ACTION OF DIPHTHERIA TOXIN IN THE GUINEA PIG*

By JOEL B. BASEMAN,‡ Ph.D., A. M. PAPPENHEIMER, Jr., Ph.D.,
D. M. GILL, Ph.D., AND ANNABEL A. HARPER

(*From The Biological Laboratories, Harvard University, Cambridge, Massachusetts
02138*)

Recent studies have demonstrated that diphtheria toxin is an enzyme of unusual type. Small amounts of the toxin, added to nicotinamide adenine dinucleotide (NAD)-containing mammalian cell extracts, block peptide-bond formation by catalyzing inactivation of the translocating enzyme, aminoacyltransferase 2 (T2) (1–3). This highly specific reaction involves the splitting of NAD with liberation of free nicotinamide and transfer of the adenosine diphosphate ribosyl moiety (ADPR) to be bound in covalent linkage to T2 (4, 5). Although the reaction is reversible, the equilibrium lies far over on the side of T2 inactivation. Only "free" T2 is inactivated by toxin; ribosome-bound T2 is not attacked (2, 6). The reaction is a highly specific one and no tissue protein other than soluble T2 has been found capable of accepting ADPR from NAD in the presence of toxin.[1] That the same reaction occurs when living cells in culture are treated with toxin was shown by Gill et al. (5) who found that inactive T2, extracted from intoxicated HeLa cells that were no longer capable of protein synthesis, could be specifically reactivated to full activity by addition of toxin in the presence of excess nicotinamide.

Bonventre and Imhoff (8) have reported that when diphtheria toxin is injected into guinea pigs, reduction in the rate of leucine incorporation into protein can be demonstrated in heart and pancreas only. In other organs from intoxicated guinea pigs in which morphological damage is known to occur, they were unable to detect a decreased leucine uptake. More recently, Bowman and Bonventre (9) have examined the rate of ^{14}C-leucine incorporation in extracts of tissue excised from intoxicated guinea pigs and once again concluded that inhibition of protein synthesis is mainly confined to heart muscle. In our laboratory we have failed to observe such organ specificity. In the present

---

‡ U.S. Public Health Service Postdoctoral Fellow. Present address: Department of Bacteriology, Harvard Medical School, Boston, Mass.

[1] Nishizuka et al. (7) have described a somewhat similar enzyme isolated from rat liver nuclei that catalyzes the transfer of adenosine diphosphate ribose from NAD to nuclear histones.

study, we are reporting on the distribution and effect on protein synthesis of ^{125}I-labeled diphtheria toxin in the tissues of guinea pigs after intravenous injection. Within a few hours the label has been found in all tissues examined (except nerve and brain) and, contrary to the findings of Bonventre and his co-workers (8, 9), we have observed a marked reduction in amino acid incorporation in all tissues where label can be found. In the tissues of both intoxicated guinea pigs and rabbits the diminished capacity to synthesize protein has been found to be associated with a reduction in T2 content.

Materials and Methods

Diphtheria Toxin and Toxoid.—Toxin lot No. 18 was received from the Antitoxin and Vaccine Laboratory, Massachusetts Department of Public Health, through the courtesy of Dr. Leo Levine. It has been partially purified by ammonium sulfate precipitation followed by dialysis. Merthiolate 1:10,000 had been added as preservative. The lot was further fractionated with ammonium sulfate, and underwent Sephadex and DEAE-cellulose chromatography. The final preparation, which eluted with 0.05 M phosphate at pH 7 from a DEAE-cellolose column, contained 7600 Lf/ml, 50 MLD/Lf, and 380 Lf per OD at 276 mμ. On polyacrylamide gels in the presence of sodium dodecyl-sulfate, over 95% of the protein moved as a single polypeptide chain with molecular weight 62,000.[2] Toxoid lot NZ 155P was also further purified by salt fractionation, and by Sephadex and DEAE-cellulose chromatography. The final product required 0.2 M phosphate at pH 7 for elution from the column and contained 7000 Lf/ml and about 350 Lf per OD at 276 mμ. Horse antitoxin 5A10 was a standard flocculating serum containing 500 units/ml.

[125]*Iodine-Labeled Toxin and Toxoid.*—30 Lf (1.2 mμmoles) of either purified toxin or toxoid were iodinated with 5 mCi (2.3 mμmoles) carrier-free [125]I (New England Nuclear Corp., Bedford, Mass.) using the method of Greenwood et al. (10) as modified by Pappenheimer and Brown (11). The yield of protein-bound iodine was at least 97%. The iodinated proteins were immediately diluted to either 3 Lf/ml or 0.5 Lf/ml (depending on the amount to be injected) in 0.05 M phosphate buffer at pH 7 containing 0.05% bovine serum albumin (BSA). In the case of toxin, 97–98% of the radioactivity was specifically precipitable by antitoxin; 92% of the toxoid label was specifically precipitable. The labeled proteins were used in animal experiments within 2–3 days of their preparation in order to minimize radiation damage. In other experiments less heavily labeled preparations were used. BSA was similarly iodinated, but was diluted in buffer containing 1% normal rabbit serum.

Experimental Animals.—Male albino guinea pigs were used. Their weights varied between 300 and 400 g depending on the type of experiment. Young white rabbits weighing approximately 1 kg were used.

Injection and Distribution of Labeled Proteins.—48 hr before injection, guinea pigs were given drinking water containing 1% potassium iodide.[3] They were fasted for 24 hr before injection into the ear vein of labeled protein diluted in buffered saline containing 0.05% BSA. At selected intervals thereafter animals were anesthetized by intraperitoneal injection of sodium pentobarbitol (2.8 mg/100 g of guinea pig). The abdominal cavity was then opened and a needle inserted into the posterior vena cava through which was perfused oxygenated,

---

[2] Gill, D. M., and L. Dinius. Reported in Proceedings 15th Oholo (Israel) Conference on Microbial Toxins. *J. Biol. Chem.* In press.

