22. EFFECT OF DIPHTHERIA TOXIN UPON TISSUE ENZYMES *IN VITRO*

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THE normal course of diphtheria is generally characterized by pronounced ketonuria and other symptoms indicating serious metabolic disturbances. Many of the regular pathological symptoms, e.g. circulatory failure, fatty changes in the heart, interference with its action and haemorrhage into the adrenals, are also capable of being traced back to a biochemical origin, whilst the last-named occurrence may obviously lead automatically to further chemical interference.

We have already shown [Peters & Cunningham, 1937] that the polyneuritic condition that occurs in the later stages is not due to induced vitamin B₁ deficiency, since the chemical changes associated therewith in the blood are not shown. Since there are no indications of lack of any other vitamins or dietary factors we conclude that the chemical disturbances are of a more profound origin. We have therefore turned our attention to a number of isolated tissue enzyme systems, and studied their behaviour in presence of diphtheria toxin.

The scope of our work embraces respiration, glycolysis, oxidations (including autoxidations) and phosphorus exchange.

Technique. For our work on respiration and oxidation we generally used the Warburg manometric technique, but macro- or semi-micro-analytical methods were substituted for the study of glycolysis and P interchange.

The toxin usually employed was a dried preparation containing 250,000 m.l.d. per g. (the gift of the Evans Biological Institute, to whom our thanks are due), free of preservatives, which had been found undesirable; it was used in large excess of the m.l.d., usually 1 or 2 mg.

Enzyme preparations were obtained as far as possible from heart muscle, which is known to be affected in diphtheria.

EXPERIMENTAL

Glycolysis

Finely chopped fowl's heart muscle was suspended in Ringer solution at pH 7.4 in a Krebs flask [Krebs & Henseleit, 1932], and incubated for 3-4 hr. at 38° in a stream of O₂ containing 5% CO₂. Flasks were set up in pairs, one containing toxin. Lactic acid was determined before and after incubation by the method of Friedemann *et al.* [1927].

Table 1 shows typical results from a large number of experiments. The increases in lactic acid per dg. of dried tissue are practically identical with and without toxin. Pyruvate was never detected in the mixture after incubation. Anaerobic experiments in an atmosphere of N₂ with 5% CO₂ also gave negative results.

Table 1. *Glycolysis*

2 g. tissue in 40 ml. Ringer medium (Ringer phosphate for Exp. 12). Glucose, 0.2%. Toxin 0.5 ml. (=25 m.l.d.) Burroughs Wellcome conc. solution, dil. 1 in 50.

Exp.	Incubation hr.		Tissue (g.) dry wt.	Lactic acid (mg.)		Increase (mg./dg. dry tissue)
				Initial	Final	
10	3	Control	0.3	1.7	2.4	0.29
		Toxin	0.3	2.0	2.1	0.03
12	3.5	Control	0.44	2.5	4.9	0.55
		Toxin	0.42	1.5	3.9	0.58

Tissue respiration

Slices of fowl's heart, suspended in phosphate Ringer at pH 7.4, were employed for a long series of manometric experiments in presence of glucose, glucose and citrate, glucose and lactoflavin, lactate, lactate and fumarate, and succinate. The manometer flasks were filled with pure O₂ and shaken for an hour at 38°. Parallel 'blank' experiments were run with the tissue but no substrate.

The results indicated that toxin caused no regular alteration in Q_{O₂}.

Oxidizing systems

A number of the oxidase systems that have been shown to exist in the body were set up *in vitro* and the effect of diphtheria toxin examined on each of them under various conditions depending on the particular enzyme being studied; these included succinic, lactic, malic, β -hydroxybutyric, α -glycerophosphoric and pyruvic dehydrogenases, and amine, amino-acid and adrenaline oxidases. The autoxidation of adrenaline in buffer was also studied.

Cozymase, when required, was prepared from yeast by a method described by Green (private communication), and further purified with CuCl [Euler *et al.* 1936]. We are indebted to Messrs Roche Products Ltd. for the gift of a sample of cocarboxylase for use in the pyruvic dehydrogenase experiments.

Acetone-dried preparations supplemented by diaphorase were used in a few experiments with lactate, malate and pyruvate.

Oxidations were generally carried out in phosphate buffer at pH 7.2-7.4 (except where otherwise stated) and an atmosphere of pure O₂. Toxin was added direct to the enzyme in some experiments, but in others it was mixed with the enzyme (and often cozymase as well) on the previous day and the mixture left overnight in the ice-box to attempt to allow for the 'latent period' observed with the toxin *in vivo*.

Tables 2-9 show typical results for all these series of experiments. The total number of experiments is indicated at the head of each table.

