STUDIES ON THE MODE OF ACTION OF DIPHTHERIA TOXIN*

III. Effect on Subcellular Components of Protein Synthesis from the Tissues of Intoxicated Guinea Pigs and Rats

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Diphtheria toxin inhibits protein synthesis by acting enzymatically on nicotinamide-adenine dinucleotide (NAD)[1] in the presence of transferase II to yield free nicotinamide and an enzymatically inactive adenosine diphosphoribose–transferase II (ADPribose-TF II) derivative (1, 2). This reaction has been demonstrated both in intact tissue-culture cells (HeLa) and in cell-free protein synthesizing systems derived from mammalian cells in culture (2). Bonventre and Imhoff (3) reported that the diphtheria-sensitive guinea pig given small doses of toxin incorporates significantly less leucine-^{3}H into muscle proteins, especially heart and skeletal muscle, than does the normal guinea pig. Inhibition of protein synthesis in tissues of the guinea pig other than the heart was not significant. Furthermore, diphtheria-resistant mice did not show an impaired ability to incorporate the radiolabeled amino acid into heart and muscle protein when given as much as 100 guinea pig minimum lethal doses (MLD) of the toxin. Species resistance or susceptibility to diphtheria toxin was also demonstrated with primary heart cell cultures derived from newborn rats and guinea pig embryos (4). Guinea pig heart cell cultures were inhibited in protein synthesis and ultimately destroyed by the toxin while the heart cell cultures derived from the rat were shown to be refractory to the action of toxin.

The experiments reported here were designed to examine the mode of action of diphtheria toxin in the intact animal by testing for the formation of inactive transferase enzymes in vivo during intoxication of guinea pigs; and to ascertain

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[1] *Abbreviations used in this article:* ADPribose-TF II, adenosine diphosphoribose–transferase II; ATP, adenosine triphosphate; DOC, sodium deoxycholate; GTP, guanosine triphosphate; HeLa and HEp-2, established tissue culture cell lines; MLD, minimum lethal dose; NAD, nicotinamide-adenine dinucleotide; PPO, 2,5-diphenyloxazole; TCA, trichloroacetic acid; tRNA, transfer ribonucleic acid.

if this inactivation takes place in specific target tissues or organs. The results suggest that the soluble components (transferases) derived from the heart and skeletal muscle of diphtheria toxin-treated guinea pigs are less active in protein synthesis in vitro than are similar components obtained from normal animals. In addition, these two tissues seem to be specifically affected when small levels of toxin are injected while the effect appears to be more generalized when overwhelming amounts of toxin are administered. The data will be discussed in terms of a tissue-specific action of diphtheria toxin in the sensitive guinea pig and a significantly different effect of the toxin in resistant animal species.

Materials and Methods

Diphtheria Toxin and Antitoxin.—Crystalline diphtheria toxin was obtained through the courtesy of Dr. M. F. Stevens and Dr. C. G. Pope (Wellcome Research Laboratories, Beckenham, England). The reconstituted toxin contained 324 L_f units per mg; 1.0 L_f unit was equivalent to approximately 60 guinea pig MLD. The crystalline toxin was used in all of the experiments. Diphtheria antitoxin (500 unts/ml) was obtained from Wyeth Laboratories, Marietta, Pa.

Animals.—Hartley strain albino guinea pigs weighing between 250 and 350 grams were used in all experiments. These were purchased from the Paul Hamm Rabbitry, Greenwood, Ind. Sprague-Dawley–derived rats of approximately 200 grams were supplied by the Laboratory Supply Co., Indianapolis, Ind.

Materials.—Creatine phosphate, guanosine triphosphate (GTP), adenosine triphosphate (ATP), and creatine-phosphokinase were obtained from Calbiochem, Los Angeles, Calif. Lubrol WX was supplied by I.C.I. Organics, Inc., Stamford, Conn. Sodium deoxycholate (DOC) was purchased from Mann Research Laboratories, Inc., New York. L-leucine-^{14}C (uniformly labeled; specific activity 278 mCi/mM) was obtained from New England Nuclear Corp., Boston, Mass.

Polyribosome Preparation.—Polyribosomes were prepared by the method of Chen and Young (5). Animals were exsanguinated by decapitation, the livers excised, immersed in cold Medium A (0.25 M KCl, 0.01 M $MgCl_2$, 0.01 M Tris buffer, pH 7.6), and homogenized in two volumes of Medium A with a Dounce homogenizer. The homogenate was centrifuged 10 min at 27,000 *g* to sediment cell debris. The supernatant was filtered through nylon cloth and the filtrate treated with a mixture of Lubrol WX dissolved in Medium A and an aqueous solution of sodium deoxycholate. The final concentrations were 0.5% (w/v) for the Lubrol WX and 1% (w/v) for the sodium deoxycholate. Three volumes of buffered Medium B (0.01 M $MgCl_2$, 0.01 M Tris buffer, pH 7.6) were added to one volume of the detergent-treated postmitochondrial supernatant to precipitate polyribosomes from single ribosomes. This was followed by a 10-min centrifugation at 27,000 *g*. The pellet was resuspended in Medium A and centrifuged for 90 min at 100,000 *g*. The pellet was again suspended in Medium A and the suspension used as the polyribosome source for amino acid incorporation experiments.