[3] When iodide was omitted from the drinking water, relatively large amounts of label were concentrated in the thyroid; other tissues showed little or no difference.

heparinized saline until the blood had been replaced. Organs were then excised, weighed, and counted in a Picker Liquimat gamma counter (Picker Nuclear, White Plains, N.Y.). Circulatory clearance was determined from counts on samples of blood collected at intervals into 25 μl disposable pipets from the retro-orbital space.

^{14}C-Leucine Uptake by Tissue Slices from Normal and Intoxicated Guinea Pigs and Rabbits.— Guinea pigs injected intravenously with 5 MLD of toxin were sacrificed at various time intervals. They were anesthetized and perfused with oxygenated, heparinized saline, and various organs were excised, as described above. Control animals were injected with toxoid or with 250 units antitoxin (intraperitoneally) followed by toxin (intravenously) 30 min later. The excised organs were immediately chilled and cut into slices approximately 1–2 mm thick; pancreatic tissue was minced. It is essential that the tissue slices be of about the same size, otherwise significant differences in the incorporation rate of leucine are observed in duplicate samples. About 1 g of sliced tissue from each organ was placed in a 10 ml Pyrex flask containing 5 ml Krebs-Ringer bicarbonate buffer with 2 mg/ml glucose and 1 μCi uniformly labeled ^{14}C-leucine. Each flask was flushed with 95% O_2:5% CO_2 and was sealed and rotated at 300 rpm in the water bath at 37°C for 2–3 hr. The flasks were then removed from the water bath, the supernate poured off, and the slices washed twice with cold bicarbonate buffer. The tissue was then homogenized in 5 ml of 10% perchloric acid containing 0.5% ^{12}C-leucine, heated to 90°C for 15 min, and centrifuged. The precipitate was dissolved in 4 ml 1 N NaOH containing cold leucine and was reprecipitated with 1 ml 50% perchloric acid. The final precipitate was dissolved in 1.5 ml 1 N NaOH and 0.3 ml samples were pipetted onto glass fiber pads (Whatman GF82), dried, and counted in a liquid scintillation counter. Incorporation was linear over the 3 hr period studied.

In other experiments, slices of tissues from normal guinea pigs were preincubated for 2½ hr in 5 ml Krebs bicarbonate-glucose buffer containing 2 Lf/ml toxin or toxoid. The tissues were then washed with buffer and 400 ± 30 mg portions were distributed into three flasks, each containing 1 μCi ^{14}C-leucine in 5 ml Krebs bicarbonate-glucose buffer. They were incubated for 30, 60, and 90 min respectively, after which the washed tissues were homogenized in perchloric acid and radioactive protein was measured as described in the preceding paragraph.

Estimation of Transferase 2 in Tissues.—Transferase 2 was allowed to react with ^{14}C-labeled NAD in the presence of excess diphtheria toxin and the labeled ADP ribosyl-transferase 2 (ADPR-T2) formed was estimated in the total cold trichloroacetic acid (TCA)-insoluble precipitate. Under the conditions used, there is negligible incorporation of label in the absence of toxin and any unlabeled ADPR-T2 which might have been formed by the action of toxin in vivo does not exchange its ADPR moiety and is not recorded.

About one gram of each tissue was weighed and homogenized in three volumes of cold 0.25 M sucrose. One volume of 2 M NH$_4$Cl was added to release T2 from the cellular particles and 0.3 g of Norit-A (decolorizing carbon) Fisher Scientific Company, Fairlawn, N. J. was added to adsorb endogenous NAD. The suspension was shaken vigorously for 30 min and then centrifuged at 300,000 g for 45 min. 50 μl of the clear supernatant was incubated for 30 min at 37°C with 150 μl of a mixture that contained 140 Lf/ml toxin, 1.4 μM (adenine-7-^{14}C) NAD (47 cpm/$\mu\mu$mole), 0.14 M 2-mercaptoethanol, 7% glycerol, and 0.013 M (tris[hydroxymethyl]-aminomethane) Tris-HCl at pH 8.0. The reaction was stopped with ice-cold 5% TCA and the precipitate was collected by filtration and counted.

Amino Acid Pool in Toxin and Toxoid-Treated Guinea Pig Tissues.—Animals were injected intravenously with 5 MLD of toxin or an equivalent amount of toxoid (0.1 Lf). 26 hr later they were sacrificed and perfused. Organs were then removed, weighed, chilled, and homogenized in 7 ml 5% TCA. The homogenate was centrifuged and the pellet washed by homogenizing a second time with 5% TCA. The combined supernates were extracted five times with 15 ml changes of ether in order to remove TCA. Finally, the aqueous layers were evaporated to

dryness *in vacuo*, resuspended in 2 ml standard citrate buffer (pH 2.2) for each gram of tissue, and analyzed in a Spinco Amino Acid Analyzer.[4]