Lecithin. The effect of the toxin was studied on the oxidation of lecithin in presence of glutathione [Hopkins, 1925]. Experiments were carried out in acetate buffer at pH 4.6, which was found to be the optimum reaction. Preliminary contact with the toxin for varying periods was tried with each reactant. Other proteins, named in Table 10, were used for comparative experiments to ascertain if they produced any effects similar to that of toxin.

The final volume of liquid in the manometer flasks was always 2 ml., about 1 ml. of enzyme suspension being used and the other reagents added in the concentrations or dry weights given. If cyanide was present the precautions of Krebs [1935] were followed with respect to the CO₂-absorbing liquid in the inner cups.

Table 2. *Succinic dehydrogenase*

1 ml. enzyme [Stotz & Hastings, 1937] from heart of pig or sheep; 0.05 *M* phosphate, pH 7.4; 0.05 *M* succinate; 1 mg. toxin overnight with enzyme. Twenty-three experiments.

Exp.		O ₂ uptake in 30 min. (μl.)	
		Control	Toxin
15	No methylene blue	276	290
	Methylene blue, 0.008 %	278	261
23	No selenite	339	342
	Selenite, $0.5 \times 10^{-3} M$	19	17
16	HCN, $2.5 \times 10^{-3} M$, methylene blue 0.008 %	50	45

Table 3. *Lactic, malic and β-hydroxybutyric dehydrogenases*

1 ml. lactic [Green & Brosteaux, 1936], malic [Green, 1936, 2], or β-hydroxybutyric dehydrogenase [Green *et al.* 1937], from pig or sheep heart; 0.025 % methylene blue, 0.015 *M* pyrophosphate; HCN, hydrazine or semicarbazide (0.015 *M*) as ketone fixative; toxin overnight with enzyme. Ninety-eight experiments.

Exp.	Substrate	O ₂ uptake per hr. (μl.)	
		Control	Toxin
46	Lactate	249	230
82*	Lactate	29	27
29	Malate	197	203
84*	Malate	24	24
79	β-Hydroxybutyrate	40	41

*Dried muscle enzyme with heart flavoprotein [Straub, 1939] as diaphorase.

O₂ uptake was not altered significantly by overnight contact between the toxin and the enzyme or enzyme-cozymase, presence or absence of pyrophosphate, or any particular ketone fixative.

Table 4. *α-Glycerophosphate dehydrogenase*

1 ml. enzyme [Green, 1936, 1] from guinea-pig skeletal muscle; 0.04 *M* α-glycerophosphate 2 mg. toxin; pH 7.2. Two experiments.

Exp.		O ₂ uptake per hr. (μl.)	
		Control	Toxin
1	No methylene blue	43	42
2	Methylene blue, 0.075 %	170	168

Table 5. *Pyruvic dehydrogenase*

0.8 ml. enzyme [Lipmann, 1939] from skeletal or heart muscle; 0.2 *M* NaF, 0.04 *M* phosphate pH 7.2, 0.04 *M* pyruvate, 4 mg. adenylic acid (yeast), 3 mg. toxin; 1 mg. cozymase and 1 mg. cocarboxylase in side-bulb with pyruvate. Seven experiments.

Exp.		O ₂ uptake per hr. (μl.)	
		Control	Toxin
2		171	176
6	0.25 mg. lactoflavin (Roche) added	27	29

Table 6. *Amine oxidase*

1 ml. enzyme [Richter, 1937] from liver; 0.5 mg. toxin added in 0.9 % NaCl, 0.001 *M* HCN as ketone fixative. Seven experiments.

Exp.	Substrate	O ₂ uptake per hr. (μl.)	
		Control	Toxin
4	Tyramine, 0.04 <i>M</i>	66	71
5	Adrenaline, 0.005 <i>M</i>	84	87

Table 7. *Amino-acid oxidase*

1 ml. enzyme [Krebs, 1933] from guinea-pig kidney; 0.1 *M* hydrazine, 1 mg. toxin added solid or in 0.9% NaCl, pH 7.4. Fourteen experiments.

Exp.	Substrate	O ₂ uptake per hr. (μl.)	
		Control	Toxin
4	<i>d</i> -Alanine, 0.1 <i>M</i>	95	82
11	<i>L</i> -Cysteine, 0.025 <i>M</i>	214*	222*
13	<i>L</i> -Cysteine, 0.025 <i>M</i> (autooxidation—no enzyme)	158	169

* Total O₂ uptake.