Preparation of Soluble Enzyme Fractions from Guinea Pig Tissues.—The partially purified pH-5.0 fractions were prepared from liver tissue according to the method of Keller and Zamecnik (6). Tissue was homogenized in two volumes of Medium C (0.35 M sucrose, 0.035 M $KHCO_3$, 0.004 M $MgCl_2$, and 0.025 M KCl; pH 7.5). After spinning out cellular debris, the supernatant was diluted with two volumes of Medium D (0.9 M sucrose, 0.025 M KCl, 0.004 M $MgCl_2$, and 0.006 M 2-mercaptoethanol). After sedimenting the polysomes for 2 hr at 100,000 *g*, the supernatant was diluted with an equal volume of Medium D and adjusted

to pH 5.2, centrifuged for 15 min at 34,000 *g*, and the pellets resuspended in Medium C and frozen. This fraction which contained partially purified transferase enzymes will be referred to as the pH-5.0 fraction (7).

The 100,000-*g* supernatant fractions were prepared according to the method of Moehring and Moehring (8). Animals were perfused with a mixture of warm Ringer's solution and 3 % sucrose to remove as much blood as possible from the viscera. The tissues were then excised rapidly and placed on ice, weighed, and homogenized in 4.0 ml of buffer (0.01 M KCl, 0.0015 M Mg acetate, 0.006 M 2-mercaptoethanol, and 0.01 M Tris buffer, pH 7.4). The cell debris was removed by centrifugation and the supernatant was then centrifuged at 100,000 *g* for 2 hr to pellet the polysomes. The supernatant fraction was used as the source of soluble enzymes in the in vitro assays for amino acid incorporation into proteins. It contains transfer ribonucleic acid (tRNA), amino acyl tRNA synthetases, and enzymes involved in elongation and termination of peptide chains. The latter enzymes include the translocase usually referred to as transferase II (9). This fraction will be designated as 100,000-*g* supernatant fraction or soluble enzyme fraction.

Cell-free Amino Acid Incorporation.—Protein synthesis was followed by measuring the amount of leucine-^{14}C incorporation into trichloroacetic acid (TCA)-insoluble polypeptides. The reaction mixture consisted of 5 μmoles ATP, 1 μmole GTP, 15 μmoles creatine phosphate, 20 μg creatine phosphokinase, 1.0 mg pH 5.0 enzyme fraction or 100,000-*g* supernatant fraction, 10 mμmoles of a complete amino acid mixture, 1 μc of uniformly labeled L-leucine-^{14}C, and polyribosomes (0.2–0.3 mg). When indicated, NAD was present at a concentration of 2×10^{-5} M. The mixture was incubated for 45 min at 37°C and the reaction then stopped by adding 5 % cold TCA. The tubes were incubated consecutively at 4°C and 95°C for 30 min at each temperature. The precipitate was washed twice with cold 5 % TCA, once with absolute alcohol, and dissolved in a mixture of 1 ml Hydroxide of Hyamine (Packard Instrument Co., Inc., Downers Grove, Ill.) and 10 ml of 0.6% 2,5-diphenyloxazole (PPO) in toluene. Counting was done in a Model 3003 Tri-Carb Liquid Scintillator Spectrometer (Packard Instrument Co., Inc.). Protein determinations were made by the method of Lowry et al. (10) as modified by Oyama and Eagle (11).

General Experimental Procedures.—Animals received diphtheria toxin intramuscularly and were killed after suitable time periods depending on the dosage of toxin injected. In all cases the animals were visibly ill but had not reached a moribund state. The 100,000-*g* supernatant fractions were prepared from tissues of the intoxicated animals and from those of healthy control animals as described above. These fractions were assayed in vitro for their ability to incorporate radiolabeled leucine into hot TCA-insoluble polypeptides. Such a procedure allowed a comparison of protein-synthesizing activity of soluble fractions from the tissues of normal and intoxicated animals.

