5 per cent phenylhydrazine containing 1 mM glutathione (GSH). Injections were omitted on the 5th and 6th days, and the rabbits were bled out on day 7. Heparin was used to prevent clotting. The blood was chilled, and after centrifugation for 15 minutes at 1500 RPM, the serum was removed and discarded. The cells, which consisted of about 90 per cent reticulocytes, were washed once with Eagle's salt solution and used immediately for preparation of cell extracts.

Preparation and Fractionation of Cell Extracts.—One to two ml of packed HeLa cells was resuspended at 0°C in 5 to 10 ml of 10 mM tris, pH 7.5, containing the following medium: 0.25 M sucrose, 60 mM KCl, 5 mM $MgCl_2$, and 5 mM GSH. The cell suspension was passed through a French pressure cell at about 3000 pounds per square inch, and the resulting extract was centrifuged at 2000 RPM for 5 minutes to remove unbroken cells and large cellular debris. The supernate was then recentrifuged at 15,000 *g* for 10 minutes to remove mitochondria and other particulate matter of similar size. The supernate from the second centrifugation, termed a "crude extract", was used as a routine without further fractionation for studies of amino acid incorporation into protein.

On occasion crude extracts from HeLa cells were separated into ribosomal (or microsomal) and supernatant fractions by centrifugation at 105,000 *g* for 90 minutes in a model L Spinco ultracentrifuge. The top three-fourths of the supernate was withdrawn and recentrifuged to remove any remaining ribosomal particles. The pellet of ribosomes was rinsed by carefully

overlayering with medium B[1] and decanting, and was then resuspended in medium B and recentrifuged at 105,000 *g*. The washed ribosomes were suspended in 0.25 M sucrose and centrifuged at 15,000 *g* for 10 minutes to remove any large aggregates of material. Both the ribosomal and supernatant fractions could be stored frozen for a period of several days without appreciable loss of activity.

Rabbit reticulocytes were extracted and fractionated according to the method of Allen and Schweet (6). The washed cells were lysed by adding 4 volumes of 2 mM $MgCl_2$, and large cellular debris was removed by centrifugation at 15,000 *g*. The supernate was then fractionated into ribosomal, soluble RNA (sRNA), and enzyme fractions as follows: Ribosomes were first separated by centrifugation at 78,000 *g* for 90 minutes; they were washed twice with medium B in the centrifuge and finally suspended in 0.25 M sucrose. Soluble RNA was separated from the ribosome-free lysate by precipitation with protamine sulfate. The precipitated sRNA was purified by phenol extraction to remove protein. After ethanol precipitation the sRNA was exhaustively dialyzed against water. Finally, the supernate from the protamine-RNA precipitation was fractionated with ammonium sulfate. The fraction precipitated between 40 and 70 per cent saturation with ammonium sulfate was collected, dissolved, and dialyzed against tris buffer containing GSH and the chelating agent, EDTA. This enzyme fraction is referred to as AS-70 (6). All of the reticulocyte fractions were stored in the frozen state.

Frozen extracts of *Escherichia coli* were a gift of Dr. J. W. Hopkins. These had been prepared by grinding bacteria with alumina and extracting the resulting paste with Mg^{++}-tris buffer (7). The supernate (crude extract) from centrifugation of this mixture at 15,000 *g* was used for amino acid incorporation studies.

Incorporation of Amino Acids in Cell-Free Systems.—Incorporation of C^{14}-amino acids by crude extracts from HeLa cells was carried out in 12 ml conical tubes as described by Allen and Schweet (6) in tris pH 7.5 buffer (50 μmoles) containing 1 μmole ATP, 0.25 μmole GTP, 10 μmoles creatine phosphate, 120 μg creatine phosphokinase, 50 μl amino acid mixture (8), 0.1 or 0.05 μmole C^{14}-amino acid, 20 μmoles GSH, 50 μmoles KCl, and 5 μmoles $MgCl_2$. The final volume was 1.4 ml after addition of 0.5 ml crude cell extract to initiate the amino acid incorporation reaction. After 40 minutes at 37°C, the reaction was stopped by addition of 1.5 ml of 10 per cent trichloroacetic acid (TCA). The precipitate was centrifuged, ground with a stirring rod, and washed 3 times with 5 ml portions of 5 per cent TCA. Five ml of 5 per cent TCA was again added, and the tubes were placed in a water bath at 90°C for 15 minutes. The precipitates were dissolved in 0.5 ml of 1 N NaOH, and exactly 2 minutes later the protein was reprecipitated with TCA. Finally, after extraction with an ethanol-ether mixture (1:1), and two washes with ether, the precipitates were filtered and weighed. Counting was carried out by liquid scintillation. In experiments in which this procedure of washing and counting was used, results are expressed as counts per minute per milligram protein.

Studies of the incorporation of C^{14}-amino acids by the cell-free reticulocyte system were carried out using reaction mixtures identical with those described above for HeLa extracts. About 5 mg of the AS-70 fraction, 100 μg of reticulocyte sRNA, and 300 μg ribosomes were normally added to each tube, although no great effort was made to keep these amounts constant in separate experiments. The higher level of incorporation of C^{14}-amino acids in this system permitted a greatly simplified washing and counting procedure to be used. Samples were precipitated with TCA, washed 3 times with 5 ml portions of 5 per cent TCA, and heated to 90°C for 15 minutes. They were then washed once again, collected on Millipore filters, and washed with TCA. The filters were glued to aluminum planchets, dried, and counted in a thin window, gas flow counter. With the usual incorporation levels of 1000 to 2000 CPM in this system, the background of about 20 to 50 CPM obtained with this washing procedure was

[1] 0.25 M sucrose, 0.0175 M $KHCO_3$, 0.002 M $MgCl_2 \cdot 6\ H_2O$ (Allen and Schweet reference 6).

not considered objectionably high. Where this washing and counting procedure was used the results are expressed as CPM per sample.

Assay for ATP.—ATP in cell-free amino acid incorporation mixtures was assayed according to the method of Slater (9), as outlined in the preceding paper.

