Ammonia toxicity and cerebral oxidative metabolism

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McKhann, GUY M. and DONALD B. Tower, Ammonia toxicity and cerebral oxidative metabolism, Am. J. Physiol. 200(3), 420-424, 1961—Effects of NH₄Cl on oxidative metabolism of cat cerebral cortex slices and mitochondria incubated in vitro were studied. In slices, addition of 10 mM NH₄Cl to the incubation medium resulted in significant (16%) reduction of O₂ uptake, doubling of lactate production and marked increase of glucose utilization compared to control slices. Mitochondria showed a 30-40% decrease of O₂ consumption in the presence of 15 mM NH₄Cl when pyruvate or α-ketoglutarate were substrates, but little or any difference from controls with succinate, glutamic acid or γ-aminobutyric acid as substrates. Pyruvate utilization by ammonia-treated mitochondria was inhibited to the same degree as O₂ consumption and was not increased by supplementing the incubation medium with excess succinate. Additions of α-lipoic acid, thiamine pyrophosphate or DPN to such preparations failed to reverse the NH₄Cl effect. Satisfactory P/O ratios were obtained for all mitochondrial preparations. It is concluded that a primary toxic effect of ammonia on the brain may be direct interference with oxidative decarboxylation of pyruvic and α-ketoglutaric acids.

The association of increased levels of blood and brain ammonia with drowsiness and coma has been reported both in man and experimental animals (1-5). The mechanisms underlying the toxic effects of ammonia have not yet been completely elucidated. Previous studies indicate that several biochemical abnormalities are present in the ammonia-intoxicated brain. Both in vivo and in vitro, there are increased levels of cerebral free glutamine, accompanied by decreased levels of free glutamic acid (5-8) and possibly also of α-ketoglutaric acid (9). In addition to these alterations of amino acid metabolism, there are changes in carbohydrate metabolism, consisting of increased glucose consumption (6) and accumulation of pyruvic acid (5, 10, 11) and lactic acid (6).

Consequently it has been suggested that the toxic effects of ammonia in brain are the result of a diversion of α-ketoglutaric acid from the Krebs citric acid cycle through glutamic acid to glutamine (9). This diversion of α-ketoglutarate would result in: a) depletion of 4-carbon dicarboxylic acids of the citric acid cycle; b) accumulation of pyruvate and lactate secondary to a relative lack of oxalacetic acid for condensation with acetyl-coenzyme A to form citrate; c) consequent reduction of oxygen consumption, and d) depletion of adenosine triphosphate (ATP) secondary to increased glutamine synthesis.

There is another biochemical mechanism which is also consistent with established biochemical findings—namely, a direct toxic effect of ammonia on the oxidative decarboxylation of pyruvic and α-ketoglutaric acids (6). In the experiments reported here, the effects of ammonia on cerebral oxygen consumption and oxidative decarboxylation of pyruvic acid have been investigated in vitro, using cat cerebral cortex slices and mitochondria.

MATERIALS AND METHODS

Stock adult cats were used. The methods of sacrifice and preparation of cortex slices for incubation have been previously described (12). Mitochondria were prepared from 0.25 M sucrose homogenates of freshly sliced cerebral cortex. The method of preparation was similar to procedure II of Brody and Bain (13) except that fractions II-R₁ and II-R₂ were brought down together by centrifugation at 755 X g for 10 minutes, and fraction II-R₃ was obtained by centrifugation at 18,800 X g for 15 minutes. The entire procedure was carried out at 0°C, using a model SS-4 Servall centrifuge. The mitochondrial pellet (fraction II-R₃) was resuspended in 9 volumes of 0.25 M sucrose and washed once and the final pellet made up in 0.25 M sucrose to a volume equal to the original tissue weight. Examination of these fractions by electron microscopy (T. Wanko and M. A. Gavin, unpublished) indicates that these modifications in procedure result in a mitochondrial fraction free of myelin and cellular debris than that obtained by the procedure originally described by Brody and Bain (13). Some residual contamination with microsomal particles remains but is not considered to have significantly affected the studies reported here.