RESULTS

Direct Effect of Diphtheria Toxin on Extracts from Sensitive and Resistant Animals.—Soluble enzymes prepared from heart muscle, skeletal muscle, liver, and brain of the diphtheria-sensitive guinea pig or the resistant rat were equally sensitive to the direct addition of diphtheria toxin (Table I). Toxin added at a concentration of 0.06 L_f/ml to a complete reaction mixture in vitro caused minimal inhibition of leucine-^{14}C–incorporation into proteins. Marked inhibition was obtained with all cell extracts except rat brain at a toxin level above 0.5 L_f/ml. These observations are an extension of those of Johnson et al. (12), who showed that diphtheria toxin inhibited protein synthesis in vitro when

added to liver extracts from both rat and guinea pig. Thus, the refractoriness seen in the intact animal cannot be attributed to a difference in the components of protein synthesis per se, but possibly to anatomic and/or permeability barriers.

Effects of Diphtheria Intoxication on the Protein-Synthesizing Components of Guinea Pig Tissues.—Bonventre and Imhoff (3) observed that in vivo protein synthesis, as measured by quantitative radioautography and incorporation of leucine-^{3}H into protein, was inhibited only in specific and relatively few tissues of guinea pigs given low levels (20 MLD) of diphtheria toxin. In order to ascer-

TABLE I

Effect on Polypeptide Synthesis In Vitro by the Direct Addition of Diphtheria Toxin to Tissue Extracts of the Guinea Pig and Rat

Toxin added (L_f/ml)	Guinea pig				Rat			
	Skeletal muscle	Brain	Heart	Liver	Skeletal muscle	Brain	Heart	Liver
0	0*	0	0	0	0	0	0	0
0.02	0	2	—	7	5	0	3	0
0.06	17	10	38	16	14	2	15	10
0.1	22	15	57	23	38	0	28	0
0.5	68	54	57	50	63	14	50	19
1.0	71	63	60	58	67	29	68	37
3.0	87	73	73	60	68	41	68	63
5.0	88	70	73	59	71	55	76	72

The 100,000-*g* supernatant fractions prepared from tissues of animal species listed. Polysomes were prepared from livers of homologous species. NAD (2×10^{-5} M) was present in all assay tubes. Toxin added immediately before beginning assays. Complete reaction mixture as described in Materials and Methods.

* Expressed as the per cent of reduction in the leucine-^{14}C–incorporating ability of the tissue extracts.

tain if selective inhibition of protein synthesis in vivo could be detected by more refined biochemical methods, experiments were designed to answer two questions: (*a*) does diphtheria toxin inhibit protein synthesis in the intact animal by acting upon soluble components (transferases) rather than particulate fractions (ribosomes) as it does in tissue culture systems; and (*b*) if the soluble enzymes are inhibited, can one detect inactive transferase enzymes in the tissue extracts of diphtheria intoxicated guinea pigs? The results of these experiments hopefully would not only tell us how the toxin acts in vivo but would also suggest target organs and tissues.

Effect on polyribosomes: Polyribosomes were prepared from the liver of guinea pigs given 1000 MLD toxin. When used in cell-free amino acid incorporation studies in combination with soluble fractions from normal animals,

polyribosomes from intoxicated guinea pigs showed no inhibition of activity. Preparations from normal or toxin-treated guinea pigs were equally active in directing the incorporation of amino acids into proteins. This is in agreement with results obtained with cell-free systems derived from KB cells and from rabbit reticulocytes (8, 13), and indicated that in animals diphtheria toxin acts on a soluble component of the cells and has no effect on the particulate fraction.

Effect on soluble enzymes: Since the ability of polyribosomes to direct protein synthesis was not impaired by diphtheritic intoxication, the protein-synthesiz-

TABLE II

*Comparison of Leucine-^{14}C Incorporation into Protein by Soluble Enzymes from Tissues of Normal and Diphtheria Toxin-Treated Guinea Pigs**

Source of 100,000-*g* supernatant	Inhibition of protein synthesis‡							
	Dose of toxin administered, MLD							
	1500	1250	300	200	150	25	10	5
	%	%	%	%	%	%	%	%
Heart	57	65	24	68	58	43	64	29
Skeletal muscle§	—	55	58	57	—	41	80	25
Liver	40	29	35	0	0	3	8	0
Lung	48	58	33	30	56	43	0	26
Spleen	81	19	64	5	0	24	0	4
Kidney	22	46	25	44	69	0	0	15
Brain	0	0	5	9	13	6	7	0

* Composite data from a total of 36 guinea pigs. All assays were conducted in duplicate. The components of the reaction mixture for the assay of protein synthesis in vitro were as described in Materials and Methods. Polyribosomes were prepared from livers of normal guinea pigs.

‡ Data is expressed as the per cent of reduction in the capacity of the 100,000-*g* supernatant fractions from tissues of intoxicated guinea pigs to incorporate leucine-^{14}C into protein as compared with those prepared from tissues of normal animals.