Assay for Breakdown of Poly U.[2]—The capacity of toxin and of HeLa extracts to degrade poly U was tested in the system of Spahr and Schlessinger (10). The solution to be assayed was added to tubes containing C^{14}–poly U. After incubation, poly U was precipitated with ethanol, and any C^{14}–nucleotides and small oligonucleotides liberated by ribonuclease action were assayed as soluble radioactivity remaining in the supernate. To each tube were added 0.2 ml of 10 mM tris, pH 7.4; 0.1 ml of 2.5 mM magnesium acetate; 0.1 ml of 0.5 M KCl; 0.1 ml of C^{14}–poly U; and 0.1 ml toxin or cell extract. After 20 minutes at 30°C, the reaction was stopped by adding 0.1 ml of yeast RNA (5 mg per ml in 0.6 M NaCl), and then immediately 1.0 ml cold ethanol. After 20 minutes in an ice bath, the C^{14}–poly U precipitate was removed by centrifuging at 3000 RPM for 10 minutes. One ml of the clear supernate was plated on 2 inch planchets, dried, and counted.

Assay for DNase.—DNase was assayed by measuring the initial rate of increase in optical density at 260 mμ of a solution of highly polymerized DNA. To each cuvette were added 100 μg DNA, 100 μmoles tris, pH 7.4, 10 μmoles $MgCl_2$, and 80 μmoles KCl, in a volume of 2.8 ml. The enzyme to be assayed was then added in a volume of 0.2 ml, and the OD 260 was followed for several minutes. The initial rate of increase in OD 260 was taken as a direct measure of the DNase activity of the enzyme.

Preparation of Soluble RNA from Rabbit Liver.—Two rabbits were sacrificed, and their livers were excised and immediately homogenized in a Waring blendor 1 to 2 minutes in ice cold 0.25 M sucrose, 50 mM tris, pH 7.5. The homogenate was centrifuged at 15,000 *g* for 10 minutes to remove cellular debris. The supernate was centrifuged for 60 minutes at 78,000 *g*. Acetic acid was added to the clear supernate to bring the pH to 5.2, and after 15 minutes in the cold, the "pH 5 precipitate" was collected by centrifugation. This precipitate was resuspended in tris buffer, neutralized, and extracted twice with equal volumes of 90 per cent redistilled phenol. The RNA was precipitated from the aqueous phase by adding one-tenth volume 20 per cent potassium acetate at pH 6, and 2 volumes of cold absolute ethanol. The RNA was then discharged of amino acids by incubation in 0.5 M tris, pH 9.5, for 45 minutes at 37°C. Finally the RNA was reprecipitated with ethanol and dialyzed exhaustively against water. It was stored in the frozen state.

Charging of Soluble RNA with C^{14}–Amino Acids.—Rabbit liver sRNA was charged with C^{14}-phenylalanine or C^{14}–algal protein hydrolysate, using reaction mixtures identical with those used in studies of amino acid incorporation into protein except that GTP, non-radioactive amino acids, and ribosomes were omitted. C^{14}–algal protein hydrolysate (1.43 μc per μg) was added to one mixture and C^{14}-phenylalanine was added to the other, each to a level of 2 μc per ml. Each mixture contained 2.5 mg sRNA per ml and 1 mg AS-70 fraction per ml. After 30 minutes at 37°C, each of the reaction mixtures was extracted with phenol by a procedure almost identical with that used in the preparation of the rabbit liver sRNA, and the aminoacyl-sRNA was then subjected to several ethanol precipitations. The final solution of aminoacyl-sRNA was dialyzed against 1 mM acetate buffer, pH 6–6.5, and was stored frozen in small portions.

EXPERIMENTAL

Incorporation of Amino Acids into Protein by HeLa Cell Extracts.—When a labeled amino acid is added to crude extracts from washed HeLa cells, the

[2] Dr. P. F. Spahr kindly carried out these tests for the breakdown of poly U.

label is incorporated into TCA-precipitable material. In the case of C^{14}-leucine, incorporation is linear for about 20 minutes at 37°C and is complete after 40 minutes. As shown in Table I, incorporation of C^{14}-leucine in such extracts requires the presence of ATP, GTP, and an ATP-generating system (creatine phosphate and creatine kinase). The system is completely inhibited by low concentrations of puromycin and pancreatic ribonuclease but is insensitive to DNase. The fact that there was some incorporation of leucine even in the absence of added ATP, GTP, and the ATP-generating system can probably be attributed to the presence of a low level of ATP in the cell extract. Since omission of non-radioactive amino acids from the reaction mixture failed to

TABLE I

Incorporation of C^{14}-Leucine into Polypeptides by Crude Extracts from HeLa Cells

	Incubation period	C^{14}-leucine incorporated	Inhibition
	min.	CPM/*mg protein*	*per cent*
Complete system	20	228	—
Complete system	40	318	—
Complete system	60	316	—
No ATP, GTP, or ATP-generating system	60	53	83
No C^{12}-amino acids	60	302	4
Plus 10 μg RNase	60	4	99
Plus 50 μg puromycin	60	6	98
Plus 50 μg DNase	60	302	4

Each tube contained the complete amino acid incorporation mixture described under Materials and Methods supplemented with 0.1 μmole (0.6 μc) C^{14}-leucine. All components, including any reagents to be tested, were mixed; then 0.5 ml of HeLa extract was added to each tube to initiate the reaction. After incubation at 37°C, TCA was added, and the precipitates were processed for liquid scintillation counting.

inhibit leucine incorporation significantly, an adequate pool of amino acids must also have been present in the cell extract. The characteristics of the system are virtually identical with those previously reported by Attardi and Smith (11) for HeLa extracts and are similar to the characteristics exhibited by most other mammalian cell-free amino acid–incorporating systems.

Amino Acid Incorporation in Crude Extracts from Intoxicated HeLa cells.— HeLa cells were incubated in spinner culture for 3.5 hours with 1 Lf (2.5 μg) toxin per ml. They were then centrifuged, washed twice with Eagle's salt solution, disrupted as usual, and incorporation of C^{14}-leucine by the crude extract was measured. Table II shows that the level of leucine incorporation into protein in the extract from intoxicated cells was only about 20 per cent that in a control extract prepared in exactly the same way from normal cells. Incorporation was measured in the presence of three different levels of added C^{14}-leucine

in order to estimate the size of the pool of leucine in each extract. Assuming that the different concentrations of labeled leucine added did not significantly alter the total amount of leucine incorporated into protein, it may be calculated from Table II that the extracts from both normal and intoxicated cells contained close to 0.42 μmole leucine per ml. Thus the inhibition of leucine incorporation in the intoxicated cell extract could not have been due to dilution of the added C^{14}-leucine by a larger pool of non-radioactive leucine. The results suggest, therefore, that the protein-synthesizing system itself is altered in some way in intoxicated cells.

