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ACTION OF TETANUS TOXIN ON BRAIN, LIVER AND MUSCLE MITOCHONDRIA FROM RESISTANT AND SUSCEPTIBLE SPECIES

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Bacterial toxins from Diphtheria (Niselovskaya, 1960), Staphylococci (Niselovskaya and Paderina, 1964), Plague (Kadis, Ajl and Rust, 1963), Shigella paradysenteriae type III-somatic antigen (Mager and Theodor, 1957) and Shigella dysenteriae (Raskova and Vanecek, 1964) have been found to affect the mitochondrial metabolism. However, no work seems to have been done on the effect of tetanus toxin on mitochondria.

Tetanus toxin is presumed to act primarily on the central nervous system, although it appears to have at least some peripheral action (Wright, 1955). We know as yet very little about the biochemical changes caused by tetanus toxin, leading to paralysis and death. Wensink & Cohen (1953) showed decreased quantities of energy-rich phosphate compounds in local tetanus and decreased glycogen synthesis with a blockade at the aldolase or triosephosphate dehydrogenase levels. However, they were unable to demonstrate gross changes in aerobic metabolism. Michelazzi, Mor and Dianzani (1955) have reported decrease in adenosine triphosphate (ATP) content of skeletal muscle of guinea-pigs poisoned with tetanus toxin. However, they report that the oxidative phosphorylation is normal in the earlier stages of tetanus but decreases slightly later on.

It is well known that mammals are highly susceptible to tetanus toxin whereas other classes of animals such as aves, amphibia and reptiles (Fulthorpe, 1956 ; Metchnikoff, 1898) are highly resistant. Davies, Morgan & Wright (1955) reported that the pigeon is nearly 1,000 times more resistant to intravenously administered tetanus toxin than the mouse. They believed that the brain tissue of birds was intrinsically much less susceptible to tetanus toxin than that of mammals. However, Van Heyningen (1959) was unable to find any significant difference between the tetanus toxin-fixing protagon from the brain of the highly resistant chicken and the susceptible bovine in its capacity to fix tetanus toxin.

The object of this investigation was to find the reasons for the resistance of the pigeon and the susceptibility of the rat to tetanus toxin. The effect of the toxin on mitochondria isolated from rats and pigeons was therefore studied. It was also of interest to determine the ATP content of skeletal muscle and brain of the rat and the pigeon injected with tetanus toxin.

METHODS

Tetanus toxin. Toxic culture filtrates from the Harvard strain of *Clostridium tetani*, grown on the protein-free medium of Latham, Bent & Levine (1962) were obtained from the Immunology Department of the Haffkine Institute. The toxin was dialysed against cold distilled water and contained 100,000 MLD per ml. where MLD represents the minimum lethal dose in mice. The lethal dose for rats weighing 100 g was about 5 mouse MLD, that of pigeons weighing about 400–450 g was about 20,000 mouse MLD.

Tetanus formol toxoid, purified by ammonium sulphate according to the method of Levine & Stone (1951) was also from the Haffkine Institute. It was dialysed against cold distilled water for three days and was adjusted to 25 Lf per ml. This was not toxic to mice when 0.5 ml. was injected subcutaneously or intravenously.

Heated tetanus toxin was prepared by heating tetanus toxin at 100° C for 30 min. This was not lethal to mice when 0.5 ml. was injected subcutaneously.

Preparation of mitochondria and measurement of their respiration. Brain mitochondria were prepared by the method of Brody & Bain (1952) and liver mitochondria as described by Schneider (1948). Muscle mitochondria were prepared according to the method of Harman & Osborne (1953). Adult albino rats (Norwegian strain), weighing about 100 g, and adult pigeons, obtained from the market, and weighing about 400–450 g, were used for the experiments. Rats and pigeons were killed by stunning and decapitation. Brain, liver and leg muscle tissues were then removed and placed in small beakers chilled with broken ice and weighed immediately. All subsequent operations were carried out at 0–3° C.