§ Muscles of upper hind leg.

ing activity of soluble fractions prepared from the tissues of guinea pigs treated with varying doses of diphtheria toxin was examined. As seen in Table II, a decrease in the ability to incorporate leucine-^{14}C into proteins was apparent when the activity of soluble enzymes prepared from the tissues of toxin-treated guinea pigs was compared with the activity of the same fraction from healthy control animals. The polyribosomal fractions in all cases were derived from livers of untreated guinea pigs and thus remained a constant parameter in each experiment.

Activity of extracts from heart and skeletal muscle was consistently inhibited at all levels of toxin used. The activity of the 100,000-*g* supernatant fractions from liver, lung, spleen, and kidney tissues was impaired when high doses of toxin were administered, but in the cases where 25 MLD or less of toxin was

given, a variable response was observed. Diphtheria intoxication did not impair the activity of soluble enzymes prepared from brain tissues. This suggests that in the intact animal, diphtheria toxin has a specificity for heart and skeletal muscle and at physiological levels of toxin does not act indiscriminately on all tissues as might be expected in view of the fact that protein synthesis is mediated by transferases in all mammalian cells.

Elimination of a Role for Nonspecific Inhibitors of Protein Synthesis.—Before the inhibition described above could be attributed solely to in vivo inactivation of soluble enzymes by diphtheria toxin, it was necessary to eliminate possible interference in the in vitro assay of protein synthesis by nonspecific factors. Three possibilities were considered: (*a*) competition in the incorporation of leucine-^{14}C by cold amino acids present in cell extracts; (*b*) presence of residual-free toxin in 100,000-*g* supernatant fractions which would decrease the amount of polypeptide synthesis measured in vitro; and (*c*) generalized inhibition due to a heat-stable serum factor. In order to eliminate the possibility that the differences in the activity of soluble enzyme fractions prepared from control and intoxicated guinea pigs was an artifact caused by differences in the size of the amino acid pools in the tissues of normal and intoxicated animals, a comparison of protein synthesis was made using two independent systems: i.e., incorporation of leucine-^{14}C in vitro into TCA-precipitable polypeptides and leucine-^{3}H incorporation in vivo into tissues during a 2.5-hr exchange period as described by Bonventre and Imhoff (3). As seen in Table III, similar results were obtained with both methods. Widespread inhibition was found when high doses of diphtheria toxin were administered, while, at low levels heart and skeletal muscle appeared to be specifically inhibited. The extent of inhibition of protein synthesis was in most cases similar whether the in vivo or the in vitro method was used. Since the degree and specificity of inhibition were essentially the same when measured in two different ways, the inhibition appears to be a specific effect of diphtheria intoxication and not an artifact attributable to the size of the amino acid pools. Further evidence to substantiate this conclusion was obtained in later experiments in which the 100,000-*g* supernatant fractions were assayed before and after passage through a Sephadex G-25 column. With the exception of spleen extracts, levels of leucine-^{14}C incorporation in both cases were comparable and thus show that the relative concentration of free amino acids in the tissue extracts was not responsible for the inhibition of protein synthesis as measured in our assay system.

Next, it was considered necessary to show that free diphtheria toxin in the circulation of the intoxicated guinea pigs did not contaminate the 100,000-*g* supernatant fractions used as the source of soluble enzymes for the in vitro assay procedure. Such residual-free toxin, if present in sufficiently high concentrations, would reduce the level of cell-free protein synthesis and this might be misinterpreted as being due to inactivation of soluble transferase enzymes in

vivo during toxemia. Approximately 3 hr before death was to occur, several of the animals received excess antitoxin intracardially to neutralize any free toxin which remained in the circulation. This had no effect on the outcome of the intoxication since the lethal action of the toxin at this point was irreversible. No difference in the degree of inhibition of protein synthesis as measured by the in vitro assay system was found in animals receiving toxin alone and those receiving toxin followed by antitoxin (Table IV). As was shown previously, the heart

TABLE III

Comparison of Protein-Synthesizing Activity of Guinea Pig Tissues as Measured In Vitro and In Vivo Subsequent to Injection of Diphtheria Toxin

Tissue	Inhibition of protein synthesis*			
	10 MLD		1250 MLD	
	Leucine-^{3}H‡ (in vivo)	Leucine-^{14}C§ (in vitro)	Leucine-^{3}H (in vivo)	Leucine-^{14}C (in vitro)
	%	%	%	%
Heart	42	64	73	65
Skeletal muscle	51	80	66	55
Liver	0	8	45	29
Lung	0	0	0	58
Spleen	12	0	42	19
Kidney	0	0	68	46
Brain	0	7	0	0

* Results are expressed as per cent of inhibition of intoxicated samples as compared to controls. Composite data of 15 guinea pigs.

‡ Determination of tritiated leucine incorporation in vivo into TCA-precipitable protein during a 2.5-hr exchange period. Guinea pigs received 10 or 1250 MLD toxin at time zero and 1 mc leucine-^{3}H (i.p.) 2.5 hr before termination of experiment.