RESULTS

Intravenous Dose Response in Guinea Pigs.—In Fig. 1 we have plotted the survival times of 300 g guinea pigs after intravenous injections of increasing
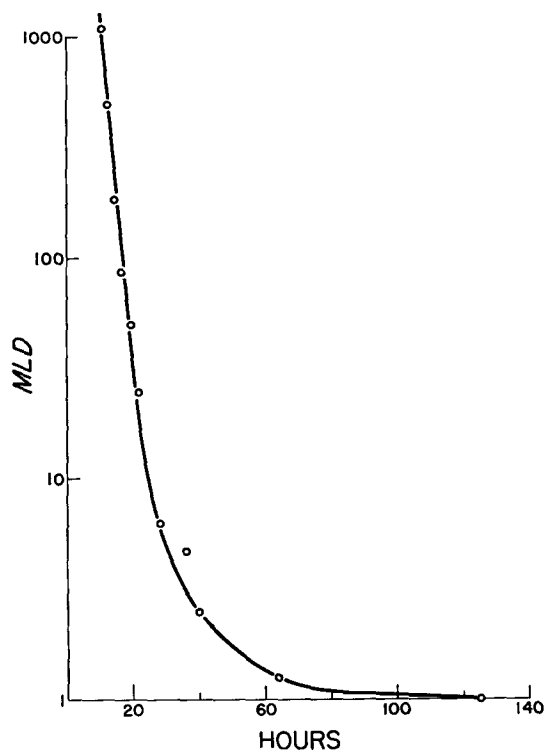

FIG. 1. Survival times of intoxicated guinea pigs. 300 g guinea pigs were injected into the ear vein with increasing doses of purified diphtheria toxin. That amount of toxin causing death in 120–125 hr was taken as one MLD. Each point represents the average of at least two animals.

doses of diphtheria toxin. The survival time at any given dose level is remarkably reproducible; with small doses in the range of 1–5 MLD (50–250 ng) a relatively small change in the amount of toxin injected has a pronounced effect on survival time. It is for this reason that the MLD for diphtheria toxin can be determined with relative precision (within 20–30%) even when only a single

---

animal is used per dose. Even very large doses of toxin, on the other hand, fail to cause death in less than 10–12 hr.

Fate of Labeled Toxin and Toxoid after Intravenous Injection.—In Fig. 2 we have compared the disappearance of 0.25 μg of ^{125}I-labeled toxin (5 MLD) and of toxoid with the disappearance of 0.25 μg of ^{125}I-labeled BSA from the circulation after intravenous injection into 400 g guinea pigs. Similar blood clearance curves were obtained following injection of 1–3 μg of the same three labeled proteins into 1 kg rabbits. In both species BSA disappears more slowly than toxin and toxoid is eliminated most rapidly of all.
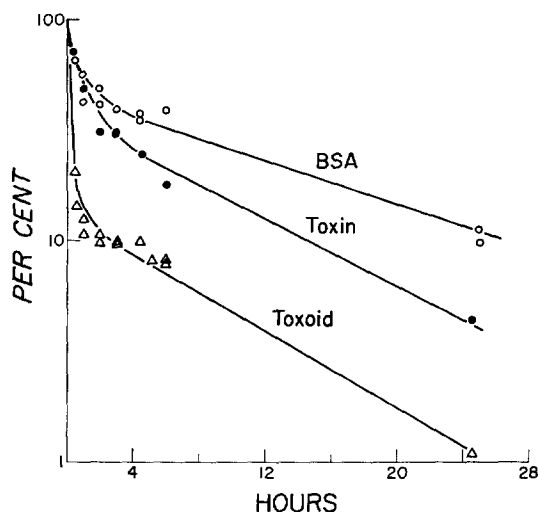

Fig. 2. Blood clearance curves of radio-iodinated proteins. 0.25 μg (*circa* 5 μCi) of each ^{125}I-labeled protein was injected intravenously into 400 g guinea pigs at time zero: open circles, ^{125}I-bovine serum albumin; closed circles, ^{125}I-toxin (5 MLD); open triangles, ^{125}I-toxoid. BSA was diluted in buffer containing 1% normal guinea pig serum. Toxin and toxoid were diluted in buffer containing 0.05% BSA.

We have observed that the survival time of guinea pigs may be prolonged for several hours when an excess of antitoxin is given intraperitoneally ½ hr after intravenous injection of 30 MLD toxin. Inspection of Fig. 2 shows that 70–80% of the injected toxin still remains in the blood stream 30 min after its injection and can presumably still be neutralized by antitoxin so as to render it ineffective. Even after 6 hr, 15–25% of the toxin remains in the circulating plasma in a relatively undegraded form, since more than 50% of the radioactivity in the serum can be coprecipitated with added unlabeled toxin by antitoxin and the amount of label attached to blood cells appears to be negligible.

We have also determined the distribution of label among various perfused organs and tissues of guinea pigs sacrificed at intervals following intravenous injection of 1.5 MLD [125]I-

labeled toxin (*circa* 5 μCi or 2 $\times$ 10^{-12} g atoms ^{125}iodine) or of an equivalent amount (0.03 Lf) ^{125}I-labeled toxoid. Other 400 g guinea pigs injected with the same amount of labeled toxin died in approximately 50 hr.