Table 8. *Adrenaline oxidase*

Enzyme [Blaschko *et al.* 1937] used crude or purified by precipitation with 30% alcohol or isoelectrically at pH 5.0; 0.013 *M* adrenaline, 0.5 mg. toxin, pH 7.4. Sixty-two experiments.

Exp.	Enzyme	Addition	O ₂ uptake per hr. (μl.)	
			Control	Toxin
35	Crude	2 mg. glutathione	302	303
28	Alcohol ppt.	HCN, 0.001 <i>M</i>	35	45
51	Ppt. at pH 5.0	0.16 mg. ascorbic acid	174	178

The presence of HCN, glutathione, or ascorbic acid, or of any two of these together, caused no significant alteration in the effect of toxin.

Table 9. *Autoxidation of adrenaline*

Adrenaline 0.006 *M* in 0.1 *M* phosphate (Na and K salts), pH 7.4; 1 mg. toxin added in water. Eighty-seven experiments.

Exp.		O ₂ uptake per hr. (μl.)	
		Control	Toxin
77		208	211
14	HCN, 0.001 <i>M</i>	96	104
42	Normal buffer	308	199
	Na buffer free of K	223	186
49	Normal buffer—toxin in isotonic NaCl	223	164
	K buffer free of Na—toxin in isotonic KCl	121	124

Table 10. *Oxidation of lecithin*

1.6 ml. lecithin suspension (ox liver) in water; toxin and other proteins added solid; glutathione (4 mg. except where otherwise stated) in water in side-bulb, pH 4.6. Forty-four experiments.

Exp.	Toxin (mg.)	O ₂ uptake per hr. (μl.)	
		Control	Toxin
30	2	105	140
(Diphtheria antitoxin, 131; toxin—antitoxin (overnight contact), 144 μl. per hr.)			
32	2	143	147
(Egg-white, 46; wheat-gluten, 110 μl. per hr.)			
36	2	76	100
(Antitoxin, 89; toxin—antitoxin (mixed direct), 88; cryst. ovalbumin, 88 μl. per hr.)			
33	2	116	177
	1	116	170
	0.5	116	156
	0.25	116	148
41	0.25 Glutathione, 1 mg.	67	79
	Glutathione, 0.5 mg.	73	106

In individual experiments in each group occurred considerable random variations from +50 to -50%, with and without toxin, but taken collectively the results with toxin showed no real deviation from the normal under most of the conditions studied.

The net O_2 uptake for every enzyme varied with each individual preparation, but duplicate experiments with a fresh preparation showed good agreements in both the control and toxin series; with an uptake of 100 μ l. or more per hour and the manometer taps frequently re-greased there was rarely obtained a deviation greater than $\pm 10\%$.

With lecithin and glutathione the toxin most frequently caused an acceleration (see Table 10). The accelerations sometimes produced by the other proteins were rarely of the same order as that from toxin.

In the autoxidation experiments with adrenaline where retardations were obtained, the most interesting results were those in absence of either Na^+ or K^+ , as shown in Table 9; the retardation in presence of Na^+ and the negative effect with K^+ were too consistent to be due to experimental error. In later experiments, however, with both ions present, consistent retardation was not found, although external conditions were precisely the same.

Phosphorus exchange

Nucleotidase activity. The toxin was tested for nucleotidase activity by incubation for periods of 3 hr. in Krebs flasks with a mixture of nucleotides prepared by hydrolysis of vegetable sodium nucleate with alkali in the cold. Free phosphoric acid was determined colorimetrically before and after incubation, and control vessels were run without toxin. When toluene was present as antiseptic the increase in free phosphoric acid was virtually zero in both vessels.

Phosphorylation. The effect of toxin on three phosphorylation systems was investigated.

For the coupled oxidation-phosphorylation system of Needham & Pillai [1937], the enzyme was prepared from rabbit or guinea-pig skeletal muscle, or fowl's liver brei; the hydrogen acceptor was pyruvate, whilst both glycerophosphates and hexosediphosphate were used as donators. The mixed nucleotides used in the nucleotidase experiments, adenylic acid, or aneurin, served as P acceptor. Experiments were again of the macro-type, the mixtures being incubated for an hour at 38° ; inorganic P was determined before and after incubation by the method of Fiske & Subbarow [1925]. Aneurin was found to interfere with the colorimetric determinations by forming a precipitate with the molybdate reagent, and was first removed by adsorption on kaolin.

Total inorganic P disappearance was in general very small, and was in most cases unaltered by toxin even after overnight contact. But with the enzyme suspensions from the muscles of one particular rabbit we obtained a series of results where the toxin caused an increase in P disappearance if left overnight in contact with the enzyme. Table 11 shows typical results from this series. We are inclined to think that these positive results originated in some degree from the particular animal, the treatment of which, however, differed in no way from that of the others except that it was killed by chloroform instead of by a blow on the neck. These active enzyme suspensions were also found to give a precipitin reaction with the toxin.