§ The 100,000-*g* supernatant fractions were prepared from normal and toxin-treated guinea pigs. Protein synthesis was measured in vitro by determining the amount of incorporation of leucine-^{14}C into TCA-precipitable polypeptides. All other components in reaction mixture present in concentrations given in Materials and Methods.

and skeletal muscle extracts from intoxicated animals were markedly inhibited in ability to participate in cell-free protein synthesis. Furthermore, direct treatment with antitoxin of the soluble components from the tissues of intoxicated guinea pigs also did not increase the level of leucine-^{14}C incorporation into protein. These results show, therefore, that there was no free toxin present in the soluble enzyme fraction which would account for the inhibition of protein synthesis noted in the tissue extracts of diphtheria toxin-treated guinea pigs.

It was noted in early experiments that the direct addition of heat-inactivated serum to the protein-synthesizing reaction mixture resulted in a significant inhibition of protein synthesis. It was considered possible, therefore, that the observed inhibition of protein synthesis might in part be due to a nonspecific

serum factor present in the soluble components of the tissue extracts of intoxicated animals. To eliminate this possibility, 100,000-*g* supernatant fractions were prepared from the tissues of normal guinea pigs and from those of animals receiving 10 and 300 MLD toxin. These fractions were assayed alone or in the presence of pH-5.0 enzyme fractions (partially purified transferase enzymes) prepared from normal guinea pig tissues. In general, mixing the two fractions resulted in a greater incorporation of leucine-^{14}C into protein than did either fraction alone (Table V). For the most part an additive effect was seen, which is what would be expected since more transferase enzyme would be available for

TABLE IV

Use of Antitoxin to Neutralize Free Diphtheria Toxin in the Circulation of Guinea Pigs

Source of 100,000-*g* fractions	Treatment of animals		
	Normal	Toxin*	Toxin-antitoxin‡
	(*cpm* × 10^{-3}/*mg RNA*)§		
Heart	8.1	2.7	2.3
Skeltal muscle	2.3	1.0	1.1
Liver	2.9	3.0	2.5
Lung	1.0	0.7	1.4
Spleen	2.4	2.3	1.2
Kidney	3.4	1.9	1.8
Brain	4.3	4.1	5.2

* The 100,000-*g* supernatants were prepared from tissues of guinea pigs receiving 200 MLD toxin intramuscularly.

‡ Guinea pigs received 200 MLD toxin; 3 hr before death excess antitoxin was given intracardially.

§ Data represents the quantity of leucine-^{14}C incorporated into proteins by the tissue extracts of the three groups of guinea pigs. Composite data from a total of six guinea pigs.

peptide elongation reactions. These results therefore negate the possibility that a nonspecific inhibitor of protein synthesis was present in the 100,000-*g* supernatant tissue fractions.

Thus, the elimination of possible artifacts which might have interfered with the in vitro assay or led to spurious results made it evident that the observed inhibition of protein synthesis was the result of the in vivo inactivation of soluble enzymes, i.e., was a specific effect of diphtheria toxin. The results suggest also that cardiac and skeletal muscle may be target tissues for the action of diphtheria toxin in vivo.

Reversal of the In Vivo-Formed Complex.—Diphtheria toxin in vitro cleaves NAD into nicotinamide and ADPribose moieties. The latter in turn binds with transferase II to form an inactive ADPribose-TF II complex (1, 2). Gill et al. (2) showed that active transferase II could be recovered from the inactive ADPribose-TF II complex by treatment with excess nicotinamide and toxin.

It was assumed that the demonstration of a similar reversal of inhibition with tissue extracts from diphtheria toxin-treated guinea pigs would constitute proof for the formation of the inactive transferase complex in vivo. Furthermore, it would show that the biochemical lesion induced in sensitive tissue culture cells by the toxin is identical with the action of the toxin in the tissues of guinea pigs.

TABLE V

*Experiment Designed to Show that Nonspecific Serum Component in Cell Extracts Is Not Responsible for Inhibition of Protein Synthesis**

Expt. No.	Source of 100,000-*g* fraction	Source of soluble enzymes		
		pH 5 fraction	100,000-*g* supernatant	pH-5 fraction + 100,000-*g* supernatant
		cpm × 10^{-3}/mg RNA‡		
1. (10 MLD)				
	Heart	14.6	2.3	18.0
	Skeletal muscle	4.2	1.4	4.1
	Liver	5.4	7.3	10.3
	Lung	16.3	2.1	15.1
	Spleen	3.5	0.9	6.9
	Kidney	14.0	6.0	17.0
	Brain	11.8	5.6	19.2
2. (300 MLD)				
	Heart	5.9	6.0	9.4
	Skeletal muscle	3.0	1.6	1.1
	Liver	2.3	3.0	3.3
	Lung	5.5	4.1	10.1
	Spleen	3.0	0.7	2.7
	Kidney	2.3	1.6	3.4