Effect of Direct Addition of Toxin to Normal Cell Extracts.—Further studies have revealed that direct addition of small amounts of toxin to extracts from *non-intoxicated* HeLa cells, causes a marked inhibition of amino acid incorpora-

TABLE II

Incorporation of C^{14}-Leucine by Crude Extracts from Intoxicated and and Non-Intoxicated HeLa Cells

C^{14}-leucine added	C^{14}-leucine incorporated		Inhibition
	Control extract	Intoxicated extract	
μmole	*cpm/mg protein*	*cpm/mg protein*	*per cent*
0.1	241	42	79
0.2	365	65	82
0.3	440	77	82

Crude extract (0.5 ml) from either intoxicated or non-intoxicated cells was added to complete reaction mixtures as in Table I. Incubation was carried out for 40 minutes at 37°C. The specific activity of C^{14}-leucine used was 6 μc per μmole.

tion. Table III shows the results obtained when increasing amounts of toxin were added to amino acid–incorporating mixtures immediately before addition of crude HeLa cell extract. Significant inhibition of C^{14}-leucine incorporation occurred in the presence of only 0.2 μg toxin per ml. A near maximum inhibition of 86 per cent was reached at a toxin concentration of about 3 μg per ml. Even 50 times this concentration failed to inhibit incorporation completely, however. It may be estimated that about 0.5 μg/ml toxin is required for 50 per cent inhibition of incorporation.

Evidence for Specificity of the Effect of Toxin.—Before investigating the mode of action of toxin in HeLa cell extracts in greater detail, it was important to obtain evidence that the observed inhibition of amino acid incorporation was in fact caused by diphtheria toxin itself and not by some minor impurity in the preparation. In the first place, it was found that a 3 times recrystallized preparation of toxin (generously supplied by Professor M. Yoneda) caused an inhibition of leucine incorporation comparable to that produced by a similar amount

of our non-crystalline preparation. Secondly, it was shown that relatively large amounts of purified toxoid (40 μg/ml) fail to inhibit amino acid incorporation significantly (see Table X). Finally, it has been found that the supernate from which toxin has been specifically precipitated by antitoxin has no effect on amino acid incorporation. It is of interest that the washed toxin-antitoxin floccules, as shown in Table IV, retain up to 20 per cent as much inhibitory activity as free toxin itself.

The effect of toxin on intact cells in tissue culture can be neutralized quantitatively and completely by prior treatment with an equivalent amount of specific antitoxin. However, the toxicity of toxin-antitoxin floccules in cell-free extracts need not surprise us, since many enzymes are known that are not neutralized by their homologous antibodies. Indeed, since the particular anti-

TABLE III

Effect of Toxin on the Incorporation of C^{14}-Leucine by Extracts from Normal HeLa Cells

Toxin added	Toxin concentration	C^{14}-leucine incorporated	Inhibition
Lf/tube	*μg/ml*	*cpm/mg protein*	*per cent*
0	0	230	—
0.1	0.18	185	19
0.3	0.54	89	61
1.5	2.7	33	86
15	27	11	95
90	161	15	93

Toxin was pipetted into reaction mixtures immediately before the cell extract was added. One-tenth μmole (0.6 μc) C^{14}-leucine was added per tube. Other conditions were the same as in Table I.

toxin used shows only a single line of precipitation on immunoelectrophoresis against purified toxin, the striking effect of the floccules on amino acid incorporation provides convincing evidence for the specificity of inhibition by toxin. Presumably, in the susceptible animal or in cell cultures, antitoxin can prevent toxin from gaining access to the target area within the cell.

Nuclease Activity of Toxin and of HeLa Cell Extracts.—All cell-free amino acid-incorporating systems that have been studied are extremely sensitive to inhibition by ribonuclease. Since RNase is a frequent contaminant of many bacterial enzyme preparations, the toxin preparation was tested for RNase activity. Two methods were used.

1. Table V shows that a relatively large amount of toxin was required to cause even slight inhibition of C^{14}-leucine incorporation into protein in an extract of *E. coli*. The table also shows that incorporation in this bacterial extract was about 50 times more sensitive to inhibition by pancreatic RNase than in a HeLa extract. Moreover, even the slight inhibition of leucine incor-

poration observed in the *E. coli* extract was probably not due to traces of RNase, but rather to a small amount of DNase known to be present in the toxin preparation.[3]

2. Using the assay system of Spahr and Schlessinger (10), it has been found that toxin does not catalyze the hydrolysis of synthetic polyuridylic acid (poly U). When 6 Lf of toxin was incubated at pH 7.4 with C^{14}–poly U for 20 minutes at 30°C, only 10 CPM above the blank were liberated as 60 per cent cold ethanol-soluble C^{14}-nucleotides and small oligonucleotides. HeLa cell extract itself shows far more RNase activity. After removal of microsomes, ribosomes, and

TABLE IV

*Effect of Antitoxin on the Inhibition by Toxin of C^{14}-Leucine Incorporation in Crude Extracts from HeLa Cells**

Additions	C^{14}-leucine incorporated	Inhibition
	CPM/*mg protein*	*per cent*
None	262	—
Toxin (2 Lf)	18	93
Toxin (1 Lf)	40	85
Supernate from flocculation‡		
20 Lf toxin + 20 units antitoxin	283	None
60 Lf toxin + 60 units antitoxin	282	None
Antitoxin (25 units)	289	None
Washed floccules§ (2 Lf)	98	63
Washed floccules (15 Lf)	40	85

* Conditions for amino acid incorporation same as in Table I. Each tube contained the standard reaction mixture + 0.5 ml HeLa extract + additions as above.

‡ 200 Lf of toxin and 200 units of antitoxin were mixed in a volume of 1 ml. After removal of floccules, 0.1 ml and 0.3 ml supernate were tested for inhibition of C^{14}-leucine uptake.

§ Floccules prepared at equivalence (one unit antitoxin per Lf), washed 3 times with 0.15 M KCl and resuspended in distilled water.

other particulate material from a crude HeLa cell extract by centrifugation at 105,000 *g* for 90 minutes, only 0.2 ml of the supernate liberated about 500 CPM from C^{14}–poly U under the same conditions as above. Addition of toxin (6 Lf) to HeLa supernate, failed to increase the ethanol-soluble nucleotides liberated from C^{14}–poly U. Thus, not only was the toxin preparation free from signifi-

[3] Unlike mammalian systems, the bacterial amino acid–incorporating system is partially dependent on DNA. An extracellular DNase seems to be released by all toxigenic strains of *Cornybacterium diphtheriae* (12) and Pope has found traces of DNase activity even in his recrystallized preparations of diphtheria toxin (13). The toxin used in these experiments contained DNase activity equivalent to about 2 μg crystalline DNase per 100 μg protein. After precipitation of the toxin with a highly specific antitoxin, all of the DNase activity was recovered in the supernate.

cant RNase activity, but its toxicity cannot be attributed to activation of a latent RNase present in HeLa supernate; nor does the HeLa supernate contain a factor which activates a latent RNase associated with toxin.