Brain and liver tissues were homogenized in 9 volumes of 0.25 M sucrose in a glass homogenizer. For the preparation of skeletal muscle homogenate, 2 g leg muscle was coarsely chopped in 15 ml. of 0.25 M sucrose, containing an added 0.15 ml. of 0.04 M $NaHCO_3$. This was homogenized in the blender for 60 sec. An additional 15 ml. of 0.25 M sucrose was then stirred in and the mixture squeezed through a muslin cloth into centrifuge tubes.

For experiments involving freezing and thawing of brain mitochondria, the procedures were as described by Hansen & Smith (1964). The mitochondrial pellet was suspended in 0.25 M sucrose containing 0.01 M Tris-acetate (*p*H 7.5), and was kept frozen for 1 hr at −20° C. After thawing, the suspension was supplemented with the following additions in the final concentrations mentioned: 1 mM potassium succinate, 1 mM ATP, 5 mM $MgCl_2$, 10 mM $MnCl_2$, and *p*H was adjusted to 7.5. The control unfrozen mitochondria were also supplemented with the above additions.

The amount of mitochondria used in each experiment consisted of mitochondrial suspension corresponding to 200 mg fresh brain, liver or skeletal muscle tissue and was put in the main compartment of the Warburg respirometer. Respiration was determined by O_2 uptake by standard techniques. The measurements were done at 30° C with air as gas phase. Phosphate esterification was determined by the disappearance of Pi in presence of a glucose-hexokinase trapping system. Pi was estimated by the method of Lowry and Lopez (1946). The assay medium was prepared according to Michelazzi (1961) with slight modifications and contained: 39.9 μM Na_2HPO_4-KH_2PO_4 buffer, *p*H 7.4; 30.0 μM Trisbuffer, *p*H 7.4; 15.0 μM $MgCl_2$; 30.0 μM KCl; 0.03 μM $MnCl_2$, 60.0 μM NaF; 90.0 μM sucrose; 6.0 μM ATP; 0.03 μM cytochrome C; 0.05 μM DPN and 30.0 μM Na-glutamate or 30.0 μM Na-succinate or 30.0 μM Na-ascorbate. The volume of this solution was 2.0 ml. The total volume in the Warburg flask was 3.0 ml.

Analysis of ATP content: Animals were killed by decapitation under $MgSO_4$ anaesthesia (Lepage & Umbreit, 1945). The brain, liver and leg muscle tissues were immediately removed and frozen. The brain and liver tissues were weighed while still frozen and were homogenized in ice-cold 10% trichloroacetic acid (TCA) using a glass homogenizer. Skeletal muscle was also weighed and ground in a mortar by suspending it in 10% TCA. The suspension was then homogenized as above. The homogenates were centrifuged at 5,000 g for 15 min. The supernatants were collected. The residues were re-extracted with 5% TCA and centrifuged. The combined TCA extracts of tissues were analysed for ATP content using the method of fractionation with barium acetate as described by Lepage & Umbriet (1945).

Determination of ATPase activity: The ATPase activity of homogenates and myofibrils was measured according to the method of DuBois and Potter (1943). Myofibrils were isolated from leg muscle of rats by using the method of Mor (1953).

RESULTS

Effect of tetanus toxin on the oxidative phosphorylation of Mitochondria

The rates of oxygen consumption and phosphorus fixation were measured with brain, liver and skeletal muscle mitochondria of the rat and the pigeon in presence of tetanus toxin using one control with tetanus toxoid and a second control with heated tetanus toxin. Glutamate was used as a substrate. The results of the experiments showed (Table 1) that tetanus toxin was able to uncouple the oxidative phosphorylation of rat brain

TABLE 1

EFFECT OF ADDED TOXIN ON THE OXIDATIVE PHOSPHORYLATION OF BRAIN, LIVER AND SKELETAL MUSCLE MITOCHONDRIA OF RATS AND PIGEONS

