ALTERATIONS IN THE LEVEL OF MUSCLE PHOSPHOCREATINE OF GUINEA PIGS PRODUCED BY THE INJECTION OF DIPHTHERIA TOXIN*

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An investigation of the acid-soluble phosphorus compounds in the muscle of guinea pigs injected with diphtheria toxin was undertaken in the hope of finding a clue to the nature of the metabolic disorders which must underlie the manifestations of such a toxemia. Although a good deal has been written about the changes in carbohydrate metabolism produced in animals by Corynebacterium diphtheriae or its toxin (1, 2), as yet there has been no demonstration of a primary metabolic defect adequate to explain death. Pappenheimer has collected evidence indicating that the diphtheria toxin is the protein part of bacterial cytochrome b, and he has suggested that the primary defect produced by the toxin in the host is inhibition of synthesis of a comparable host enzyme (3–6).

Since weakness is a major symptom of this and other infectious diseases, and since death may be looked upon as a failure either to utilize or produce energy adequately, the high energy bond organic phosphorus compounds seemed a logical place to look for a fundamental metabolic defect leading to death. It is well recognized that peripheral circulatory collapse or shock is a frequent finding in terminal infectious states, and it has been shown by Bollman and Flock (7) that shock decreases the muscle stores of high energy phosphate bonds. Studies of arterial and venous blood oxygen content were, therefore, included so that if a change was found in the energy reserves it might be possible to determine whether this was caused by a failure of oxygen transport to the tissues, or by inability of the tissues to utilize adequately the oxygen transported to them because of a defect such as that postulated by Pappenheimer.

Materials and Methods

Guinea pigs, weighing between 360 and 450 gm., before fasting, and fed a diet of Purina rabbit chow supplemented with daily injections of 20 mg.

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of ascorbic acid for at least 6 days immediately before use, were the experimental animals. Acid-soluble phosphorus compounds in the gastrocnemius muscle were studied in four groups of eight animals each. Uninjected animals served as controls; a second group was injected subcutaneously in the abdominal region with 0.25 ml. of a solution of crude diphtheria toxin\(^1\) in physiological saline, each ml. of which contained 3.7 Lf units. To rule out the possibility that effects observed with crude toxin were the results of impurities, a third group received comparable doses of an 85 per cent pure toxin given us by Dr. A. M. Pappenheimer, Jr., while the fourth group was injected with the same dose of purified toxin neutralized with commercial antitoxin. The dose of toxin was selected to give maximum sublethal toxicity in the 18 to 20 hour fast allowed after injection. The animals which received toxin alone all suffered from mild to severe toxicity as evidenced by weakness, ataxia, ruffling of the fur, and in a few cases coma and death. Only those animals which were still alive were used in these studies. Postmortem examination revealed congested adrenals and congestion and hemorrhage in the gut walls and the abdominal lymph glands. The animals which received the neutralized toxin had no clinical or postmortem evidences of toxicity.

Following injection with toxin the animals were fasted from 18 to 20 hours, but allowed water. Nembutal was used as the anesthetic, under which rectal temperatures were taken and one gastrocnemius muscle was removed for acid-soluble phosphorus fraction analysis. Fractionation was carried out by a modification of the method of Umbreit, Burris, and Stauffer (8) described below. Phosphorus concentration was determined in the fractions by the method of Fiske and Subbarow (9) with an Evelyn colorimeter and a 660 m\(\mu\) filter.

Two other groups of eight guinea pigs each were submitted to studies of arterial and venous blood oxygen and hemoglobin content. An uninjected group served as the controls, while the experimental group received crude diphtheria toxin as above. Blood was taken under nembutal anesthesia from the aorta and common iliac vein. Hemoglobin was determined by the method of Evelyn (10), blood oxygen content by the method of Roughton and Scholander (11), and oxygen capacity was calculated by the hemoglobin-combining capacity as given by Peters and Van Slyke (12).

Statistical analyses were carried out by the methods of Snedecor (13).

**EXPERIMENTAL**

Since the main object of this study was to measure changes in the high energy phosphorus compounds of muscle in diphtherial toxemia, it was

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\(^1\) Obtained through the kindness of the Lederle Laboratories Division, American Cyanamid Company.
felt that the usual methods of separating the phosphorus fractions by employing barium were unnecessarily complex. Simplifications were made and tested in rats so that our results could be compared with those already in the literature. Stimulated muscles with or without a recovery period, as well as resting muscles, were used in order to test the reliability of the simplified method in a variety of situations.