Determination of ATP in a Cell-Free Amino Acid–Incorporating System Containing Toxin.—It was shown in the preceding paper (1) that ATP levels in intoxicated HeLa cells remain normal for some time after protein synthesis has ceased. Nevertheless, it seemed worthwhile to carry out determinations of ATP at intervals during amino acid incorporation by HeLa cell extracts in order to make absolutely certain that toxin does not inhibit the ATP-generating

TABLE V

Effect of Toxin and Pancreatic Ribonuclease on C^{14}-Leucine Incorporation by Crude Extracts from E. coli and from HeLa Cells

	Toxin added	RNase added	C^{14}-leucine incorporated	Inhibition
	μg/tube	μg/tube	CPM/mg *protein*	*per cent*
E. coli extract	None	None	1101	—
	38	None	1022	7
	187	None	885	20
	None	0.001	869	21
	None	0.008	586	47
	None	0.04	256	78
HeLa cell extract	None	None	170	—
	5	None	30	82
	None	0.2	168	1
	None	1.2	13	92

Toxin or RNase was pipetted into reaction mixtures immediately before adding cell extract. Each tube contained one-tenth μmole (0.6 μc) C^{14}-leucine. Other conditions were the same as in Table I.

system or otherwise affect the level of ATP. A parallel series of tubes was prepared, each containing the complete amino acid–incorporating system including one μmole ATP and the ATP-generating system. Control and toxin-containing (10 Lf/ml) tubes were analysed for ATP content after 10, 25, and 40 minutes' incubation at 37°C using the enzymatic method (9) described in the preceding paper (1). Recoveries were between 84 and 88 per cent of the added ATP whether or not toxin had been added. It seems reasonable to conclude that toxin does not inhibit protein synthesis either by causing the degradation of ATP or by preventing its formation.

Effect of Toxin on the Poly U–Directed Synthesis of Polyphenyl-Alanine.—When poly U is added to a cell-free amino acid–incorporating system, there is an increased uptake of phenylalanine into TCA-precipitable material (14). This increased incorporation of phenylalanine represents the formation of poly-

phenylalanine on the poly U messenger template. Table VI shows that toxin strongly inhibits the poly U–directed formation of polyphenylalanine from C^{14}-phenylalanine in crude extracts from HeLa cells. This finding is of interest because it suggests that toxin probably does not act by specifically inhibiting the incorporation of a single amino acid into protein. In a system containing only natural messenger RNA, one might expect that inhibition of one amino acid would prevent incorporation of other amino acids as well, since polypeptide synthesis is known to be a sequential process, proceeding from the amino-terminal to the carboxyl-terminal end of the chain (15). The synthesis of polyphenylalanine requires only a single amino acid, however. Thus the finding that the poly U–directed formation of polyphenylalanine is inhibited by toxin suggests that if the incorporation of only one amino acid is blocked by toxin, then that amino acid must be phenylalanine.

TABLE VI

Effect of Toxin on Poly U–Directed Synthesis of Polyphenylalanine in HeLa Extracts

Additions	C^{14}-phenylalanine incorporated
	cpm/*sample*
None	275
50 μg poly U	1402
50 μg poly U + 6 Lf toxin	206

Poly U and toxin were added to reaction mixtures before HeLa extract was added. Each tube contained 0.1 μmole (0.6 μc) C^{14}-phenylalanine. The TCA precipitates were processed for planchet counting.

Effect of Toxin on Amino Acid Incorporation in a Cell-Free System from Rabbit Reticulocytes.—Because of the effort and expense involved in growing large amounts of HeLa cells, a more easily obtainable source of material was sought for further study of the mode of action of toxin. The cell-free system prepared from rabbit reticulocytes was selected because hemoglobin synthesis in this system has already been thoroughly investigated. The system is easily fractionated and the almost complete absence of nuclease activity makes it possible to store the purified fractions in the frozen state for long periods of time.

Rabbit reticulocytes were prepared, extracted, and fractionated according to the method of Allen and Schweet (6). Incorporation of C^{14}-leucine was followed in a reaction mixture containing sRNA, washed ribosomes, and a dialyzed ammonium sulfate–precipitated enzyme-containing fraction (AS-70) from reticulocytes together with the usual cofactors that are added to the HeLa-incorporating extract. Leucine incorporation in this system, as in HeLa cell extracts, was found to be dependent on ATP, GTP, and an ATP-generating system and was inhibited by puromycin and by ribonuclease. However, when diphtheria toxin was tested as an inhibitor, it was found to be almost without

effect; even 50 Lf (125 μg) of toxin per ml caused less than 15 per cent inhibition of leucine uptake. This result was unexpected in view of the fact that rabbits are highly sensitive to diphtheria toxin.

In an attempt to find an explanation for the insensitivity of the reticulocyte system to toxin, fractions prepared from HeLa cells were mixed with fractions from reticulocytes, and the effect of toxin on incorporation of C^{14}-leucine in the mixtures was investigated. In the one case, the supernate after centrifuging crude HeLa cell extract at 105,000 *g* for 90 minutes was added to washed reticulocyte ribosomes and in the other, the washed HeLa ribosomal sediment was mixed with sRNA and AS-70 fractions from reticulocytes. In such mixed systems, the level of amino acid incorporation was determined by the specific

TABLE VII

Sensitivity to Toxin of C^{14}-Leucine Incorporation in Systems Composed of Mixed HeLa and Reticulocyte Fractions

Supernatant fraction	Ribosomes	C^{14}-leucine Incorporated		Inhibition
		Control	Toxin (6 Lf)	
		cpm/sample	*cpm/sample*	*per cent*
Reticulocyte*	Reticulocyte	1414	1418	None
Reticulocyte	HeLa	292	311	None
HeLa	Reticulocyte	1776	355	80
HeLa + reticulocyte	Reticulocyte	2166	672	69

Fifty mμmoles (0.6 μc) C^{14}-leucine was added per tube. Other conditions were the same as in Table I. Ribosomes were always added last.

* AS70 + sRNA fractions.

ribosomes, reticulocyte ribosomes showing a considerably higher level of amino acid uptake than ribosomes from HeLa cells.[4] It can be seen from Table VII, that only those systems which contained HeLa supernate were sensitive to inhibition by toxin. The table also shows that sensitivity to toxin is retained in mixtures containing both HeLa supernate and the reticulocyte sRNA and AS-70 fractions. Thus the presence, in reticulocyte fractions, of a factor that inactivates toxin appears unlikely. The results suggest, therefore, that some factor that is present in HeLa supernate but absent from reticulocyte fractions is required for inhibition of amino acid incorporation by toxin.