Results are given as mean ± S.D. The medium used was the same as described in the methods. One side-arm contained 60 μM of glucose and 150 Kunitz-MacDonald units of hexokinase. The other side-arm contained 0·5 ml. of tetanus toxin or 0·5 ml. of tetanus toxoid or 0·5 ml. of heated toxin. The flasks were allowed to equilibrate for 10 min. at 30° C and were incubated for 30 min. at 30° C. Central well contained 0·2 ml. of 20% NaOH and fluted filter paper to absorb the CO_2 produced

Source of mitochondria	No. of experiments	Addition	Uptake		P : O ratio
			△O μatoms	△P μmoles	
Rat brain	10	Nil	1·8±0·10	4·5±0·15	2·5±0·20
	10	25,000 MLD toxin	2·0±0·08	3·3±0·21	1·6±0·18
Rat liver	8	Nil	2·1±0·17	5·6±0·26	2·6±0·12
	8	25,000 MLD toxin	2·2±0·14	5·3±0·21	2·4±0·10
Rat skeletal muscle	6	Nil	1·7±0·13	4·6±0·14	2·7±0·16
	6	25,000 MLD toxin	1·8±0·11	4·5±0·18	2·5±0·13
Pigeon brain	8	Nil	1·7±0·12	4·1±0·25	2·4±0·08
	8	25,000 MLD	1·9±0·13	4·2±0·21	2·2±0·10
Pigeon liver	8	Nil	2·0±0·10	5·2±0·24	2·6±0·14
	6	25,000 MLD toxin	2·1±0·14	5·4±0·28	2·5±0·020
Pigeon skeletal muscle	6	Nil	1·6±0·16	4·1±0·21	2·5±0·12
	6	25,000 MLD toxin	1·5±0·13	3·9±0·15	2·6±0·08

mitochondria. Tetanus toxin lowered the P : O ratio of rat brain mitochondria from 2.5 in control experiments to 1.6 ($P<0.01$). However, the P : O ratio of pigeon brain mitochondria was not significantly altered in presence of the toxin. The decrease in the P : O ratio of rat brain mitochondria was due to a simultaneous stimulation of oxygen uptake (1.15 times) and a decrease in the phosphorylation process. With pigeon brain mitochondria there was a slight stimulation of oxygen uptake but no decrease in the phosphorus fixation.

Tetanus toxin did not show any significant change in the oxygen uptake or the phosphorylation of the liver and skeletal muscle mitochondria from susceptible rat as well as resistant pigeon.

Effect of tetanus toxin on frozen-thawed brain mitochondria

As tetanus toxin uncoupled the oxidative phosphorylation of rat brain mitochondria but not that of pigeon brain mitochondria, it was considered likely that mitochondrial membrane of pigeon brain may be impermeable to tetanus toxin. If such is the case, then use of disrupted pigeon brain mitochondria should result in the uncoupling of oxidative phosphorylation upon the addition of tetanus toxin.

The mitochondria prepared from rat and pigeon brain were disrupted by freezing and thawing as described in the methods. This preparation gave consistently a P:O ratio of 1.95–2.15 with glutamate as substrate. The respiration and the oxidative phosphorylation of the disrupted mitochondria were then measured in presence of tetanus toxin. Non-disrupted mitochondria incubated in the presence and in the absence of toxin were used as controls. The results of the experiments described in Table 2 showed that the

TABLE 2

EFFECT OF TETANUS TOXIN ON DISRUPTED BRAIN MITOCHONDRIA FROM RATS AND PIGEONS

Results are given as mean ± S.D. The medium used was the same as described in the methods. Gutamate was used as a substrate. One side-arm contained 60 μM of glucose and 150 Kunitz-MacDonald units of hexokinase. The other side-arm contained 0·5 ml. of tetanus toxin. The flasks were allowed to equilibrate for 10 min at 30° C and were incubated for 30 min at 30° C. Central well contained 0·2 ml. of 20% NaOH and fluted filter paper to absorb the CO_2 produced