Fractionation was carried out in the following manner. After careful removal under nembutal anesthesia, the gastrocnemius muscle was immediately frozen between two previously flattened blocks of dry ice. An appropriate amount of tissue was then chipped off and weighed on the chilled pan of a micro torsion balance and then ground in 6 per cent trichloroacetic acid in a glass tissue grinder. Grinding and all subsequent steps except hydrolysis and phosphorus determination were performed in the cold room at 3-5°. Following centrifugation, an aliquot of the supernatant was used for 180 and 7 minute phosphorus determinations (180 minute P and 7 minute P), in the usual way, by hydrolyzing in a boiling water bath with 1 \( N \) hydrochloric acid and determining the phosphorus content. The difference between these two values is reported as ester phosphorus (ester P). Another aliquot of the deproteinized extract was neutralized with 0.5 \( N \) sodium hydroxide to phenolphthalein and the inorganic phosphorus (inorganic P) was precipitated with 10 per cent calcium chloride. The precipitate was taken up in dilute hydrochloric acid and phosphorus was determined. Orthophosphorus (ortho P), comprising phosphocreatine (PCP) and inorganic P, was determined on an aliquot of the neutralized extract by allowing a 20 minute hydrolysis period at room temperature in the acid molybdate solution. Adenosine triphosphate (ATP) was calculated as the difference between the 7 minute P and the ortho P, PCP as the difference between ortho P and inorganic P, and total high energy bond phosphorus (\( \sim P \)) was the sum of ATP and PCP. All results were calculated as mg. of phosphorus per 100 gm. of muscle (wet weight), except in the case of ATP for which the 2 labile phosphorus atoms only are reported.

Results

In several cases, phosphocreatine was determined directly on the supernatant fluid after precipitation of inorganic P as a check on the method by difference. The maximum error was 5.2 per cent. The adequacy of the precipitation of inorganic P by calcium chloride was checked by adding known amounts of inorganic phosphate to neutralized aliquots of tissue extracts containing known amounts of inorganic P. Recoveries ranged from 96.2 to 104 per cent of the added phosphorus.

In Table I are presented the results obtained in three groups of eleven
rats each. The first group was resting animals, the second was stimulated through the sciatic nerve by an electronic stimulator 180 times in 1 minute with a 100 gm. weight attached to the severed Achilles tendon and allowed

| Table I |

**Results of Phosphorus Determinations on Eleven Rats As Mg. of P Per 100 Gm. of Tissue**

Means and standard errors are reported.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Resting rats</th>
<th>Stimulated rats, 5 min. recovery</th>
<th>Stimulated rats, no recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>180 min. P</td>
<td>156.3 ± 3.4</td>
<td>146.7 ± 2.6</td>
<td>146.7 ± 2.4</td>
</tr>
<tr>
<td>Ester P</td>
<td>24.3 ± 1.9</td>
<td>24.3 ± 1.1</td>
<td>25.2 ± 1.8</td>
</tr>
<tr>
<td>ATP*</td>
<td>41.1 ± 2.7</td>
<td>37.9 ± 1.2</td>
<td>38.4 ± 1.5</td>
</tr>
<tr>
<td>PCP</td>
<td>60.3 ± 1.7</td>
<td>60.0 ± 2.7</td>
<td>20.0 ± 1.3</td>
</tr>
<tr>
<td>Inorganic P</td>
<td>30.6 ± 1.9</td>
<td>24.4 ± 2.9</td>
<td>63.2 ± 1.3</td>
</tr>
<tr>
<td>~P</td>
<td>101.4 ± 2.2</td>
<td>97.9 ± 3.1</td>
<td>68.4 ± 1.9</td>
</tr>
</tbody>
</table>

* The two high energy bond P's only are reported.

| Table II |

**Acid-Soluble Phosphorus Fractions in Gastrocnemius Muscle of Guinea Pigs Receiving Purified and Crude Diphtheria Toxin and Purified Toxin Plus Antitoxin**

Probability of variations occurring by chance (P values) recorded between values to which they apply.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Controls, P values</th>
<th>Crude toxin P values</th>
<th>Pure toxin P values</th>
<th>Pure toxin and antitoxin P values</th>
<th>Pure controls, P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>180 min.</td>
<td>137.2 ± 4.8</td>
<td>133.5 ± 4.5</td>
<td>135.4 ± 2.1</td>
<td>149.6 ± 7.6</td>
<td>0.2-0.1</td>
</tr>
<tr>
<td>Ester</td>
<td>18.8 ± 1.8</td>
<td>18.8 ± 0.5-0.4</td>
<td>20.5 ± 1.1</td>
<td>23.1 ± 1.9</td>
<td>0.2-0.1</td>
</tr>
<tr>
<td>ATP</td>
<td>34.2 ± 2.0</td>
<td>32.8 ± 0.5-0.5</td>
<td>33.9 ± 1.9</td>
<td>31.2 ± 2.8</td>
<td>0.4-0.3</td>
</tr>
<tr>
<td>PCP</td>
<td>58.1 ± 1.2</td>
<td>34.8 ± 0.5-0.5</td>
<td>34.1 ± 0.1</td>
<td>65.9 ± 3.7</td>
<td>0.1-0.05</td>
</tr>
<tr>
<td>Inorganic P</td>
<td>26.1 ± 2.1</td>
<td>47.2 ± 0.5-0.5</td>
<td>46.9 ± 0.1</td>
<td>29.5 ± 2.9</td>
<td>0.4-0.3</td>
</tr>
<tr>
<td>~P</td>
<td>92.3 ± 2.1</td>
<td>67.6 ± 0.5-0.5</td>
<td>68.0 ± 0.1</td>
<td>97.0 ± 4.7</td>
<td>0.4-0.3</td>
</tr>
</tbody>
</table>

a 5 minute recovery period before removal of the muscle, while the third was similarly stimulated but allowed no recovery. There is, in general, good agreement between our results and the results of other investigators.
on resting rat and guinea pig muscle, and on stimulated rat muscle with and without a recovery period (7, 14-17). The simplifications are therefore justified for this study, since the method gives reliable results both in resting and stimulated animals.

