THE IN VIVO INACTIVATION BY CYANIDE OF BRAIN CYTOCHROME OXIDASE AND ITS EFFECT ON GLYCOLYSIS AND ON THE HIGH ENERGY PHOSPHORUS COMPOUNDS IN BRAIN

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The symptomatology of cyanide poisoning has been known for well over 100 years. Claude Bernard (1) was among the first to stress the inhibitory effect on the respiration of higher animals. The work of Keilin (2) and of Stotz (3) has shown that cyanide ion combines in vitro with cytochrome oxidase and thereby interferes with the utilization of molecular oxygen by the tissue oxidation-reduction systems. It has also been demonstrated that the utilization of molecular oxygen is coupled with phosphorylation reactions (4–7), and Lipmann (8) has emphasized the rôle of aerobic metabolism in the resynthesis of high energy phosphorus compounds. LePage\(^1\) analyzed the tissues of rats subjected to experimental shock in a Noble-Collip rotating drum and observed elevated lactic acid and inorganic phosphate, low glycogen, adenosine triphosphate depletion, some phosphocreatine depletion, and an abnormal accumulation of phosphopyruvate. Proger, Decaneas, and Schmidt (9) have recently found that the readily hydrolyzable phosphorus fraction of kidney and heart tissue was decreased when rats were exposed to low oxygen tensions. These results indicate that in the intact animal also the resynthesis of high energy phosphorus compounds is coupled with oxidative processes.

The present experiments were undertaken, first, to determine whether brain tissue from cyanide-poisoned rats showed a diminution in cytochrome oxidase activity and, secondly, to study in some detail the distribution of glycogen, lactic acid, and phosphorylated intermediates in such tissue, particularly with reference to the distribution pattern of high energy phosphorus compounds.

**EXPERIMENTAL**

*Measurement of Brain Cytochrome Oxidase Activity after Cyanide Injection*—A dose of 5 mg. per kilo of NaCN in aqueous solution was injected intraperitoneally into each of a series of adult male rats ranging in weight from about 250 to 400 gm. Rats so injected showed a rapid respiratory

\(^1\) LePage, G. A., personal communication.
stimulation, followed by agitation, incoordination, convulsions, and ultimate cessation of respiration. At time intervals varying from 3½ to 8 minutes after injection, when respiration had just ceased, the animals were decapitated and the brain quickly removed and weighed on a torsion balance. The brain was then dropped into a Waring blender and enough iced, distilled water was added to make a 2 per cent final homogenate. The tissue was then homogenized for 2 minutes and strained through several layers of gauze to remove gross particles. Immediately before use, each homogenate was diluted with an equal volume of iced, distilled water. Control animals not injected with cyanide were treated in a similar fashion. Cytochrome oxidase activity was measured spectrophotometrically, as previously described.2

Preparation of Brain Tissue for Analysis—The methods and scheme of LePage and Umbreit (10) were followed in preparing the brain tissue for analysis and in analyzing for the various phosphorylated components. To minimize changes in the labile components attendant upon the sacrifice of the rats, the control or experimental animal was anesthetized by intraperitoneal injection of 50 mg. of pentobarbital per kilo, and immersed in liquid nitrogen.

The control animals were placed in liquid nitrogen 14 minutes after the injection of pentobarbital, when most of them were in surgical anesthesia; rats showing movement on immersion were not used. In the cyanide-poisoned animals, 5 mg. of NaCN per kilo were injected intraperitoneally 10 minutes after the administration of pentobarbital. These cyanide poisoned, anesthetized rats, in contrast to the cyanide-injected, unanesthetized animals, did not show any convulsions. Observations on a group of rats treated in this manner had shown that almost immediately after the administration of cyanide they exhibited a respiratory stimulation, followed within 1½ to 2 minutes by the onset of apnea of about 1½ to 2 minutes duration. The apnea was succeeded by gasping, irregular breathing, cessation of respiration, and death at about 8 to 13 minutes after the injection of cyanide, or about 18 to 23 minutes after the injection of pentobarbital. The rats were allowed to remain in the liquid nitrogen for 1½ to 2 minutes, at the end of which time they were removed and decapitated with a sharp axe.