Source of mitochondria	No. of experiments	Addition	Uptake		P:O ratio
			△O μatoms	△P μmoles	
Rat brain	6	Nil	1·9±0·08	5·0±0·25	2·6±0·08
(intact)	6	25,000 MLD toxin	2·2±0·12	3·3±0·23	1·5±0·10
Rat brain	6	Nil	2·0±0·12	4·4±0·18	2·2±0·10
(frozen-thawed)	6	25,000 MLD toxin	2·1±0·12	3·1±0·20	1·4±0·07
Pigeon brain	6	Nil	1·8±0·12	4·9±0·16	2·7±0·13
(intact)	6	25,000 MLD toxin	2·0±0·09	4·8±0·15	2·4±0·08
Pigeon brain	8	Nil	1·9±0·10	3·9±0·18	2·0±0·10
(frozen-thawed)	8	25,000 MLD toxin	2·0±0·12	3·0±0·18	1·5±0·08

addition of toxin to the frozen-thawed mitochondria of pigeon led to uncoupling of oxidative phosphorylation. In case of rat brain mitochondria, tetanus toxin lowered the P:O ratio with disrupted as well as intact preparation to more or less the same extent.

Oxidative phosphorylation of brain and liver mitochondria from rats injected with tetanus toxin

In order to correlate the results obtained in *in vitro* experiments with the changes produced by tetanus toxin in the metabolism of the cell in the intact animal, experiments were made on mitochondria isolated from rats injected subcutaneously with two rat lethal doses of tetanus toxin. After 48–72 hr, when the rats came down with paralysis, they were sacrificed, and brain and liver tissues were removed for isolation of mitochondria. Control rats injected with 0.5 ml. of tetanus toxoid or 0.5 ml. of saline were also sacrificed after 48–72 hr. P:O ratios were then determined in the mitochondria isolated from the brain and liver of these animals. The results of the experiments showed

(Table 3) that there was a statistically significant ($P<0.01$) decrease in the P:O ratio of brain mitochondria from rats which had received tetanus toxin as compared with the control rats. The reason for not getting the same decrease in the phosphorylation of brain mitochondria *in vivo* as in *in vitro* is probably because the amount of toxin injected was little (2 rat lethal doses) as compared to that in *in vitro* experiments, when about 25,000 mouse MLD toxin was used.

The liver mitochondria did not show any change in the oxygen uptake as well as the phosphorylation, and the P:O ratio remained unaltered.

TABLE 3

OXIDATIVE PHOSPHORYLATION OF BRAIN AND LIVER MITOCHONDRIA OF RATS *IN VIVO*

Results are given as mean ± S.D. The medium used was the same as described in the methods. Glutamate was used as a substrate. One side-arm contained 60 μM of glucose and 150 Kunitz-MacDonald units of hexokinase. The flasks were allowed to equilibrate for 10 min at 30° C and were incubated for 30 min at 30° C. Central well contained 0·2 ml. of 20% NaOH and fluted filter paper to absorb the CO_2 produced

Source of mitochondria	No. of experiments	Type of injection	Uptake		P:O ratio
			△O μatoms	△P μmoles	
Rat brain	8	0·5 ml. saline	1·7±0·12	4·3±0·27	2·5±0·10
	8	0·5 ml. toxoid	1·6±0·18	4·3±0·22	2·6±0·18
	8	2 rat lethal doses of toxin	1·7±0·13	3·5±0·22	2·0±0·10
Rat liver	6	0·5 ml. saline	2·0±0·06	5·2±0·25	2·6±0·12
	6	0·5 ml. toxoid	1·9±0·09	5·3±0·25	2·7±0·16
	6	2 rat lethal doses of toxin	2·0±0·07	5·0±0·23	2·5±0·07

The stage of uncoupling of oxidative phosphorylation

It was of interest to find out the part of the respiratory chain where the uncoupling of oxidative phosphorylation of rat brain mitochondria takes place in the presence of tetanus toxin. It is well established that three molecules of ATP are formed during the transfer of a pair of electrons from diphosphopyridine (DPN)-linked substrates such as glutamate to oxygen. In the case of succinate, which is linked to flavoprotein, two moles of phosphate are fixed per atom of oxygen, and with ascorbate, which is linked to cytochrome C, one mole of phosphate is fixed per atom of oxygen taken up. The effect of the toxin on esterification of inorganic phosphate with glutamate has been done in the first experiment.