The results of the phosphorus determinations in animals injected with diphtheria toxin are shown in Table II. It will be seen that both crude and pure diphtheria toxin produced almost identical statistically significant decreases in PCP, with a comparable rise in inorganic P. A comparable fall in $\sim P$ took place since the ATP values were unchanged. There were no significant differences in the results produced by the pure and crude toxins, and injection of pure toxin neutralized with antitoxin caused no significant changes from the normal values.

The results of the blood oxygen studies are reported in Table III. Injection of crude diphtheria toxin produced a marked hemoconcentration with an associated marked increase in arterial oxygen content, as there was only a slight decrease from the control value in the arterial oxygen saturation. On the venous side there was a marked fall in oxygen saturation, although the oxygen content was only slightly below the normal level. The arteriovenous oxygen difference rose markedly following the injection of toxin.

Rectal temperatures in the animals which received unneutralized toxin ranged from 26.7-37.8°, while the controls varied from 34.8-38.8°. The marked fall below the normal range in some of the controls is probably the result of anesthesia (18).
DISCUSSION

The fall in PCP and consequently in \( \sim \text{P} \) represents a significant lowering of the muscle energy reserves. The fact that the crude and pure toxin produced the same change and that this could be prevented by neutralization of the toxin with antitoxin before injection indicates that this effect was the result of the toxin itself and not of some impurity carried over from the culture medium.

Since the high energy phosphorus compounds are the only known source of energy for muscular and chemical work in the body, a reduction in the reserve of these compounds is obviously a serious metabolic defect. This is particularly true in resting muscle in which the rate of utilization of ATP and PCP is at a minimum, and consequently implies a much larger percentage decrease in the maximum rate of synthesis of \( \sim \text{P} \) than the percentage fall in \( \sim \text{P} \) content. The significance of this change is enhanced by the fact that it occurred, in most of the animals at least, before they were moribund, and several hours before they would be expected to die. Thus the phenomenon is not merely a terminal one, and may have some causal relationship to the manifestations of toxemia and ultimately to death from the toxemia.

The arteriovenous oxygen differences might at first glance suggest that the fall in \( \sim \text{P} \) may be the result of shock, since a high arteriovenous oxygen difference, which the toxemic animals exhibited, is one of the characteristics of shock (19), and since it is known that shock causes a fall in muscle \( \sim \text{P} \) (7). This latter finding is to be expected because shock is essentially a failure of oxygen transportation to the tissues, and because the synthesis of high energy bond phosphate is predominantly an aerobic process. The real question is then whether or not these figures represent a failure of oxygen transportation to the tissues with consequent tissue anoxia.

The adequacy of oxygen supply to the tissues depends on the relative rates of tissue utilization and blood transportation of oxygen. There is no evidence of an increased rate of tissue utilization of oxygen in the experimental animals. In fact quite the reverse is true, since the muscles are at rest and there is a fall in body temperature at this stage of the toxemia. The fact that the venous content of oxygen is nearly normal indicates that more oxygen is being transported to the tissues than they need, since in tissue anoxia caused by shock the venous blood draining the leg has a markedly lowered oxygen content frequently approaching zero (20–22). It is conceivable that tissue anoxia might have existed in these animals in the presence of considerable amounts of venous oxygen if the rate of blood flow through the capillaries was too rapid to allow adequate
time for diffusion of oxygen into the tissues. In the animals receiving diphtheria toxin the circulation was slowed rather than speeded up, however, as shown by the fact that the veins were narrower than normal and that blood could be withdrawn from the aorta or common iliac veins only at a much slower rate than in the controls.

It would seem likely, therefore, that defective oxygen transportation and tissue anoxia were not the cause of lowered muscle \( \sim P \) reserves, and that the explanation must be sought in either defective tissue utilization of oxygen because of some such defect as that postulated by Pappenheimer, or in defective coupling of inorganic phosphorus uptake to oxidation.

**SUMMARY**

Diphtheria toxin, both crude and purified, when injected into guinea pigs, produced a fall in phosphocreatine and, since adenosine triphosphate remained unchanged, a comparable fall in total high energy phosphates in the gastrocnemius muscle. Toxin neutralized with antitoxin failed to produce these changes. Toxin also produced a marked hemoconcentration and increase in the arteriovenous oxygen difference in the blood supplying the hind leg. The oxygen content of the venous blood was only slightly reduced, however. The causal relationships between the changes in circulatory status and the acid-soluble phosphorus compounds, as well as the relationship between these findings and the manifestations of toxemia, are discussed.

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**BIBLIOGRAPHY**

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