The results, summarized graphically in Fig. 3, are expressed in terms of molecules fixed per gram of tissue, neglecting any possible degradation of the toxin molecules. It can be seen that, at any given time, there was relatively little variation from one tissue to another in the amount of toxin label per gram
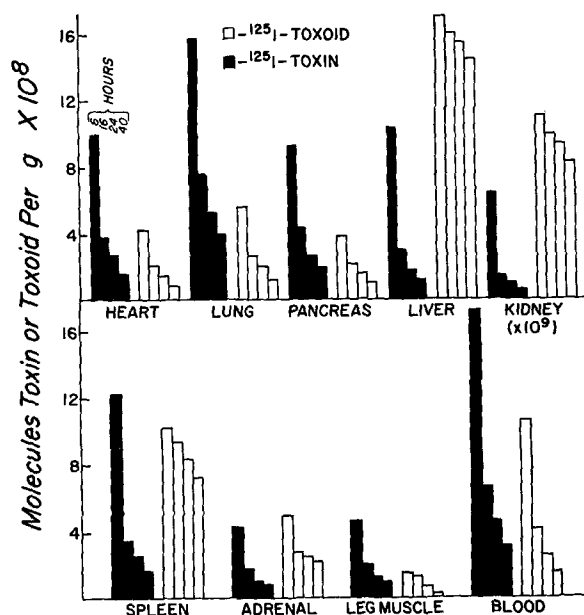

Fig. 3. Distribution of label in organs of guinea pigs after i.v. injection of ^{125}I-toxin or ^{125}I-toxoid. At zero time, 1.5 MLD of ^{125}I-toxin (5 μCi, ca. 2 $\times$ 10^{-12} moles toxin) or an equivalent amount of ^{125}I-toxoid was injected into the ear veins of 400 g guinea pigs. Animals were perfused and sacrificed after 6, 16, 24, and 40 hr at which times organs were excised, weighed, and counted. Note that for the kidney, the ordinate scale has been reduced 10-fold.

except for kidney which showed up to 3–5 times as many counts as any other organ. In every case, after 4–6 hr, the disappearance of label from the tissues parallels its rate of disappearance from the blood stream, suggesting that there is an equilibrium between plasma and tissues. Somewhat to our surprise, label was found in tissues to be distributed similarly relative to the blood level when comparable amounts of ^{125}I-BSA were injected and the guinea pigs sacrificed 24 hr later. There was thus no evidence for any special affinity by tissues for diphtheria toxin nor was there any indication of a specific "target" organ, at least not among the larger organs.

Diphtheria toxoid was handled in a strikingly different manner and is presumably degraded rapidly in liver, kidney, and spleen. Labeled toxoid disappears more rapidly from the circulation than either toxin or BSA and what label does remain cannot be coprecipitated from the serum upon addition of cold toxin and antitoxin. Most of the toxoid label soon appears fixed in kidney,
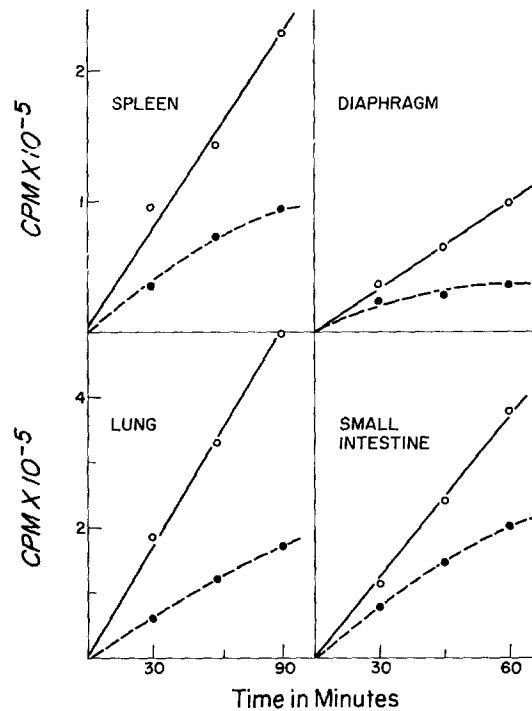

Fig. 4. Effect of diphtheria toxin on protein synthesis in tissue slices from normal guinea pigs. Weighed slices of tissue, 1–2 mm thick, from exsanguinated normal guinea pigs were incubated for 2.5 hr under 95% O_2–5% CO_2 in Krebs-Ringer bicarbonate buffer at 37°C with 2 Lf/ml diphtheria toxin or toxoid. 0.2 μCi ^{14}C-leucine per ml was then added. At 30, 60, and 90 min, flasks were removed from the rotating water bath and leucine incorporation into protein was determined. Open circles, toxoid control; closed circles, toxin.

liver, and spleen where it remains at high levels that bear no relation to its disappearance from the blood and other tissues.

Effect of Added Toxin on ^{14}C-Leucine Incorporation by Normal Tissue Slices.— In the presence of relatively low toxin concentrations, cultured cells derived from a variety of sensitive tissues and animal species lose their ability to incorporate labeled amino acids into protein after a lag period, the duration of which depends on the particular cell line being studied, on the concentration of toxin, and on other factors (12–14). Figs. 4 a–d show that protein synthesis
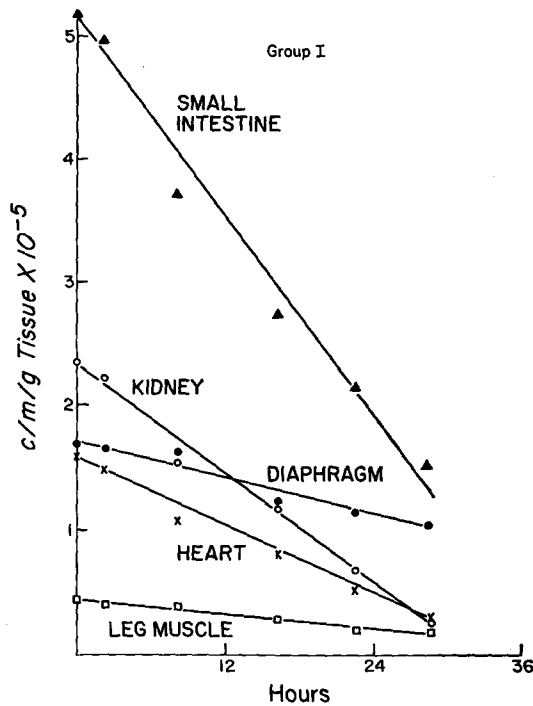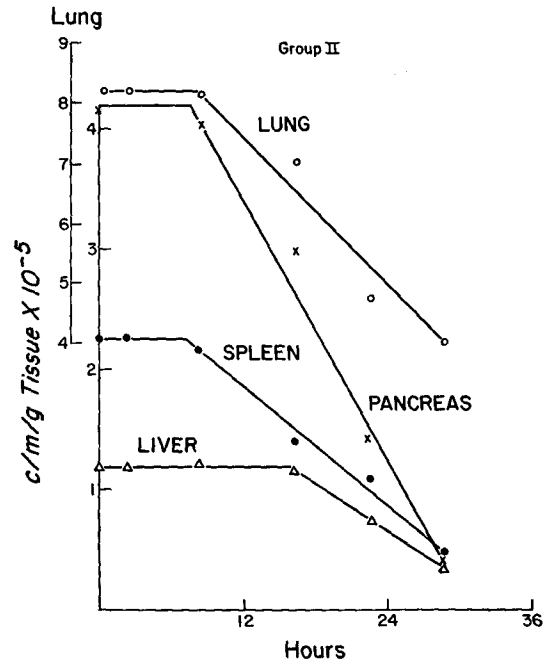
FIG. 5a