* The pH-5.0 fractions (transferases) prepared from normal guinea pig liver and the 100,000-*g* supernatant fractions from tissues of guinea pigs given toxin in the amounts indicated for each of the two experiments. The pH-5 fraction and the supernatant fractions were assayed separately or combined. 1 milligram of 100,000-*g* supernatant fraction and/or 1 mg pH-5 fraction was present in all assays. Polyribosomes in all cases were derived from normal guinea pig livers. The data of the two experiments are from a total of eight guinea pigs.

‡ Protein synthesis measured by leucine-^{14}C incorporation into TCA-insoluble material by the soluble enzyme preparations.

For these experiments the 100,000-*g* supernatant fractions were applied to a small Sephadex G-25 column and eluted with pH 7.4 Tris(hydroxymethyl)-aminomethane buffer (8). This was done to remove any NAD which might be present in the preparations. Reversal cannot be affected in the presence of NAD since equilibrium of the reaction lies very strongly in the direction of formation of the inactive transferase II-ADP-ribose complex (2).

In the experiment summarized in Table VI, 100,000-*g* supernatant fractions from the heart and skeletal muscle of toxin-treated guinea pigs were incubated

with nicotinamide and toxin. It is seen that either substance alone did not relieve the inhibition but that a mixture of nicotinamide and toxin restored soluble enzyme activity to the level found in extracts prepared from comparable tissues of normal untreated animals. Such reactivation indicates that the inactive transferase II-ADPribose complex is formed and accumulated to some extent in cardiac and skeletal muscle of diphtheria toxin-treated guinea pigs.

Effect of Diphtheria Intoxication on the Resistant Rat.—Since the rat is known to be resistant to the action of diphtheria toxin, it was of interest to examine the effect of fatal diphtheria intoxication on the activity of the soluble components involved in protein synthesis prepared from these animals, and also to establish

TABLE VI

*Restoration of Soluble Enzyme Activity by Diphtheria Toxin and Nicotinamide in Heart and Muscle Tissue Extracts from Intoxicated Guinea Pigs**

Additions to reaction mixture‡	Heart	Skeletal muscle
	cpm × 10^{-3}/*mg RNA*§	
A. 100,000-*g* fraction from tissues of untreated guinea pig	22.9	11.1
B. 100,000-*g* fraction from tissues of intoxicated guinea pig	13.3	6.3
C. Same as B + nicotinamide (40 mM)	13.4	7.2
D. Same as B + toxin (20 L_f/ml)	9.7	4.3
E. Same as B + nicotinamide + toxin	19.5	11.4

* Soluble enzymes (100,000-*g* fractions) were prepared from muscle tissues of normal and toxin treated (1200 MLD) guinea pigs. Polyribosomes were prepared from livers of normal animals.

‡ Additions were made to the standard components of the in vitro protein synthesizing reaction mixture described in Materials and Methods.

§ Data in the two columns represent leucine-^{14}C incorporation into protein by the respective tissue extracts.

whether the site(s) of action of the toxin is the same in resistant and sensitive animal species. Rats were given 300 or 10,000 guinea pig MLD of toxin intramuscularly. The smaller dosage had no apparent effect on the animals, while the latter proved to be fatal in 20–24 hr. Since the larger dose of toxin was sufficient to kill the resistant rat, it was reasoned that an examination of the protein-synthesizing capacity of the soluble enzymes from the tissues of such animals might provide information concerning the nature of resistance to the toxin. Particularly we were interested in determining if the soluble enzymes of heart and muscle extracts from rats given lethal or nonlethal amounts of diphtheria toxin were impaired in their ability to participate in protein synthesis as were those from the sensitive guinea pig given as little as five MLD. Soluble enzymes were prepared and assayed as described for the previous guinea pig experiments. Protein-synthesizing activity was essentially normal in the tissue

extracts of rats given the lower (nonlethal) level of toxin. The high (lethal) dose resulted in inhibition of the activity of soluble enzyme fractions from several tissues. It is noteworthy, however, that contrary to the results with guinea pigs, inhibition was not observed in the 100,000-*g* supernatant fractions prepared from cardiac tissue of rats given either high or low dosages of diphtheria toxin. This suggests that the specificity of the toxin, and thus the lethal biochemical lesion, may be different in sensitive and resistant animal species.