Heat-Stable, Dialyzable Factor in HeLa Supernates.—Since HeLa supernates were not dialyzed before use, they contained numerous amino acids, cofactors, and other low molecular weight cell components. Reticulocyte fractions, on

[4] Levels of incorporation of amino acids by reticulocyte ribosomes were consistently higher in the presence of HeLa supernate than in the presence of reticulocyte enzymes and sRNA. The reason for this difference is not known.

the other hand, had been freed from all dialyzable components during fractionation. It was found that after only 6 hours' dialysis in the cold, crude HeLa extracts lost most of their sensitivity to toxin although their capacity to promote amino acid incorporation remained unimpaired. Next, a freshly prepared HeLa supernate was passed through a G-25 sephadex column which had been equilibrated with 30 mM tris buffer, pH 7.4, containing 60 mM KCl and 5 mM $MgCl_2$. The protein-RNA fraction emerging from this column was almost completely free from low molecular weight components. This fraction was mixed with washed ribosomes from reticulocytes and the effect of toxin on C^{14}-leucine uptake in the mixture was tested. Table VIII shows that the in-

TABLE VIII

Effect of Gel Filtration on Sensitivity of HeLa Supernate to Toxin

Supernatant fraction*	C^{14}-leucine incorporated		Inhibition
	Control	Toxin (6 Lf)	
	CPM/*sample*	CPM/*sample*	*per cent*
HeLa supernate (untreated)	1091	185	83
HeLa supernate filtered through sephadex G-25	1263	1105	12
HeLa supernate filtered through sephadex G-25 + boiled HeLa extract‡	1270	323	75
Reticulocyte AS-70 and sRNA	670	699	0
Reticulocyte AS-70 and sRNA + boiled HeLa extract	1080	249	77

* Each tube contained reticulocyte ribosomes plus the supernatant fraction indicated. Other conditions were the same as in Table VII.

‡ Prepared by heating HeLa supernate to 100°C for 10 minutes and removing the coagulated protein.

corporation of leucine was only slightly inhibited by 6 Lf toxin in the presence of the sephadex-treated HeLa supernate. Moreover, it was found that sensitivity to toxin could be restored, both to this system and to the complete reticulocyte system, by adding a small amount (0.2 ml) of a boiled HeLa extract prepared by heating HeLa supernate to 100°C for 10 minutes and then removing the coagulated protein. These findings, summarized in Table VIII, clearly demonstrate that HeLa extracts contain a heat-stable, dialyzable factor that is required for the action of toxin.

Identification of the Cofactor Required for Inhibition of Amino Acid Incorporation by Toxin.—Before making any attempt to fractionate boiled extracts from HeLa cells, a number of known substances were tested for their capacity to confer sensitivity to toxin on the reticulocyte system. The various cofactors tested, both singly and in mixtures, included flavin adenine dinucleotide, pyridoxal phosphate, the oxidized and reduced forms of nicotinamide adenine

dinucleotide (NAD and NADH), yeast concentrate (Sigma Chemical Company), and Eagle's vitamin mixture. They were added together with toxin to the complete reticulocyte cell-free system and the incorporation of C^{14}-leucine into protein was then measured. Toxin inhibited leucine uptake only in those reaction mixtures to which NAD, NADH, or yeast concentrate had been added. The results clearly suggested, therefore, that NAD might be the cofactor present in crude HeLa cell extracts that is necessary for the action of toxin.

Table IX shows the effect of adding increasing amounts of NAD and NADP on the incorporation of C^{14}-leucine in the reticulocyte system in the presence of 6 Lf of toxin. Significant inhibition of amino acid uptake by toxin occurred in the presence of as little as 0.01 μg NAD per ml, and reached 75 to 80 per cent

TABLE IX

Sensitivity of the Reticulocyte Cell-Free System to Toxin in the Presence of Varying Amounts of NAD and NADP

Amount of cofactor	C^{14}-leucine incorporated in the presence of 6 Lf toxin plus:	
	NAD	NADP
μg/tube	*cpm/sample*	*cpm/sample*
0	549*	—
0	543	543
0.1	489	539
0.5	371	534
2.5	213	489
50	142	333
50	539*	—

* Toxin omitted from these tubes.

in the presence of 50 μg per ml. Parallel titrations revealed that NADP is only 1 to 2 per cent as effective as NAD in conferring sensitivity to toxin on the reticulocyte system. Moreover, it is not unlikely that the requirement for NAD is even more specific than the figures indicate, since we have not been able to exclude the possibility that the NADP preparation used contained traces of NAD or alternatively, that NADP may have been slowly converted to NAD by enzymes present in the cell extracts.

Table X summarizes the effects of increasing concentrations of toxin and of purified diphtheria toxoid on leucine incorporation in the reticulocyte system containing a constant amount (50 μg per ml) of added NAD. Significant inhibition of leucine uptake was caused by only a fraction of a microgram of toxin whereas high concentrations of toxoid showed no appreciable inhibition.

The possibility that some trace contaminant present in the NAD preparation might have been responsible for conferring sensitivity to toxin upon the in-

corporating system was ruled out by the following experiment. NAD (2.5 mg) was applied to a column of dowex-1 anion exchange resin and then eluted with 0.05 M ammonium bicarbonate. Fractions of 40 drops each were collected and each fraction was tested for its ability to render reticulocyte extracts sensitive to toxin and for its absorption at 260 and 280 mμ. The profile of sensitivity to toxin was found to correlate closely with the NAD content of the fractions as measured spectrophotometrically.

Finally, we have used a highly purified preparation[5] of streptococcal NADase to demonstrate that specific degradation of NAD in crude extracts from HeLa cells, results in almost complete loss in sensitivity to toxin. Table XI shows

TABLE X

Effect of Purified Diphtheria Toxin and Toxoid on Incorporation of C^{14}-Leucine in the Complete System from Rabbit Reticulocytes

Toxin or toxoid added	NAD, 50 μg	C^{14}-leucine incorporation in the presence of			
		Toxin	Inhibition	Toxoid	Inhibition
Lf		CPM	*per cent*	CPM	*per cent*
0	Not added	1772	—	1772	—
0	+	1901	—	1901	—
16	Not added	2052	—	1725	—
16	+	505	74	1489	14
8	+	589	69	1651	4
2	+	766	60	1654	4
1	+	1208	37	1660	4
0.5	+	1313	31	1617	6
0.25	+	1509	21	1823	0
0.125	+	1579	17	—	—

that after treatment of HeLa extracts with 900 units NADase per ml at 0°C for 1 hour, leucine incorporation was inhibited only 5 per cent in the presence of 12 Lf toxin per ml. Moreover, sensitivity to toxin could be partially restored in NADase-treated extracts by adding excess NAD to reaction mixtures at the time of initiation of the amino acid incorporation reaction. Other experiments have shown that treatment with NADase destroys the capacity of boiled HeLa extract and of NAD itself to render the reticulocyte system sensitive to toxin.