FIG. 5b

FIGS. 5 a and b. Protein synthesis in tissue slices from intoxicated guinea pigs. 5 MLD (0.25 µg) toxin was injected i.v. into guinea pigs weighing 350 ± 20 g. At time intervals thereafter, organs from the perfused animals were removed and tissue slices were prepared and incubated for 3 hr at 37°C under 95% O_2–5% CO_2 in Krebs-Ringer bicarbonate buffer containing 0.2 µCi/ml ^{14}C-leucine. Perchloric acid insoluble counts were determined and are expressed per gram wet weight of tissue. c/m/g-counts per minute per gram.

in tissue slices from freshly excised organs taken from normal guinea pigs is likewise inhibited by toxin. Presumably because toxin diffuses slowly into the slices, relatively high toxin concentrations (5 µg/ml) were needed and some incorporation of ^{14}C-leucine into protein still continued, although at a greatly reduced rate, 4 hr after addition of toxin.

Protein Synthesis in Tissue Slices from Intoxicated Animals.—Guinea pigs weighing 350–400 g were injected intravenously with 5 MLD of diphtheria toxin, a dose that would ordinarily cause death in 28–32 hr.[5] They were sacrificed at intervals, exsanguinated by perfusion, and tissue slices were prepared from the various organs as described in Materials and Methods. ^{14}C-leucine incorporation into protein at 37°C was measured in Krebs-Ringer buffer over a 3 hr period.

It will be seen from Figs. 5 a and b that as animals approach the terminal

---

[5] Since these animals were heavier than those used to determine the survival curve shown in Fig. 1, their life expectancy was 3–4 hr longer.

stages of diphtheria intoxication, the ability of every tissue examined to incorporate leucine decreases drastically. Moreover, in several tissues (Group 1), decreased leucine uptake can be detected within 2–3 hr as shown in Fig. 5 a. Group 1 tissues include kidney and various types of muscle tissue. In other tissues (Group 2), such as liver, lung, pancreas, and spleen, there is a pronounced lag of several hours before a significant decrease in ^{14}C-leucine incorporation can be observed (Fig. 5 b). As will be discussed in the following section, the leucine pool size either shows relatively little change or tends to be lower in tissues from severely intoxicated animals. We may therefore conclude that the

TABLE I

Protein Synthesis in Normal and Intoxicated Tissues

	Guinea pigs		Rabbits	
Tissue	Leu uptake % normal	T2 content % normal	Leu uptake % normal	T2 content % normal
Heart	47	62	49	44
Liver	99	86	60 (33)	20
Kidney	81	74	31	39
Pancreas	74	67	4	*
Spleen	45	33	27	34
Lung	48	47	—	—
Small intestine	—	—	73	71

Guinea pigs (400 g) received 5 MLD toxin i.v. 20–24 hr previously; rabbits (0.7–1.3 kg) were injected i.v. with 4 guinea pig MLD per kg on the day preceding sacrifice. All animals were severely ill when sacrificed. Results are expressed as per cent of values observed in normal tissues.

* In rabbit pancreas extracts the proteolytic activity is so high that reproducible results cannot be obtained.

observed decreased rate of ^{14}C-leucine uptake in tissues from intoxicated animals reflects a decreased capacity to synthesize protein.

It was of interest to find out whether or not the decreased rate of protein synthesis observed in tissues from intoxicated animals was associated with a concomitant reduction in aminoacyl transferase 2 (T2) content. It is now possible to assay T2 on a molar basis in tissues with reasonable reliability by reacting the extracted enzyme, freed from endogenous NAD, with NAD labeled in the ADP ribose moiety, using diphtheria toxin as the catalyst. We have therefore estimated, simultaneously, the ability of various organ slices to incorporate leucine in vitro and the residual non-ADPribosylated T2 contents in other samples from the same organs. As can be seen in Table I, in tissues from both rabbits and guinea pigs sacrificed shortly before death from diphtheria intoxication, the observed decreases in ^{14}C-leucine incorporation were accompanied by a significant drop in active T2 content. The apparent failure

of inhibition of ^{14}C-leucine incorporation to match the drop in T2 content in the livers of intoxicated animals needs a word of comment. As seen from Fig. 5 b, of all the tissues tested liver showed the greatest time interval before inhibition of leucine incorporation could be detected in intoxicated animals. Intoxicated liver moreover may be presumed to contain a smaller leucine pool size than normal liver, as shown below for lung and spleen. Increasing the specific activity of added ^{14}C-leucine would tend to mask an inhibition of leucine incorporation. In any case, we conclude that inactivation of T2 is the primary effect of toxin in tissues of the living animal just as it is in cultures of sensitive mammalian cells.