DISCUSSION

In spite of enormous progress made in the last decade, the manner in which diphtheria toxin brings about the death of sensitive animal hosts remains unclear. The recent evidence that the toxin enzymatically cleaves nicotinamide-adenine dinucleotide (NAD) with the subsequent formation of an inactive transferase-adenosine diphosphoribose product is unequivocal (1, 2). The catalytic inactivation of the transferase enzymes within sensitive cells is presumed to be the lethal biochemical lesion induced by the toxin. The extent to which this reaction occurs in vivo and contributes to the demise of the host is at the moment entirely conjectural. We consider that the experiments described here constitute preliminary probings into this area and that the results provide a reasonable basis for discussion and speculation concerning the in vivo mode and site of action of diphtheria toxin.

As is the case with polyribosomes from HeLa cells and rabbit reticulocytes (13), diphtheria toxin does not exert any deleterious effect on the polyribosomes derived from the tissues of toxin-treated guinea pigs. The ability of polyribosomes prepared from the tissues of moribund guinea pigs to participate in reactions leading to polypeptide synthesis in vitro was unimpaired. On the other hand, the soluble components of protein synthesis were shown to be quite sensitive to diphtheria toxin. The *direct* addition in vitro of toxin to extracts prepared from tissues of normal guinea pigs or rats inhibited the incorporation of leucine-^{14}C into proteins. This inhibitory effect was noted with the tissue extracts from heart, skeletal muscle, brain, and liver. Presumably the inhibition would occur when toxin is incubated with soluble enzyme fractions from all guinea pig or rat tissues. These observations are compatible with those of Johnson et al. (12), who demonstrated inhibition of protein synthesis by diphtheria toxin when incubated with cell extracts of liver tissue of both sensitive and resistant animal species. These and previous results obtained with HeLa cells and rabbit reticulocytes argue strongly that the reaction inhibited by diphtheria toxin is common to all mammalian cells.

Having established that the soluble components of protein synthesis derived from guinea pig tissues were inhibited by the direct addition of diphtheria toxin, it became important to evaluate the extent to which this inhibition occurred in the tissues of guinea pigs given the toxin parenterally. Earlier work

by Bonventre and Imhoff (3, 4) suggested that the inhibition of protein synthesis was not a general phenomenon demonstrable in all guinea pig tissues, but rather seemed to be restricted primarily to heart and skeletal muscle and very rarely involved other organs. The present set of experiments show that in the intact guinea pig, relatively small amounts of toxin (less than 25 MLD) cause inactivation of soluble enzymes involved in protein synthesis specifically in the heart and skeletal muscle. Only when large doses of toxin are administered does a reduction in the activity of soluble enzymes derived from tissues other than the heart and muscle occur. That the inhibition of soluble enzyme activity noted was a specific effect of diphtheria toxin and not an artifact was established by control experiments which ruled out (*a*) the presence of free toxin in the soluble tissue extracts, (*b*) a nonspecific inhibition due to a serum component, and (*c*) effects attributable to differences in the size of the free amino acid pools of the different tissue extracts. This set of observations can be interpreted to mean that toxin levels compatible with a diphtheritic infection cause a circumscribed effect in the cells of specific organs and tissues, whereas overwhelming amounts are required to produce a more generalized inhibition. It is tempting to speculate that cells which are resistant in the presence of small quantities of toxin become sensitive if anatomic and/or permeability barriers are breached by high extracellular concentrations of toxin.

It is likely that the reaction inhibited by diphtheria toxin in vivo is identical with that which occurs in HeLa cell cultures and in vitro, namely the ADP-ribosylation of aminoacyl transferase II (1, 2). This supposition is based on the fact that in our experiments with intact animals, we were able to restore the activity of soluble enzymes prepared from heart and skeletal muscle of intoxicated animals. Addition of excess nicotinamide and diphtheria toxin to skeletal muscle and heart tissue 100,000-*g* fractions with impaired enzyme activity restored the activity to control levels. One may conclude, therefore, that the toxin catalyzed the formation of the inactive ADP-ribose–Transferase II complex in vivo.

In view of the fact that the heart and skeletal muscle seem to be "target" tissues when physiological quantities of diphtheria toxin are administered, it becomes important to consider if the biochemical mode of action of the toxin, namely, inhibition of transferase enzymes, and the presumed site of action of the toxin in vivo can be reconciled. Myocarditis and cardiac failure are recognized clinical sequelae of human diphtheritic infections (14). In addition, fatty degeneration of the myocardium has been reported frequently subsequent to fatal diphtheria infections of man (15). Lesions in the heart tissues of guinea pigs have also been reported after acute diphtheritic toxemia. Jaffe (cited in reference 14) after an extensive study with guinea pigs concluded that parenchymatous lesions of the heart are the direct result of diphtheria toxin and that pathological changes and degeneration may occur during the first 24 hr of a