Effect of Diphtheria Toxin on the Formation of Aminoacyl-sRNA.—The first steps in the biosynthesis of protein involve the formation of aminoacyl derivatives of sRNA. The reactions require ATP, and the sRNA's and activating enzymes specific for each amino acid (16). As a first step towards localizing the

[5] We are indebted to Professor Alan W. Bernheimer for a generous gift of purified streptococcal NADase.

site of action of toxin, we have tested its effect on the formation of aminoacyl-sRNA. The usual reaction mixtures containing amino acids, ATP, and ATP-generating system were prepared with and without toxin; to each was added AS-70 as a source of activating enzymes, reticulocyte sRNA, 50 μg NAD per ml and C^{14}-algal protein hydrolysate, but no ribosomes or GTP. A low concentration of the AS-70 fraction was used and the reaction was carried out at 15°C in order to slow the reaction to a rate that would permit the kinetics of amino acid uptake into aminoacyl-sRNA to be followed. Samples were removed at intervals and the RNA and protein were precipitated with ice cold 5 per cent TCA. The precipitates were washed 5 times with ice cold TCA, collected on Millipore filters, and counted. Curve *A* in Fig. 1 shows that the uptake of C^{14}-amino acids into aminoacyl-sRNA remained linear for the 1st hour and

TABLE XI

Effect of NADase on the Toxin Sensitivity of HeLa Crude Extracts

HeLa extract	C^{14}-leucine incorporated into protein		Inhibition
	Control	Toxin (12 Lf)	
	cpm/sample	*cpm/sample*	*per cent*
Untreated	767	50	93
Pretreated with NADase*	761	720	5
Pretreated with NADase; 250 μg NAD added at time of initiation of incorporation reaction	802	320	60

* These extracts were pretreated with 900 units/ml NADase for 1 hour at 0°C. before addition to amino acid incorporation mixtures.

reached a plateau after about 1.5 hours. Addition of 6 Lf toxin per ml failed to alter significantly, either the kinetics or the extent of amino acid uptake into RNA. No uptake of amino acids was observed in tubes from which ATP had been omitted and the radioactivity of the cold TCA precipitates was reduced to the background count after heating at 90°C in 5 per cent TCA for 15 minutes. Curves *B* and *C* in Fig. 1 show that toxin inhibited the incorporation of amino acids into protein, as usual, in tubes to which ribosomes and GTP were added. These results suggest that there is no effect of toxin either on the activation of amino acids or on transfer of amino acids to sRNA.

Due to limitations in the sensitivity of the assay method used, the results of the preceding experiment do not rigorously exclude the possibility that toxin may block the formation of aminoacyl-sRNA from some *particular* amino acid. To rule out this possibility, kinetic studies on the uptake of C^{14}-phenylalanine into sRNA were carried out by a method similar to that described above. Again, toxin caused no detectable alteration in either the kinetics or extent of forma-

tion of phenylalanyl-sRNA. Since toxin has already been shown to inhibit the poly U-directed synthesis of polyphenylalanine, its failure to inhibit phenylalanyl-sRNA formation, provides strong evidence that toxin does not act by inhibiting the formation of any single amino acid.

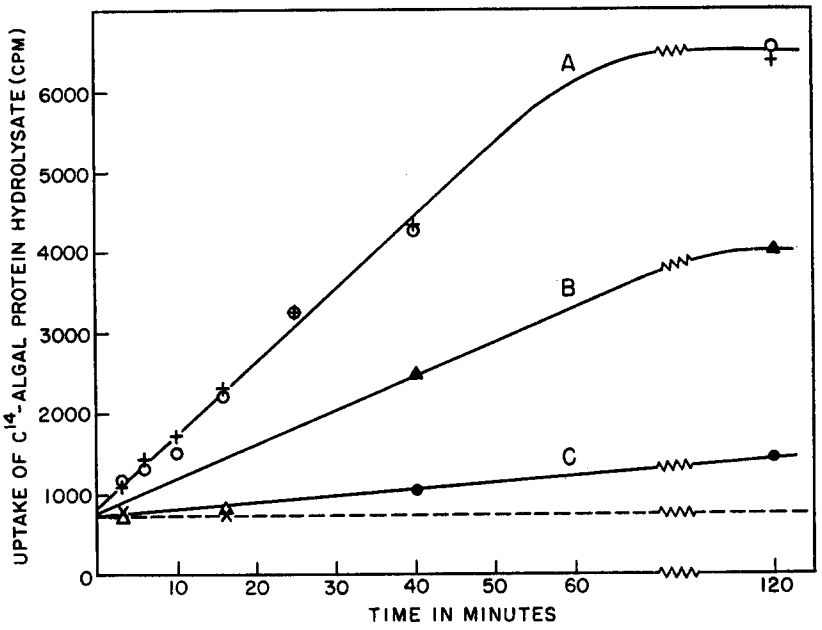

FIG. 1. Kinetics of aminoacyl-sRNA formation in the presence and absence of toxin: Each tube contained 0.6 μc C^{14}-algal protein hydrolysate.

Curve *A*: Incorporation of C^{14} into sRNA (cold TCA precipitate). ○, control; +, toxin (6 Lf/ml). No ribosomes or GTP added.

Curves *B* and *C*: Incorporation of C^{14} into polypeptides (hot TCA precipitate). ▲, control; ●, toxin (6 Lf/ml). Ribosomes and GTP added.

----------, Same as curve A but extracted with *hot* TCA.