TABLE II

Amino acid	Heart			Kidney			Small intestine			Pancreas			Lung		
	To	Tx	%	To	Tx	%	To	Tx	%	To	Tx	%	To	Tx	%
Lysine	0.31	0.27	87	0.28	0.26	93	0.29	0.32	110	0.41	0.22	54	0.20	0.11	55
Histidine	0.29	0.32	110	0.16	0.21	130	0.16	0.17	107	0.20	0.13	65	0.09	0.04	45
Arginine	0.14	0.12	86	0.08	0.07	88	0.18	0.13	72	0.11	0.06	55	0.09	0.02	22
Aspartic acid	1.15	1.71	150	1.18	1.78	150	1.14	1.92	169	1.20	0.88	74	1.00	0.89	89
Threonine + serine	3.3	3.48	105	2.0	1.86	93	1.84	1.24	68	2.2	1.12	50	1.59	0.86	54
Glutamic acid	3.74	4.32	115	4.78	5.65	118	2.92	2.88	99	5.28	2.56	48	1.45	1.12	77
Proline	—	—	—	—	—	—	0.6	0.62	103	0.56	0.34	61	—	—	—
Glycine	0.81	1.16	143	2.28	5.4	236	2.36	6.1	258	5.3	5.7	107	2.4	2.78	116
Alanine	4.71	4.38	93	1.14	0.88	77	2.06	2.08	99	4.23	1.32	31	0.79	0.42	54
Valine	0.31	0.30	97	0.38	0.20	53	0.56	0.36	65	0.38	0.20	53	0.17	0.12	71
Isoleucine	0.13	0.14	107	0.16	0.14	88	0.34	0.28	83	0.18	0.08	45	0.08	0.05	63
Leucine	0.21	0.18	86	0.22	0.24	110	0.50	0.42	84	0.26	0.14	54	0.15	0.09	60

Guinea pigs (400 g) that had received either 5 MLD toxin (Tx) or 0.1 Lf toxoid (To) 26 hr previously were sacrificed and perfused. Pool sizes are expressed as μmoles per gram wet weight of tissue.

Amino Acid Pools in Normal and Intoxicated Tissues.—We have determined the pool sizes for a number of amino acids (including leucine) in five tissues taken from severely intoxicated and from toxoid-treated control guinea pigs. They are compared in Table II. The free amino acid contents found for the five perfused tissues from the animals given toxoid do not differ appreciably from those reported in the literature for the same tissues in other animal species (Tallan et al., 15). In the case of heart, small intestine, and kidney, for most amino acids there was little if any difference in pool size between the intoxicated and control animals. On the other hand, severe intoxication does seem to cause an appreciable decrease in the free amino acid contents of lung and pancreas. It may be of significance that these latter tissues are among those showing a pronounced lag between administration of toxin and decreased rate of leucine incorporation. It will also be noted from Table II that, in contrast to all other amino acids, the free glycine content of extracts of all five tissues is

actually increased in severe intoxication, especially in tissues of Group 1. The aspartic acid pool also is significantly elevated in intoxicated heart, intestine, and kidney. While these observations are interesting, their significance in the mechanism of intoxication is not known.

DISCUSSION

Following intravenous injection of ^{125}I-labeled bovine serum albumin, diphtheria toxin, or toxoid into guinea pigs and rabbits, label disappears rapidly from the blood stream. In guinea pigs a constant ratio of radioactivity in the blood to that in the tissues is reached within about 4 hr after injection. At this time the amount of label remaining in the circulation corresponds to about 35–40% of the injected BSA, about 25% of the injected toxin, but only 8–9% of the toxoid. Thereafter, the half-lives in the circulation of the labeled proteins are respectively 11–12, 8, and 6 hr. The rates of disappearance from the tissues both of labeled BSA and of toxin follow closely their rates of disappearance from the blood stream. Thus the half-life of toxin label found in heart, lung, and pancreas was about 8–10 hr in guinea pigs that had received 1.5 MLD ^{125}I-toxin (circa 1.5×10^{-12} moles). The finding that toxin is taken up by the tissnes only slightly faster than BSA and that elimination of the toxin label from the tissues follows almost the same time course as nontoxic labeled BSA was surprising. We had expected toxin to be removed rapidly from the circulation and become fixed to the tissues. Yet at a time (40 hr) when the guinea pigs were obviously in a state of severe intoxication, were losing weight rapidly, and the capacity of their tissues to synthesize protein had fallen drastically, ^{125}I-label equivalent to only 1 or 2×10^8 molecules remained per gram of tissue. We estimate that for a tissue such as liver this corresponds to no more than one toxin molecule per cell. Moreover, if the toxin in tissues were in true equilibrium with the blood plasma, we would expect that excess antitoxin could still save the animals if given 6 hr after injection of toxin. Such is not the case. These apparent discrepancies might be explained if, as a result of interaction with the sensitive membrane, a small fragment containing all of the enzymatic activity were split from the toxin molecule and taken up by the cell, leaving most of the radioactivity on a larger nontoxic fragment to be metabolized in the same manner as any nontoxic protein such as BSA. Both Gill and Pappenheimer[6] and Drazin et al (16) have recently demonstrated that following reduction of disulfide bonds and mild treatment with trypsin, all of the T2-inactivating enzymatic activity can, in fact, be recovered on a relatively small fragment of the toxin molecule; they have suggested that fragmentation of the toxin molecule may take place at the cell membrane. However, when the enzymatically active fragment was isolated from ^{125}I-labeled toxin, it was