Cerebral cortex slices were incubated at 37°C in either bicarbonate saline or phosphate-saline media containing...
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glucose 10 mM and K+ 27 mM (12). Mitochondrial preparations were incubated at 25°C in a standard phosphate-saline medium of final volume 2.0 μl containing (final concentrations) KCl 50 mM, MgCl2 8 mM, Na-phosphate buffer (pH 7.2) 40 mM, ATP 2.5 mM, substrates as specified under results, and mitochondria equivalent to 0.5–1.0 gm of original tissue, in sucrose 0.95 M.

The methods for measurement of oxygen uptake and determination of P/O ratios have been previously described (14). Mitochondrial incubations were carried out in specially designed Warburg flasks fitted with a rubber-sealed port which permitted addition of acid through the rubber seal at the end of the desired incubation period (15). Collection of C14O2 from metabolized C14-labeled substrates was effected with Hyamine in vessel center-wells and counting was done in a Tri-Carb liquid scintillation counter (16). Diphosphopyridine nucleotide (DPN) assays were carried out by the method of Lowry et al. (17), using a Farrand fluorimeter.

All substrates and cofactors were obtained from commercial sources. The α-lipoic acid was kindly provided by Dr. Roscoe Brady and pyruvic acid-1-C14 by Dr. Lemone Yielding.

RESULTS

O2 consumption. The addition of 10 mM NH4Cl to the incubation media of cerebral cortex slices is associated with a significant decrease of oxygen consumption

**TABLE 1. Effects of 10 mM NH4Cl on O2 Consumption and Glucose Metabolism by Incubated Slices of Cat Cerebral Cortex**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control</th>
<th>NH4Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen uptake, μl/O2</td>
<td>72.7</td>
<td>72.7</td>
</tr>
<tr>
<td>Glucose uptake, μl/O2</td>
<td>72.7</td>
<td>72.7</td>
</tr>
<tr>
<td>Lactate production, μl/O2</td>
<td>72.7</td>
<td>72.7</td>
</tr>
</tbody>
</table>

Values are given ± S.D. Incubation conditions as specified in text. Media for O2 uptake: phosphate-saline-glucose; for glucose and lactate metabolism: bicarbonate saline-glucose (6, 12).

**TABLE 2. Effect of NH4Cl on O2 Uptake by Cat Cerebral Cortex Mitochondria**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration</th>
<th>Control</th>
<th>NH4Cl 10 mM</th>
<th>NH4Cl 15 mM</th>
<th>NH4Cl 40 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>10 mM</td>
<td>3.0</td>
<td>2.25</td>
<td>2.15</td>
<td>0.55</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>2.00</td>
<td>0.75</td>
<td>1.0</td>
<td>1.00</td>
<td>1.0</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.5</td>
<td>1.75</td>
<td>1.65</td>
<td>1.65</td>
<td>1.65</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>7.5</td>
<td>9.65</td>
<td>9.65</td>
<td>9.65</td>
<td>9.65</td>
</tr>
<tr>
<td>γ-Aminobutyrate</td>
<td>15 mm</td>
<td>3.5</td>
<td>2.05</td>
<td>2.05</td>
<td>2.05</td>
</tr>
</tbody>
</table>

Incubation conditions as detailed in text.

**TABLE 3. Effect of NH4Cl 15 mM on Pyruvate Oxidation by Cat Cerebral Cortex Mitochondria**

<table>
<thead>
<tr>
<th>Exp No.</th>
<th>Control</th>
<th>NH4Cl 15 mM</th>
<th>% of Control</th>
<th>Control</th>
<th>NH4Cl 15 mM</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.65</td>
<td>1.2</td>
<td>72.7</td>
<td>0.55</td>
<td>0.4</td>
<td>72.7</td>
</tr>
<tr>
<td>2</td>
<td>2.8</td>
<td>1.95</td>
<td>72.7</td>
<td>0.9</td>
<td>0.65</td>
<td>72.2</td>
</tr>
<tr>
<td>3</td>
<td>4.7</td>
<td>3.25</td>
<td>72.7</td>
<td>1.6</td>
<td>1.0</td>
<td>72.5</td>
</tr>
<tr>
<td>4</td>
<td>3.4</td>
<td>2.1</td>
<td>72.7</td>
<td>1.15</td>
<td>0.75</td>
<td>72.5</td>
</tr>
</tbody>
</table>

Incubation conditions as detailed in text. O2 uptake measured manometrically. Pyruvate utilization calculated from C14O2 evolved with pyruvate-1-C14 added to unlabeled pyruvate (10 mM) plus succinate (0.5 mM) as substrate. Per mole of pyruvate oxidized, 3 moles of O2 would be required.