Analyses of Chemical Components—The brain was extracted in trichloroacetic acid, according to the procedure of LePage and Umbreit (10). The trichloroacetic acid extract was quickly neutralized to pH 8.2 and used for the determination of the following constituents: lactic acid, inorganic phosphate, phosphocreatine, phosphopyruvic acid, and total acid-soluble phosphorus. The tissue residue remaining after extraction was used for

The procedures used were in accordance with those described in the analytical scheme of LePage and Umbreit (10), with two exceptions. In the determination of phosphopyruvate, no correction was made for the small amount of triose phosphate released in alkaline solution. The method used for the determination of glucose was that of Nelson (11).

The fractionation of the neutralized trichloroacetic acid filtrate was also carried out in accordance with the analytical scheme of LePage and Umbreit (10). To this filtrate were added a 25 per cent solution of barium acetate (0.05 ml. per mg. of phosphorus) and 4 volumes of cold 95 per cent ethanol. After standing overnight in the refrigerator, the mixture was centrifuged. The supernatant fluid (alcohol-soluble fraction) was poured off. The precipitate was dissolved in a few drops of N HCl, several ml. of water were added, and the pH was adjusted to 8.2 with 2 N NaOH. The resulting suspension was chilled for 20 minutes and then centrifuged. The supernatant fluid (alcohol-insoluble, barium-soluble fraction) was poured off. The precipitate (barium-insoluble fraction) was dissolved in a few drops of N HCl, several ml. of water were added, and the barium was removed by precipitation with 10 N H2SO4. The supernatant fluid was decanted, the barium sulfate precipitate washed once with water and centrifuged, and the washings added to the supernatant fluid from the first centrifugation. The combined supernatants, representing the barium-insoluble fraction, were neutralized and made up to volume. The first fraction (alcohol-soluble fraction) contains compounds of negligible interest and hence was not analyzed in the present work. The second fraction (alcohol-insoluble, barium-soluble fraction) contains, among other phosphorylated intermediates, phosphocreatine and phosphopyruvate. However, since these compounds had been determined in aliquots from the neutralized trichloroacetic acid filtrate before treatment with barium acetate, this second fraction was not subjected to analysis. The third fraction (barium-insoluble fraction) contains inorganic phosphate, adenosine triphosphate, adenosine diphosphate, hexose diphosphate, and phosphoglycerate; these compounds were determined in accordance with LePage and Umbreit's scheme (10). All three fractions were analyzed for total phosphorus as a measure of the fractionation procedure. In the various experiments carried out, the sum of the total phosphorus of these three fractions ranged from 92 to 99 per cent of the total phosphorus in the original trichloroacetic acid filtrate.

Results

Cytochrome Oxidase Activity of Brain after Cyanide Injection—Table I shows that the average cytochrome oxidase activity of brain, as measured
by the monomolecular reaction constant for the oxidation of cytochrome \( c \), was \( 0.243 \times 10^{-8} \) for nine normal rats. The average activity for the brains from six rats poisoned with NaCN was \( 0.113 \times 10^{-8} \). There was thus an average decrease of 54 per cent in the cytochrome oxidase activity in the brain.

**Anaerobic Glycolysis and High Energy Phosphorus Compounds after Cyanide Injection**—In Table II are shown the results of four separate experiments, each carried out with two control rats and two rats injected with NaCN. The concentrations of lactic acid, hexose diphosphate, phosphoglycerate, and phosphopyruvate were increased in the brains of the cyanide-poisoned animals. These average increases were marked in the case of lactic acid and phosphopyruvate. Although the changes were more variable for phosphoglycerate and hexose diphosphate, statistical treatment showed that the concentrations of these substances were also significantly increased. The concentration of glycogen in the brain of the cyanide-poisoned animals was significantly and markedly decreased. All these changes indicate quite clearly that in the rat brain, where the cytochrome oxidase has been partially inactivated by cyanide, there is a marked increase in glycolysis.