In this study, the fixation of phosphorus with rat brain mitochondria, using succinate and ascorbate as substrates, was therefore studied. The results given in Table 4 show that tetanus toxin stimulates respiration with these substrates also. However, the esterification of inorganic phosphate was not significantly changed in presence of the toxin. There was observed a slight statistically significant decrease in the P:O ratio with succinate and ascorbate in presence of the toxin. This slight decrease in the P:O ratio was due to stimulation of the respiration, since the phosphorylation was not significantly changed in presence of the toxin.

ATP content of different tissues of rats and pigeons injected with tetanus toxin

Tetanus toxin produces local or general paralysis in susceptible animals. Muscles in tetanic contracture show decreased elasticity and inability to relax after contraction,

TABLE 4

EFFECT OF ADDED TOXIN ON THE OXIDATIVE PHOSPHORYLATION OF RAT BRAIN MITOCHONDRIA WITH SUCCINATE AND ASCORBATE AS SUBSTRATES

Results are given as mean ± S.D. The medium used was the same as described in the methods. One side-arm contained 60 μM of glucose and 150 Kunitz-MacDonald units of hexokinase. The other side-arm contained 0·5 ml. of tetanus toxin. The flasks were allowed to equilibrate for 10 min at 30° C and were incubated for 30 min at 30° C. Central well contained 0·2 ml. of 20% NaOH and fluted filter paper to absorb the CO_2 produced

Substrate	No. of experiments	Addition	Uptake		P : O ratio
			△O μatoms	△P μmoles	
Succinate	6	Nil	1·5±0·17	2·6±0·34	1·7 ±0·10
	6	25,000 MLD toxin	1·7±0·18	2·4±0·26	1·4 ±0·12
Ascorbate	6	Nil	1·4±0·08	1·1±0·17	0·80±0·07
	6	25,000 MLD toxin	1·7±0·14	1·0±0·14	0·60±0·05

which is suggestive of deficiency of ATP. Experiments with rats were conducted by giving intramuscular injections of sublethal doses of tetanus toxin. Our interest in using a sublethal dose of toxin was to produce local paralysis; the amount of toxin being presumably insufficient to reach the brain.

One group of 8 albino rats, each weighing about 100 g, was injected with sublethal dose (¾ lethal dose) of tetanus toxin in 0.5 ml. saline into right hind leg muscle. The rats developed local paralysis after about 72 hr but recovered after about seven to eight days. Six rats were sacrificed after about 96 hr and the ATP content of brain and skeletal muscle was determined. A second group of rats, each weighing about 100 g, was injected intramuscularly with twice the lethal dose of tetanus toxin. Eight rats in this group were killed after 24 hr before they developed paralysis, and 11 rats were sacrificed after about 48 hr, when they had developed paralysis in the leg muscles of the injected limb. After killing, the brain, liver and leg muscles (of both the injected and non-injected side) were removed and the ATP content was determined as described in the methods.

One group of pigeons, each weighing about 400–450 g, was injected intramuscularly into leg muscles with about 10 times the rat lethal dose of the toxin. Another group of pigeons, also each weighing about 400–450 g, was injected intramuscularly with twice the pigeon lethal dose (about 8,000 times the rat lethal dose). In the first group of pigeons, paralysis was not observed. In the second group, the pigeons developed paralysis of the injected limbs after about 48 hr. Both the groups were killed after 48 hr and the ATP content of brain and leg muscle of the injected side was measured.

Control groups of rats and pigeons were injected with 0.5 ml. saline and were killed after about 48 hr. The results of the experiments are given in Table 5.