---

[6] Gill, D. M., and A. M. Pappenheimer. *J. Biol. Chem.* In press.

found to contain at least as much label as the larger inactive remainder of the molecule, and so far no smaller enzymatically active fragments have been found. Therefore, by whatever mechanism toxin acts in living tissues, it seems quite possible that a major portion of the ^{125}I-label measured in vivo is not associated with molecules directly involved in blocking protein synthesis. It should not, of course, be inferred from this statement that two atoms of iodine per toxin molecule reduce its toxicity to any significant extent[7] (see reference 11). From in vitro kinetic studies on T2 inactivation by the enzymatically active fragment, we have calculated that, at the levels of NAD found in normal cells, a single toxin molecule or its fragment per cell would suffice to inactivate all of the transferase 2 within 36 hr or less.

As we have seen from Fig. 5, 5 MLD of toxin injected intravenously is sufficient to cause appreciable inhibition of protein synthesis within a few hours in almost all the tissues. Slices from the various organs, removed from intoxicated guinea pigs sacrificed at intervals after receiving 5 MLD of toxin, showed decreasing capacity to incorporate ^{14}C-leucine into protein. In tissues from both intoxicated rabbits and guinea pigs, the decreased rate of protein synthesis was accompanied by a reduced tissue transferase 2 content. The tissues studied from guinea pigs that had received 5 MLD fell into two groups. In those of Group 1, including kidney and various types of muscle tissue, inhibition of leucine incorporation into protein became apparent within 3–4 hr. Tissues of Group 2, which included liver, lung, spleen, and pancreas, showed little or no decrease in rate of leucine incorporation for 10–12 hr, after which the rate of leucine uptake fell off rapidly. The latter group are tissues rich in ribosomes and show a high rate of protein turnover (Schimke et al., 17). In tissues of both groups, hemorrhagic and necrotic lesions appear characteristically during severe intoxication, as has been observed repeatedly since the time of Loeffler (for review see Andrewes, 18). Only brain and nerve tissues fail to show gross pathologic changes following parenteral injection of one or more MLD of diphtheria toxin by routes other than intracerebral or intraneural (Jacobs et al., 19). Our experiments with labeled toxin indicate that the amount of toxin that crosses the blood–brain barrier is negligible. However, not all cells reached by the toxin within a susceptible species are necessarily sensitive to its action. We have been unable to demonstrate inhibition of protein synthesis in reticulocytes by incubation with toxin in vitro. Even 6 hr of exposure to 250 μg toxin per ml fails to inhibit protein synthesis in rabbit reticulocytes despite the fact that rabbits are extremely sensitive to diphtheria toxin and that toxin blocks

---

[7] It may be calculated that for an average of two randomly bound iodine atoms per toxin molecule, only 13% of the toxin would have no iodine, and only 15% would have more than three iodine atoms per molecule. Since an amount of labeled toxin equivalent to 1.5 MLD unlabeled toxin killed guinea pigs within the expected time interval, the cell damage observed cannot be attributed solely to toxin that failed to become iodinated.

amino acid incorporation in reticulocyte extracts. Presumably rabbit reticulo-cytes, like mouse L-cells (11, 20), either lack receptor sites or are unable to activate the toxin molecule.

We may consider two possible explanations for the different behavior of intoxicated tissues of Groups 1 and 2. It may well be that those tissues which show no decrease in rate of leucine incorporation until several hours after in-jection of the toxin (Group 2) are tissues which, like HeLa cells (4, 6), contain an excess of T2. Until this excess has been depleted protein synthesis would continue at its normal rate. It is also possible that the decrease in leucine pool size that we have observed in lung and pancreas (Group 2) taken from animals shortly before death may actually occur relatively soon after injection of the toxin. If this were the case the specific radioactivity of added ^{14}C-leucine would be increased and could thus mask an inhibition of protein synthesis. A similar decreased pool size was not observed for amino acids in intoxicated tissues of Group 1, with the possible exception of valine; in fact the free aspartic acid and glycine contents of intoxicated heart, kidney, and intestinal muscle actually increased to as much as 2.5 times the normal level. It is of interest that Fazzini et al. (21) observed a reduced ^{14}C-glycine incorporation in heart and kidney slices, but not in liver slices taken from guinea pigs sacrificed only 24 hr after injection of one MLD of toxin. It is possible that the reduced glycine uptake which they observed reflected an increased glycine pool size rather than de-creased protein synthesis.

The accumulation of inactive ADP ribosyl-T2 in intoxicated HeLa cells was proved by reactivation of T2 in dialyzed cell extracts following addition of excess nicotinamide and catalytic amounts of toxin (Gill et al., 4). Bowman and Bonventre (9) have since demonstrated a similar reactivation of transferase in heart muscle extracts from intoxicated guinea pigs. The present studies have indicated that inhibition of protein synthesis is accompanied by a decreased T2 concentration in many tissues of intoxicated guinea pigs, even at toxin concentrations which may approximate no more than one or two molecules per cell. We conclude that the primary action of diphtheria toxin in guinea pigs and rabbits, just as in HeLa cells, is to bring about the conversion of trans-ferase 2 to its inactive ADP ribosyl derivative in all tissues reached by the toxin. By what steps this primary inhibition of protein synthesis leads to morphologic damage and eventual death of the animal remains to be deter-mined.