For investigation of the pyruvate-adenylic acid system of Lipmann [1939] (see Table 5), and the glucose-kidney-extract system of Kalckar [1939], O_2 uptake was measured manometrically and inorganic P afterwards determined in the contents of the flasks. In neither case was the toxin found to affect P esterification.

Table 11. *Phosphorylation of nucleotides*

2 ml. enzyme suspension from muscle of chloroformed rabbit; 0.01 *M* glycerophosphate*, 0.02 *M* pyruvate, 0.01 *M* fluoride, 0.03 *M* phosphate, pH 7.4; 5 mg. toxin, overnight with enzyme, 0.5 mg. mixed nucleotides. Total volume, 4 ml. Incubation, 30 min. at 38°.

Exp.		Phosphorus (mg.)		
		Before incubation	After incubation	Esterified
4	Control	5.26	4.65	0.61
	Toxin	5.71	4.17	1.54
8	Control	4.35	4.35	0.0
	Toxin	5.00	4.56	0.44

* B.D.H.—largely β - with some α -isomeride.

DISCUSSION

The results of this investigation up to the point reached in these experiments indicate that the enzyme systems studied are not directly affected *in vitro* by diphtheria toxin. This may be regarded as conclusive in the cases of succinic, lactic, malic and β -hydroxybutyric dehydrogenases, but for the other systems some further investigation to a degree depending on the system concerned might be advantageous.

On the other hand, the symptoms observed in diphtheria which suggested these investigations are still unexplained. The interference with the action of the heart is apparently not the result of inhibition of the muscle oxidases. So far as lactic dehydrogenase is concerned our results form a corollary to those of Corkill & Ochoa [1934], who showed that a toxæmic liver will not permit the resynthesis of glycogen from lactate. It follows therefore that all lactate formed in the body in diphtheria has to be oxidized in the muscles, so that any interference with the action of the lactic dehydrogenase would lead to a pronounced lactacidaemia.

We have so far made no attempt to study the effect of toxin on the oxidation of fats, and it must be remembered that the other symptoms of diphtheria that might be traced back to a biochemical origin—ketonuria and accumulation of fat in the heart—can quite conceivably be the result of interference with these processes.

The 'latent period' between the injection of toxin *in vivo* and the manifestation of its effect constitutes another factor which must be considered in connexion with the chemical effects observed. It is apparently the same length (about 48 hr.) under all conditions. It is known that the toxin on injection passes direct into the muscle tissue, so that one of two processes, or possibly a combination of both, may take place during the latent period. Either the toxin may pass into the interior of the cells and seriously disturb their internal equilibrium by its presence, or a further chemical change of the toxin is necessary before it becomes actually toxic, and occurs in the tissues during the latent period. On the face of it, there is a strong argument against the first possibility in that the appearance of the symptoms is very rapid at the end of the period, whereas if the toxin passed slowly into the tissue it would do its harmful work as it proceeded, so that the symptoms should appear gradually. As regards the second possibility, there is no real proof that the 'toxin' as produced from cultures of *C. diphtheriae* is the actual toxic substance, since it is grown on synthetic media *in vitro*, and has never been isolated from diphtheritic tissue. Further, Pappenheimer [1938] has shown that the toxic properties are destroyed by acetylation

with ketene, the number of amino-groups acetylated corresponding closely with the number of ϵ -amino-groups of lysine in the molecule. He was careful to point out that the toxic properties might equally possibly be due to the spatial arrangement of the ϵ -amino-groups as to their actual presence, but whichever is the correct postulation the free amino-groups form a basis for further chemical changes to the molecule in the body. If such a reaction were to occur in the body, it is quite conceivable that the 'super-toxin' formed might affect these enzymes in a way which the substance grown *in vitro* would be unable to do.

A serious disadvantage with all these investigations is that up to the present a 'chronic' diphtheritic condition has not been achieved in any animal for *in vivo* study.

SUMMARY

Experiments have been carried out *in vitro* to study the effect of diphtheria toxin on tissue respiration, glycolysis and a number of dehydrogenase and phosphorylation systems under varied conditions.

The dehydrogenase systems for succinic, lactic, malic and β -hydroxybutyric acids have been shown conclusively to be unaffected by toxin, even after preliminary contact between the enzyme and toxin, whilst the indications are that other systems studied are also unaffected. Oxidation of lecithin by glutathione is to some extent accelerated. The effect on autoxidations is inconclusive.

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