It will be seen from Table 5 that the ATP content of rat brain and skeletal muscle decreased by about 30% and 46% respectively when the animals received twice the lethal dose of the toxin, while the ATP content of liver was not significantly changed. The decrease in the ATP content of muscle was more pronounced in the injected limb than in the contralateral non-injected limb, which did not show paralysis. The ATP content of skeletal muscle of rat decreased as the paralytic symptoms appeared. There was also some reduction in the ATP content of skeletal muscle of rats injected with sublethal dose of tetanus toxin. However, the amount in the brain tissue was not affected in such

TABLE 5

ATP CONTENT OF BRAIN, LIVER AND SKELETAL MUSCLE OF RATS AND PIGEONS INJECTED INTRAMUSCULARLY WITH TETANUS TOXIN

Values are expressed in μmoles ATP/100 g tissue. Numbers in the parentheses represent the number of estimations. Mean values and S.D. are also given for each experiment

		Rat				Pigeon	
				Skeletal muscle			
Injection	Time of killing after the injection	Brain	Liver	Injected limb	Contralateral limb	Brain	Skeletal muscle
0·5 ml. saline (control)	48 hr	124±22 (10)	210±28 (8)	450±38 (8)		140±28 (6)	490±40 (6)
Sublethal dose	96 hr	130±18 (6)		338±24 (6)			
2 rat lethal doses	24 hr			364±29 (8)	416±25 (8)		
	48 hr	84±26 (11)	195±24 (8)	246±41 (8)	362±37 (8)		
10 rat lethal doses	48 hr					130±32 (6)	480±36 (6)
8,000 rat lethal doses	48 hr					100±30 (6)	325±43 (6)

animals. The ATP content of brain and skeletal muscle of pigeons remained unaffected when they were injected with 10 rat lethal doses, as this amount was not sufficient to produce paralysis in pigeons. However, when pigeons were injected with twice the pigeon lethal dose, the ATP content of brain and skeletal muscle decreased by about 29% and 34% respectively.

ATPase activity of homogenates and myofibrils

The studies of many workers have demonstrated that there is an intimate relationship between oxidative phosphorylation and ATPase activity (Schneider, 1959). The uncoupling action is often associated with increased ATPase activity (Schneider, 1959). As tetanus toxin has an uncoupling action on the oxidative phosphorylation of rat brain mitochondria but not on the muscle mitochondria, the decrease in the ATP content of skeletal muscle may be due to stimulation of ATPase activity. The ATPase activity of homogenates of brain and skeletal muscle and myofibrils isolated from skeletal muscle of rats sacrificed 48 hr after injection of twice the rat lethal dose of toxin was estimated.

The results of the experiments described in Table 6 show that tetanus toxin does not affect the ATPase activity of rat brain and skeletal muscle homogenates. These results confirm the findings of Pillemer & Muntz (1949), who were unable to detect any effect of crystalline tetanus toxin upon the ATPase activity of nerve and muscle tissue of mice

TABLE 6

ATPASE ACTIVITY OF HOMOGENATES AND MYOFIBRILS ISOLATED FROM NORMAL AND TETANIZED SKELETAL MUSCLE OF RATS

Values are expressed as μmoles Pi released/mg enzyme N/hr. Mean and S.D. are also given for each experiment

Injection	No. of experiments	Brain homogenate	Skeletal muscle homogenate	Myofibrils
0·5 ml. saline (control)	6	6·6±0·80	32·7±4·0	40·5±12·0
2 lethal doses of toxin	6	6·8±0·70	34·2±5·0	68·2±10·0

poisoned with massive doses of toxin. However, our results show that tetanus toxin increases the ATPase activity of myofibrils isolated from the skeletal muscle of rats injected with tetanus toxin.