Effect of Toxin on Transfer of C^{14}-Amino Acids from sRNA to the Growing Polypeptide Chain.—Since toxin had no effect on the formation of aminoacyl-sRNA, it seemed likely that it might inhibit one of the steps in protein synthesis involving transfer of amino acids from sRNA to protein. This hypothesis has been confirmed by studies on polypeptide formation in which purified sRNA, charged with C^{14}-amino acids, was used. Soluble RNA was prepared from rabbit liver as described under Materials and Methods and was charged with either C^{14}-phenylalanine or with C^{14}-algal protein hydrolysate using the AS-70 fraction from reticulocytes as a source of amino acid activating enzymes. After purification by phenol extraction and ethanol precipitation, the labeled

aminoacyl-sRNA was added to the usual reaction mixture (but containing no amino acids) together with either a crude HeLa cell extract or the AS-70 fraction plus reticulocyte ribosomes. The transfer of C^{14}-labeled amino acids from sRNA to protein (hot TCA-precipitable material) was then measured. As shown in Table XII, 6 Lf per ml of toxin inhibited the reaction by 60 to 75 per cent, provided NAD was present. Identical results were obtained using sRNA charged with either C^{14}-phenylalanine or C^{14}-algal protein hydrolysate. Although the inhibition of the transfer reaction is somewhat less than that observed using toxin to inhibit incorporation of free amino acids into protein,

TABLE XII

Effect of Toxin on the Transfer of C^{14}-Amino Acids from Rabbit Liver sRNA to Protein

	Extract	Additions	C^{14}-amino acids incorporated into protein		Inhibition
			Control	Toxin (6 Lf)	
			cpm/*sample*	cpm/*sample*	*per cent*
C^{14}-algal protein hydrolysate sRNA	HeLa	None	247	68	72
	Reticulocyte	None	204	207	None
	Reticulocyte	50 μg NAD	211	72	65
	Reticulocyte	50 μg NAD + 1 μg each of 18 amino acids	108	33	69
C^{14}-phenylalanyl-sRNA	Reticulocyte	None	166	174	None
	Reticulocyte	50 μg NAD	146	53	64

Reaction mixtures contained the usual components except that amino acids were omitted. 20 to 30 per cent of the labeled amino acids attached to sRNA were transferred to hot TCA-precipitable material in control tubes without toxin.

the results provide convincing evidence that it is the transfer step in protein biosynthesis that is affected by diphtheria toxin.

DISCUSSION

In this paper we have clearly demonstrated that the capacity to incorporate amino acids into polypeptides, *in vitro*, is markedly reduced in crude extracts from HeLa cells previously incubated for 3.5 hours with a saturating dose of purified diphtheria toxin before extraction. Moreover, addition of small amounts of toxin to extracts from normal HeLa cells has been found to cause a rapid and almost complete inhibition of cell-free incorporation of amino acids into TCA-precipitable polypeptides. Since the inhibition occurs in the presence of adequate supplies of ATP, GTP, and amino acids, it seems reasonable to conclude that the action of the toxin is directed specifically against some component

required for protein synthesis. We believe that an understanding of the exact mechanism by which toxin inhibits amino acid uptake in the *in vitro* system will elucidate the mechanism by which toxin brings about its striking inhibition of protein synthesis in living cells.

All of the evidence accumulated thus far supports the conclusion that the inhibition of amino acid incorporation is, in fact, attributable to the toxin itself, and not to some impurity present in the preparation. Three times recrystallized toxin is a potent inhibitor, whereas even relatively high concentrations of purified toxoid are inactive. Of primary importance, however, is the immunological evidence which has shown that, after precipitation of the toxin with a highly specific horse antitoxin, the supernate shows no capacity whatsoever to inhibit cell-free amino acid incorporation. Because of the high specificity of the antitoxin used and the fact that with flocculating antibody of this type precipitation of any antigen occurs only within a narrow zone near equivalence, it is unlikely that impurities would coprecipitate with the toxin. Indeed, one such impurity, DNase, has been found to be quantitatively recoverable from the supernate after precipitation of the toxin. Still further evidence for specificity is the finding that washed toxin-antitoxin floccules retain their toxicity in cell-free systems to a considerable degree. Since the toxin-antitoxin complex is completely non-toxic when injected into susceptible animals, it seems probable that the ability of antitoxin to neutralize an equivalent amount of toxin *in vivo* depends on preventing it from reaching the target area within the cell.

Since all protein-synthesizing systems are dependent on the presence of RNA, they are highly sensitive to inactivation by ribonuclease. The fact that a cell-free amino acid–incorporating system from *E. coli* was insensitive to toxin although it was some 50 times more readily inactivated by pancreatic RNase than the HeLa cell system, suggests that the toxin preparation contained little or no ribonuclease activity. Crude extracts from HeLa cells were found to contain traces of a ribonuclease capable of degrading polyuridylic acid. Not only did our toxin preparation fail to attack poly U, but addition of toxin to the soluble enzyme-containing fraction from HeLa cells failed to increase its ribonuclease activity against the poly U substrate. It thus seems most improbable that the inhibition of protein synthesis by toxin can be explained in terms of activation of an intracellular ribonuclease.

The evidence that toxin requires NAD for its action on cell-free amino acid incorporation seems quite conclusive. After degradation of the NAD in HeLa extracts by treatment with highly specific purified streptococcal NADase or when NAD was removed by gel filtration through sephadex, inhibition by toxin of amino acid incorporation in the NAD-free extracts was negligible. Sensitivity could be fully restored in such extracts or in a cell-free system from rabbit reticulocytes by adding NAD to levels of about 50 μg per ml. The slight ac-

tivity of NADP in restoring sensitivity to NAD-free extracts may well have been due to trace contamination with NAD or to conversion of NADP to NAD by enzymes present in the cell extracts. The levels of NAD required for maximal sensitivity to toxin are well within the range of concentrations that have been determined in mammalian tissues. Most tissues, in fact, have been found to contain more than 100 μg NAD per gm (17).

In the presence of excess NAD, significant inhibition of amino acid uptake into polypeptides, both in the HeLa and in the rabbit reticulocyte systems, can be demonstrated when the concentration of toxin added is less than 10^{-8} M. It may be calculated from the data of Lennox and Kaplan (18) and of Gabliks and Solotorovsky (19) that concentrations of diphtheria toxin as low as 10^{-13} M (200 to 400 molecules per cell) are lethal for cultures of certain highly susceptible mammalian cell lines within a period of 3 to 4 days. However, if all the toxin in their experiments was actually taken up by the cells during this period, the intracellular concentration of toxin might have reached about 10^{-10} M which begins to approach in order to magnitude, the concentrations that we have found to be effective *in vitro*.