(table 1). This decrease has been observed with slices of both gray and white matter and occurs when either bicarbonate- or phosphate-buffered media are used (6).

Previous studies in this laboratory have demonstrated that cerebral cortex cells actively accumulate NH4+ ions intracellularly at the expense of K+ ions (6). It has been calculated that the incubation of cortical slices in the presence of 10 mM NH4Cl results in an intracellular concentration of approximately 15 mM NH4+ ions. Accordingly in the present studies exposure of incubated slices to 10 mM NH4Cl is considered roughly comparable to exposure of mitochondrial preparations to 15 mM NH4Cl.

The effect of various concentrations of NH4Cl (10 to 40 mM) on the oxygen consumption of mitochondrial preparations is given in table 2. When 10 mM pyruvate or 10 mM α-ketoglutarate served as substrates, lower concentrations of NH4Cl (10–15 mM) were associated with a 30–40% decrease in oxygen consumptions. These concentrations of NH4Cl did not affect oxygen uptake with 10 mM succinic acid as substrate and only minimally affected metabolism supported by 10 mM L-glutamic acid or 10 mM γ-aminobutyric acid.

Carbohydrate metabolism. In the presence of 10 mM NH4Cl the rate of aerobic glycolysis (lactic acid production) of incubated cortex slices doubled over that found in controls (table 1). A significant increase of slice glucose utilization was associated with this rise in lactate production.

Pyruvate utilization. Pyruvate utilization by mitochondria was calculated from the evolution of C14O2 from added pyruvate-1-C14. When cortical mitochondria were incubated with 10 mM pyruvate plus 0.5 mM succinate as substrates, the ratio between the oxygen consumption observed manometrically and the oxygen consumption, calculated by multiplying pyruvate utilization by 3, was 0.99 (±0.17) for 1 1 experiments. The addition of 15 mM NH4Cl to these preparations resulted in a decrease of pyruvate utilization, as well as a decreased oxygen consumption (table 3). The ratio of observed oxygen uptake to calculated oxygen consumption (from pyruvate utilization) by ammonia-treated mitochondrial preparations was 1.04 (±0.17) for 1 1 experiments.
The metabolic activity of various control mitochondrial preparations varied considerably. However, the percentage inhibition associated with the presence of 15 mM NH₄Cl was quite constant (table 3). For different mitochondrial preparations, the oxygen uptake in the presence of 15 mM NH₄Cl was 60 (± 9)% of control oxygen uptakes and pyruvate utilization was 72 (± 7)% of controls.

Decreased utilization of pyruvic acid in the presence of ammonia might be accounted for by inadequate levels of 4-carbon intermediates which could condense with acetyl-coenzyme A (derived from pyruvate decarboxylation) to form citrate. However, when succinic acid was added in excess concentration (5 mM), a significant decrease in pyruvate utilization still occurred under these conditions, despite an increased oxygen uptake (table 4).

Effects of added cofactors. Attempts to reverse the NH₄Cl inhibition were carried out by addition of some of the cofactors associated with the oxidative decarboxylation of pyruvate (table 5). Neither α-lipoic acid nor thiamine pyrophosphate exhibited any protective effect, at the concentrations employed, against the inhibition of oxygen consumption and pyruvate utilization associated with the presence of 15 mM NH₄Cl.

On the basis of studies on the effects of ammonia on plant metabolism, Wedding and Vines (18) suggested that ammonia toxicity is related to interference with re-oxidation of reduced diphosphopyridine nucleotide (DPNH). The levels of oxidized diphosphopyridine nucleotide (DPN) found in control and ammonia-treated mitochondrial preparations varied considerably. However, the satisfactory P/O ratios were observed for both the intact and swollen mitochondria (table 7).