SUMMARY

The blood clearance and distribution in the tissues of ^{125}I after intravenous injection of small doses (1.5–5 MLD or 0.08–0.25 μg) of ^{125}I-labeled diphtheria toxin has been followed in guinea pigs and rabbits and compared with the fate of equivalent amounts of injected ^{125}I-labeled toxoid and bovine serum al-

bumin. Toxoid disappeared most rapidly from the blood stream and label accumulated and was retained in liver, spleen, and especially in kidney. Both toxin and BSA behaved differently. Label was found widely distributed among all the organs except the nervous system and its rate of disappearance from the tissues paralleled its disappearance from the circulation. There was no evidence for any particular affinity of toxin for muscle tissue or for a "target" organ. Previous reports by others that toxin causes specific and selective impairment of protein synthesis in muscle tissue were not confirmed. On the contrary, both in guinea pigs and rabbits, a reduced rate of protein synthesis was observed in all tissues that had taken up the toxin label. In tissues removed from intoxicated animals of both species there was an associated reduction in aminoacyl transferase 2 content. It is concluded that the primary action of diphtheria toxin in the living animal is to effect the inactivation of aminoacyl transferase 2. The resulting inhibition in rate of protein synthesis leads to morphologic damage in all tissues reached by the toxin and ultimately to death of the animal.

BIBLIOGRAPHY

1. Collier, R. J. 1967. Effect of diphtheria toxin on protein synthesis: inactivation of one of the transfer factors. *J. Mol. Biol.* **25**:83.
2. Goor, R. S., and A. M. Pappenheimer, Jr. 1967. Studies on the mode of action of diphtheria toxin. III. Site of toxin action in cell-free extracts. *J. Exp. Med.* **126**:899.
3. Moehring, T. J., and J. M. Moehring. 1968. Response of cultured mammalian cells to diphtheria toxin. III. Inhibition of protein synthesis studied at the subcellular level. *J. Bacteriol.* **96**:91.
4. Honjo, T., Y. Nishizuka, O. Hayaishi, and I. Kato. 1968. Diphtheria toxin-dependent adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis. *J. Biol. Chem.* **243**:3553.
5. Gill, D. M., A. M. Pappenheimer, Jr., R. Brown, and J. T. Kurnick. 1969. Studies on the mode of action of diphtheria toxin. VII. Toxin-stimulated hydrolysis of nicotinamide adenine dinucleotide in mammalian cell extracts. *J. Exp. Med.* **129**:1.
6. Gill, D. M., A. M. Pappenheimer, Jr., and J. B. Baseman. 1969. Studies on transferase 2 using diphtheria toxin. *Cold Spring Harbor Symp. Quant. Biol.* **34**:595.
7. Nishizuka, Y., K. Ureda, T. Honjo, and O. Hayaishi. 1968. Enzymic adenosine diphosphate ribosylation of histone and polyadenosine diphosphate ribose synthesis in rat liver nuclei. *J. Biol. Chem.* **243**:3765.
8. Bonventre, P. F., and J. G. Imhoff. 1966. Studies on the mode of action of diphtheria toxin. I. Protein synthesis in guinea pig tissues. *J. Exp. Med.* **124**:1107.
9. Bowman, C. G., and P. F. Bonventre. 1970. 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. *J. Exp. Med.* **131**:659.
10. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of

^{125}I-labeled human growth hormone of high specific radioactivity. *Biochem. J.* **89:**114.

11. Pappenheimer, A. M., Jr., and R. Brown. 1968. Studies on the mode of action of diphtheria toxin. VI. Site of action of toxin in living cells. *J. Exp. Med.* **127:** 1073.

12. Strauss, N., and E. D. Hendee. 1959. The effect of diphtheria toxin on the metabolism of HeLa cells. *J. Exp. Med.* **109:**145.

13. Kato, I., and A. M. Pappenheimer, Jr. 1960. An early effect of diphtheria toxin on the metabolism of mammalian cells growing in culture. *J. Exp. Med.* **112:**329.

14. Moehring, T. J., J. M. Moehring, R. J. Kuchler, and M. Solotorofsky. 1967. The response of cultured mammalian cells to diphtheria toxin. I. Amino acid transport, accumulation, and incorporation in normal and intoxicated sensitive cells. *J. Exp. Med.* **126:**407.

15. Tallan, H. H., S. Moore, and W. H. Stein. 1954. Studies on the free amino acids and related compounds in the tissues of the cat. *J. Biol. Chem.* **211:**927.

16. Drazin, R., J. Kandel, and R. J. Collier. 1970. *J. Biol. Chem.* In press.

17. Schimke, R. T., R. Ganschow, D. Doyle, and I. M. Arias. 1968. Regulation of protein turnover in mammalian tissues. *Fed. Proc.* **27:**1223.

18. Andrewes, F. W., editor. 1923. Diphtheria. *Med. Res. Counc. (Gt. Brit.) Spec. Rep. Ser.*

19. Jacobs, J. M., J. B. Cavanagh, and R. S. Mellick. 1966. In Lutra neural injection of diphtheria toxin. *Brit. J. Exp. Pathol.* **47:**507.

20. Moehring, J. M., and T. J. Moehring. 1968. The response of cultured mammalian cells to diphtheria toxin. II. The resistant cell: enhancement of toxin action by poly-L-ornithine. *J. Exp. Med.* **127:**541.

21. Fazzini, G., P. Cioni, and C. Fonnesu-Severi. 1966. Incorporazione di glicina-1-^{14}C in vitro nelle protein di fegato, rene e cuore di animali trattati con tossine batteriche. *Sperimentale.* **116:**235.