DISCUSSION

We have observed a slight stimulation of respiration of rat and pigeon brain mitochondria in presence of tetanus toxin. A similar increase in the respiration of guinea-pig liver mitochondria was observed by Niselovskaya & Paderina (1964) with Staphylococcol toxin. As tetanus toxin uncouples the oxidative phosphorylation of rat brain mitochondria but not that of pigeon brain mitochondria, it seems likely that pigeon brain mitochondrial membrane is capable of excluding the toxin. This probability was demonstrated here from the observation that, the oxidative phosphorylation of intact brain mitochondria from pigeon was not significantly affected by the toxin whereas the oxidative phosphorylation of mitochondria damaged by freezing and thawing was uncoupled in presence of the added toxin. Similar observations were made by Kadis *et al.* (1963) on plague murine toxin from *Pasteurella pestis*, which did not affect the respiration of intact heart mitochondria of resistant rabbit, but the respiration of mitochondria, disrupted by deoxycholate or sonic vibration, was inhibited.

The absence of any effect of tetanus toxin on the liver and skeletal muscle mitochondria of rats and pigeons shows that the toxin has action principally on the brain tissue.

Many uncouplers of oxidative phosphorylation either swell or shrink mitochondria. The effect of tetanus toxin on the swelling properties of mitochondria isolated from brain and liver of rat was therefore studied by measuring the decrease in O.D. at 520 mμ as described by Tapley (1956). It was found that tetanus toxin, unlike many other uncouplers, did not affect the swelling properties of rat brain and liver mitochondria. Tetanus toxin also did not increase the ATPase activity of rat brain homogenate. In these respects, the toxin resembles staphylococcal toxin (Niselovskaya & Paderina, 1964), which uncouples the oxidative phosphorylation of guinea-pig liver mitochondria but has no effect on ATPase and swelling of the liver mitochondria.

Rats showing paralysis in the injected limb 48 hr after the intramuscular injection of twice the lethal doses of toxin showed decreases in the ATP content of their brain and skeletal muscle. The low ATP levels in brain and skeletal muscle were also observed in rats injected intracerebrally with twice the lethal dose of tetanus toxin. However, intracerebral injection of tetanus toxin produced paralysis of all the four limbs after 48 hr, thus giving rise to generalized form of tetanus. The reduction in the ATP content of brain and skeletal muscle was also observed in pigeons when they were injected with twice the lethal dose of toxin. The decrease in the ATP content of brain in presence of the toxin may be due to its uncoupling action on the oxidative phosphorylation of rat brain mitochondria and not due to increase in the ATPase activity, since tetanus toxin does not affect the ATPase activity of rat brain. However, our results show that the lower ATP levels in injected muscle as compared with the non-injected limb is not due to uncoupling of oxidative phosphorylation of muscle mitochondria but to increased ATPase activity of myofibrils. These results confirm those of Michelazzi *et al.* (1955). The myofibrils preparation used was practically free from mitochondria. It must be noted, therefore, that in the absence of any effect of toxin on the ATPase activity of whole

homogenates of the injected leg muscle, the decrease in ATP content of muscle is due only to the increase in the enzyme activity of myofibrils.

It may well be argued that injections of many uncouplers, such as dinitrophenol, even in lethal doses, do not lead to paralysis as observed in tetanus. Such uncouplers, however, have no specificity to any particular tissue and in small concentration may be eliminated from the body by way of detoxication, especially by the liver or by other means. In contrast, the action of tetanus toxin is purely localized in the appropriate neurones or the brain tissue. The fixation of tetanus toxin in the subcellular fractions of brain is discussed in the next paper, in which are also discussed the mode of action of tetanus toxin and the role of gangliosides.

SUMMARY

1. Tetanus toxin uncouples the oxidative phosphorylation of brain mitochondria of rat, which is susceptible, but not that of pigeon, which is resistant.

2. The toxin had no action on the P:O ratio of liver and muscle mitochondria of the rat or the pigeon.

3. The uncoupling of oxidative phosphorylation in rat brain mitochondria takes place in the DPN-requiring step only.

4. The resistance of the pigeon to tetanus is probably due to the impermeability of its brain mitochondrial membrane to the toxin, since the toxin decreased the P:O ratio of frozen-thawed pigeon brain mitochondria.

5. The local tetanus caused by the intramuscular injection of sublethal doses of tetanus toxin seems to be due to decreased ATP levels in the injected muscle due to activation of ATPase of myofibrils.

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