fatal toxemia. The in vivo inhibition of protein synthesis in cardiac tissues observed in previous studies (3) and in the present experiments using subcellular components are consistent with the clinical and pathological observations. The inhibition of protein synthesis in heart muscle may represent the primary event which leads to pathological changes, dysfunction, and death. In view of the fact that the turnover rate of protein in muscle tissue is slow, the toxin probably inhibits synthesis either at a critical anatomic region (sino-auricular node?) or of specific enzymes which are essential for the maintenance of normal cardiac function. Wittels and Bressler (16) showed that the heart tissues of diphtheria-intoxicated guinea pigs were normal with respect to the enzymes of the electron transport system, glycolysis, and the tricarboxylic acid cycle; rather a step in the oxidation of long chain fatty acids was inhibited significantly. Since heart muscle obtains energy primarily by the oxidation of fatty acids, an abrupt cessation in the synthesis of appropriate enzymes might lead to the cardiac involvement seen in diphtheria. This, however, is still conjectural. Experiments are now in progress to determine specific proteins and enzymes associated with heart tissue whose synthesis may be inhibited by the toxin.

The data obtained with the diphtheria-resistant rat are provocative. The evidence suggests that soluble enzymes prepared from the cardiac tissue of rats given lethal quantities of toxin are as active as the enzymes prepared from the heart tissues of normal rats. Other experiments with the equally resistant mouse[2] show that incorporation of leucine-^{3}H in vivo is not impaired in any of the tissues of animals given lethal quantities of toxin. One might assume a priori that resistant animal species given overwhelming (lethal) amounts of diphtheria toxin would exhibit an impaired protein synthesis in the heart and skeletal muscle as does the guinea pig. This, however, does not appear to occur and thus suggests that the site of toxin action is not identical in sensitive and resistant species. It is likely that in resistant animals the heart and muscle tissues are not target organs and that the toxin acts elsewhere. It may be that structural components or substances produced in the central nervous system are involved since the rat is sensitive to diphtheria toxin when injected intracranially (17). An educated guess might be a substance produced in minute quantities such as a hormone since our experiments with the rat and mouse do not show evidence of impaired protein synthesis in brain tissues as measured by our procedures.

Finally, comment on another facet of resistance or susceptibility to diphtheria toxin is warranted. The concept of a "target" organ in the sensitive animal requires an explanation. Why does cardiac tissue seem to be impaired with respect to protein synthesis and not other tissues where proteins are turning over at a much faster rate? One possible explanation considered was that muscle tissue has a predilection for binding the protein toxin in amounts much higher

[2] Bonventre, P. F. Unpublished observations.

than other tissues. This was shown not to be the case in experiments utilizing ^{125}I toxin and fluorescent antibody techniques.[3] The toxin becomes associated with all tissues in very minute amounts. Another possibility is that resistant cells exclude the toxin from the cell interior while sensitive cells allow the toxin to reach the cytoplasmic constituents involved in the lethal reaction. Pappenheimer and Brown (18) obtained radioautographic evidence that the toxin becomes associated with the cell membrane of sensitive tissue culture cells and concluded that the toxin does not reach the cytoplasm. On the other hand, the observations of the Moehrings (19) that poly-L-ornithine converts the diphtheria-resistant L cell to a sensitive cell presumably by stimulating pinocytic activity of the membrane would suggest that the toxin must be interiorized to act. Preliminary experiments in our laboratory[4] suggest that toxin-sensitive HEp-2 tissue culture cells take in ^{125}I toxin in very small but significant amounts and that poly-L-ornithine stimulates this cell-associated radioactivity several-fold. Whether or not this represents true pinocytic uptake of protein toxin remains to be proven. These and other observations will be discussed in a subsequent publication.

SUMMARY

The effect of diphtheria toxin on subcellular components of protein synthesis was determined. Polyribosomes prepared from intoxicated guinea pigs functioned normally in an in vitro assay system, while the activity of soluble enzymes (transferases) from toxin-treated animals was significantly reduced. At high toxin dosages, this reduction was widespread, but when levels of toxin comparable to those which might be generated in a natural infection were given, inhibition of soluble enzyme activity was found only in extracts from heart and skeletal muscle. Possible nonspecific inhibition in the assay system due to interference by free toxin or by a serum component was eliminated.

Since it was possible to demonstrate reactivation of soluble enzyme activity with nicotinamide and toxin, it was suggested that diphtheria toxin acts in the intact sensitive animal in a manner analogous to its action in tissue culture or in cell-free systems. It was hypothesized that the lethal biochemical lesion of the toxin in sensitive animals was the inactivation of transferase enzymes, principally in the heart. It was also suggested that the lethal lesion induced in diphtheria-sensitive and resistant species may not be identical.

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[3] Bonventre, P. F., and J. G. Imhoff. Unpublished observations.

[4] Amorini, M. F., and P. F. Bonventre. Unpublished observations.

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