Kato (20) and Kato and Sato (21) have recently reported that diphtheria toxin inhibits amino acid incorporation into protein in cell-free preparations from guinea pig liver. However, their system required about 0.5 mg toxin per ml for 50 per cent inhibition and maximum inhibition of 85 per cent could only be achieved by adding 1.5 mg toxin per ml. These concentrations are about 1000 times greater than those used in the present experiments. It is quite possible that the relative insensitivity of the system from guinea pig liver can be explained by the fact that the methods used for preparing the ribosomal and soluble enzyme fractions would have removed most of the NAD present in the original extracts. However, at such high concentrations it would be difficult to rule out the possibility that traces of ribonuclease or some other substance present as impurity might be responsible for the inhibition of protein synthesis.

Some progress has been made towards locating the site that is affected by the toxin. There is clear-cut evidence that the toxin does not inhibit the early steps in protein synthesis which involve the formation of aminoacyl-sRNA. In the presence of NAD, toxin had no effect on either the rate or the extent of phenylalanyl-sRNA formation from C^{14}-phenylalanine or on mixed aminoacyl-sRNA formation from C^{14}–algal protein hydrolysate. On the other hand, the transfer of C^{14}-phenylalanine or of a mixture of C^{14}–amino acids from sRNA to the growing peptide chain was inhibited 65 to 70 per cent in systems containing NAD, reticulocyte enzymes, and reticulocyte ribosomes.

At the present time there is still insufficient evidence on which to propose a reasonable mechanism explaining how toxin blocks the transfer of amino acids from sRNA to the peptide chain. Nor can we provide a clue to explain why NAD is specifically required for this inhibition. It is possible that toxin, acti-

vated by NAD, is an enzyme that inactivates some essential component required for amino acid transfer. Alternatively, toxin may activate a latent NAD-requiring enzyme which interferes with the transfer reaction. It is hoped that experiments now in progress will throw further light on this problem.

SUMMARY

It has been demonstrated that low concentrations of highly purified diphtheria toxin specifically inhibit incorporation of labeled amino acids into polypeptides in extracts from HeLa cells and from rabbit reticulocytes. No inhibition of incorporation occurs in the absence of a specific cofactor. This cofactor has been identified as nicotinamide adenine dinucleotide (NAD). It has been shown that it is one of the steps involving transfer of amino acids from soluble ribonucleic acid to the growing polypeptide chain that is affected by the toxin in the presence of NAD.

BIBLIOGRAPHY

1. Collier, R. J., and Pappenheimer, A. M., Jr., Studies on the mode of action of diphtheria toxin. I. Phosphorylated intermediates in normal and intoxicated HeLa cells, *J. Exp. Med.*, 1964, **120,** 1007.
2. Strauss, N., and Hendee, E. D., The effect of diphtheria toxin on the metabolism of HeLa cells, *J. Exp. Med.*, 1959, **109,** 145.
3. Kato, I., and Pappenheimer, A. M., Jr., An early effect of diphtheria toxin on the metabolism of mammalian cells growing in culture, *J. Exp. Med.*, 1960, **112,** 329.
4. Strauss, N., The effect of diphtheria toxin on the metabolism of HeLa cells. II. Effect on nucleic acid metabolism, *J. Exp. Med.*, 1960, **112,** 351.
5. Eagle, H., Amino acid metabolism in mammalian cell cultures, *Science*, 1959, **130,** 432.
6. Allen, E. H., and Schweet, R. S., Synthesis of hemoglobin in a cell-free system. I. Properties of the complete system, *J. Biol. Chem.*, 1962, **237,** 760.
7. Tissieres, A., and Hopkins, J. W., Factors affecting amino acid incorporation into proteins by *Escherichia coli* ribosomes, *Proc. Nat. Acad. Sc.*, 1961, **47,** 2015.
8. Boorsook, H., Fischer, E. H., and Keighley, G., Factors affecting protein synthesis *in vitro* in rabbit reticulocytes, *J. Biol. Chem.*, 1957, **229,** 1059.
9. Slater, E. C., Spectrophotometric determination of fructose-1:6-diphosphate, hexosemonophosphates, adenosinetriphosphate, and adenosinediphosphate, *Biochem. J.*, 1952, **53,** 157.
10. Spahr, P. F., and Schlessinger, D., Breakdown of messenger ribonucleic acid by a potassium-activated phosphodiesterase from *Escherichia coli*, *J. Biol. Chem.*, 1963, **238,** PC2251.
11. Attardi, G., and Smith, J., Virus specific protein and a ribonucleic acid associated with ribosomes in poliovirus infected HeLa cells, *Cold Spring Harbor Symp. Quant. Biol.*, 1962, **27,** 271.
12. Messinova, O. V., Yusupova, D. V., and Shamusutdinov, N. S., DNase activity

of Corynebacteria and its connection with toxigenicity, *Zh. Mikrobiol. Epidemiol. i Immunobiol.*, 1963, **40**, 20.

13. Pope, C. G., Observations on the diphtheria toxin-antitoxin reaction, *Brit. J. Exp. Path.*, 1957, **38**, 207.
14. Nirenberg, M. W., and Matthaei, J. H., The dependence of cell-free protein synthesis in E. coli upon naturally occurring or synthetic polyribonucleotides, *Proc. Nat. Acad. Sc.*, 1961, **47**, 1588.
15. Dintzis, H. M., Assembly of the peptide chains of hemoglobin, *Proc. Nat. Acad. Sc.*, 1961, **47**, 247.
16. Hoagland, M. G., Stephenson, M. L., Scott, J. F., Hecht, L. I., and Zamecnik, P. C., A soluble ribonucleic acid intermeidate in protein synthesis, *J. Biol. Chem.*, 1958, **231**, 241.
17. Glock, G. E., and McLean, P., Levels of oxidized and reduced diphosphopyridine nucleotide and triphosphopyridine nucleotide in animal tissues, *Biochem. J.*, 1955, **61**, 388.
18. Lennox, E. S., and Kaplan, A. S., Action of diphtheria toxin on cells cultivated *in vitro*, *Proc. Soc. Exp. Biol. and Med.*, 1957, **95**, 700.
19. Gabliks, J., and Solotorovsky, M., Cell culture reactivity to diphtheria, staphylococcus, tetanus, and *Escherichia coli* toxins, *J. Immunol.*, 1962, **88**, 505.
20. Kato, I., Mode of action of diphtheria toxin on protein synthesis. I. Effect of diphtheria toxin on C14-amino acid incorporation into microsomes and mitochondria *in vitro*, *Japan. J. Exp. Med.*, 1962, **32**, 335.
21. Kato, I., and Sato, H., Mode of action of diphtheria toxin on protein synthesis. II. Effect of diphtheria toxin on biosynthesis of serum albumin in cell-free systems, *Japan. J. Exp. Med.*, 1962, **32**, 495.