In contrast to the increase in the intermediaries in the glycolytic chain, the two high energy phosphorylated intermediaries, adenosine triphos-

### Table I

**Cytochrome Oxidase Activity of Brain Homogenates from Control Animals and Animals Injected Intraperitoneally with NaCN (5 Mg. per Kilo)**

The final reaction mixture had a volume of 3 ml. and contained 0.04 ml. of 1 per cent tissue homogenate, 0.01 M phosphate buffer, pH 7.4, and \( 2.31 \times 10^{-5} \) M cytochrome \( c \).

<table>
<thead>
<tr>
<th>Control rats</th>
<th>Cyanide-injected rats</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Animal No.</strong></td>
<td><strong>Monomolecular reaction constant</strong> ( (K \times 10^8) )</td>
</tr>
<tr>
<td>1</td>
<td>0.271</td>
</tr>
<tr>
<td>2</td>
<td>0.275</td>
</tr>
<tr>
<td>3</td>
<td>0.210</td>
</tr>
<tr>
<td>4</td>
<td>0.190</td>
</tr>
<tr>
<td>5</td>
<td>0.265</td>
</tr>
<tr>
<td>6</td>
<td>0.282</td>
</tr>
<tr>
<td>7</td>
<td>0.258</td>
</tr>
<tr>
<td>9</td>
<td>0.210</td>
</tr>
</tbody>
</table>

Average .......... 0.243 0.113
**Table 11**

**Concentrations of Glycogen, Lactic Acid, and Phosphorus Compounds in Brains of Normal and Cyanide-Poisoned Rats**

Glycogen as mg. per 100 gm. of tissue. Inorganic phosphate as mg. of P per 100 gm. of tissue. All other values are calculated as mg. of acid per 100 gm. of tissue.

<table>
<thead>
<tr>
<th>Phosphocreatine</th>
<th>Inorganic P</th>
<th>Lactic acid</th>
<th>Glycogen</th>
<th>Hexose diphosphate</th>
<th>Adenosine triphosphate</th>
<th>Adenosine diphosphate</th>
<th>Phosphoglycerate</th>
<th>Phosphopyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Cyanide</td>
<td>Control</td>
<td>Cyanide</td>
<td>Control</td>
<td>Cyanide</td>
<td>Control</td>
<td>Cyanide</td>
</tr>
<tr>
<td>61.8</td>
<td>33.2</td>
<td>Cyanide</td>
<td>16.5</td>
<td>48.8</td>
<td>24.7</td>
<td>106.3</td>
<td>88.6</td>
<td>34.0</td>
</tr>
<tr>
<td>56.0</td>
<td>39.8</td>
<td>Cyanide</td>
<td>11.0</td>
<td>65.2</td>
<td>33.2</td>
<td>109.5</td>
<td>82.0</td>
<td>55.6</td>
</tr>
<tr>
<td>64.0</td>
<td>43.4</td>
<td>Cyanide</td>
<td>16.1</td>
<td>29.9</td>
<td>25.2</td>
<td>145.0</td>
<td>94.2</td>
<td>77.6</td>
</tr>
<tr>
<td>65.4</td>
<td>47.6</td>
<td>Cyanide</td>
<td>14.0</td>
<td>23.6</td>
<td>20.9</td>
<td>99.2</td>
<td>96.2</td>
<td>75.2</td>
</tr>
<tr>
<td>78.0</td>
<td>43.7</td>
<td>Cyanide</td>
<td>16.3</td>
<td>78.1</td>
<td>39.5</td>
<td>131.0</td>
<td>74.8</td>
<td>38.4</td>
</tr>
<tr>
<td>61.7</td>
<td>38.1</td>
<td>Cyanide</td>
<td>13.8</td>
<td>34.8</td>
<td>20.7</td>
<td>150.0</td>
<td>73.6</td>
<td>43.5</td>
</tr>
<tr>
<td>68.8</td>
<td>22.6</td>
<td>Cyanide</td>
<td>11.8</td>
<td>24.5</td>
<td>28.7</td>
<td>133.0</td>
<td>85.0</td>
<td>44.0</td>
</tr>
<tr>
<td>68.7</td>
<td>32.8</td>
<td>Cyanide</td>
<td>17.7</td>
<td>31.1</td>
<td>74.2</td>
<td></td>
<td>74.0</td>
<td>4.7</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>65.5</strong></td>
<td><strong>39.8</strong></td>
<td><strong>14.9</strong></td>
<td><strong>40.7</strong></td>
<td><strong>28.0</strong></td>
<td><strong>118.6</strong></td>
<td><strong>85.0</strong></td>
<td><strong>55.3</strong></td>
</tr>
</tbody>
</table>

\[ t^* \] 7.0  8.3  8.2  3.9  3.3  5.6  2.9  2.0  4.3
\[ P^\dagger \] <0.01 <0.01 <0.01 <0.01 <0.01 <0.01 0.01 0.06 <0.01

* Difference between the means divided by the standard error of the difference of the means.

† Probability value.
phate and phosphocreatine, show a marked and significant decrease. Adenosine diphosphate, as might be expected, increases as the adenosine triphosphate content of the tissue is lowered. Increases in the concentration of inorganic phosphate were also very marked.

**DISCUSSION**

The present work shows that inactivation of cytochrome oxidase by cyanide, previously demonstrated in vitro, may also be demonstrated to exist in vivo. It would appear that, in the intact animal tissue, anoxia, whether brought about by arterial undersaturation (9), by inadequate peripheral circulation, or by inactivation of cytochrome oxidase, results in a shift from aerobic to anaerobic metabolism and a depletion of high energy phosphorus compounds.

The question arises, however, whether there may not be differences in the degree of these changes in different tissues in the various types of anoxia. LePage found that the concentration of lactic acid was higher by 20 to 75 per cent in moribund shocked rats than in rats killed by asphyxiation and incubated for 20 minutes. In non-moribund, shocked rats the decrease in concentration of the high energy phosphorus compounds and the increases in concentration of inorganic phosphate and lactic acid were much greater in the liver than in the brain. Comparisons of our results with those of LePage reveals that the decrease in the concentration of phosphocreatine and the increases in lactic acid and inorganic phosphate were much more marked in the brain of the cyanide-poisoned rat than in that of the non-moribund, shocked rat.

Further experiments are indicated, however, to define more precisely the pattern of chemical changes in tissue in anoxia due to cytochrome oxidase inactivation ("histotoxic" anoxia). Cyanide poisoning involves disturbances in respiration which may contribute an element of "anoxic" anoxia, and at certain stages may affect cardiac function, and thus lead to inadequate peripheral circulation and an element of "stagnant" anoxia. As has been noted, in stagnant anoxia due to shock, the extent and pattern of chemical changes in tissue vary with the particular tissue. There are no data available on the pattern of chemical changes in tissue in anoxic anoxia. The pattern of chemical changes in different tissues in cyanide poisoning, the extent to which these are complicated by incidental elements of anoxic and stagnant anoxia, and the possible relation of these changes in the various tissues to the degree of cytochrome oxidase inactivation remain to be studied.

**SUMMARY**

Rats, injected intraperitoneally with 5 mg. of NaCN per kilo, showed approximately a 50 per cent decrease in cytochrome oxidase activity in

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the brain. The brains of these cyanide-poisoned rats showed significant decreases in the concentrations of glycogen, phosphocreatine, and adenosine triphosphate, and significant increases in the concentrations of inorganic phosphate, lactic acid, hexose diphosphate, phosphoglycerate, and phosphopyruvate.

These results indicate that anoxia in tissue induced by inactivation of cytochrome oxidase results in a shift from aerobic to anaerobic metabolism and a depletion of high energy phosphorus compounds.

BIBLIOGRAPHY

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