No depletion of DPN was observed in either preparation. The rise of DPN levels observed in the slices may reflect the increased conversion of pyruvic acid to lactic acid known to be occurring (table 1).

Previous studies in this laboratory and by others have indicated that mitochondria are relatively impermeable to exogenous DPN (19–22). However, pretreatment of mitochondria with 0.075 M sucrose for 10 minutes alters mitochondrial permeability and permits entry of DPN (20, 21). Despite this manipulation, reversal of NH₄Cl effects could not be observed upon addition of DPN to 'swollen' mitochondria (table 7).

P/O ratios. Under the experimental conditions used here, satisfactory P/O ratios were observed for both the control and ammonia-treated mitochondrial preparations (table 8).

### DISCUSSION

In these experiments, effects of NH₄⁺ ion on aerobic glycolysis, oxygen consumption, and pyruvate utilization by brain tissue have been observed in vitro. The increased aerobic glycolysis reported here is consistent with previous in vitro studies (22, 23). Gore and McIlwain (23) found stimulation of oxygen consumption in incubated cerebral cortex slices at lower NH₄Cl concentrations and depression at higher concentrations. Earlier Weil-Malherbe (22) had reported stimulation of oxygen consumption by brain slices incubated with NH₄Cl re-

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**TABLE 4. Effects of Added Succinate on NH₄Cl Inhibition of Pyruvate Oxidation by Cat Cerebral Cortex Mitochondria**

<table>
<thead>
<tr>
<th>Pyruvate</th>
<th>Oxygen Uptake</th>
<th>Pyruvate Utilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
<td>3.4</td>
<td>1.15</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.5 mM</td>
<td>2.1</td>
</tr>
<tr>
<td>NH₄Cl 15 mM</td>
<td></td>
<td>0.75</td>
</tr>
<tr>
<td>NH₄Cl 15 mM and Succinate 5 mM</td>
<td>4.55</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Conditions as described in text and table 7.

**TABLE 5. Effects of Added Cofactors on NH₄Cl Inhibition of Pyruvate Oxidation by Cat Cerebral Cortex Mitochondria**

<table>
<thead>
<tr>
<th>Substrates and Cofactors</th>
<th>Oxygen Uptake</th>
<th>Pyruvate Utilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate 10 mM, Succinate 0.5 mM</td>
<td>11</td>
<td>69.1 ± 9.2 71.9 ± 7.2</td>
</tr>
<tr>
<td>+ α-Lipoic acid 5 x 10⁻⁴ M</td>
<td>2</td>
<td>2.1 0.75 2 x 10⁻³ M</td>
</tr>
<tr>
<td>+ Thiamine pyrophosphate 5 x 10⁻⁴ M</td>
<td>6</td>
<td>65.8 ± 4.4 72.5 ± 3.9</td>
</tr>
</tbody>
</table>

Values are given ± S.D. Conditions as described in text and table 3.
In the present study, oxygen consumption by cerebral cortex slices in the presence of 10 mM NH₄Cl was consistently depressed. Although direct comparisons cannot be made, it is of interest that in vivo studies of patients with hepatic insufficiency and increased blood ammonia levels have demonstrated decreased cerebral oxygen consumption (24–26).

Inhibition by ammonia of oxygen consumption in mitochondrial preparations was selective, depending upon the substrate being utilized. This selective toxicity by ammonia has been previously reported in plants (18). Pyruvate and α-ketoglutarate, the two substrates most susceptible to ammonia inhibition, undergo oxidative decarboxylation by very similar reaction sequences, involving thiamine pyrophosphate, α-lipoic acid, DPN, coenzyme A, and Mg⁺⁺ as cofactors (27, 28). The effects of arsenite and of thiamine deficiency on utilization of these two substrates are well known (29) and not unlike the effects reported here for ammonia. It is of interest that Dr. K. S. Warren has recently reported to us that in unpublished studies he has found thiamine deficiency comparable decreases of oxygen consumption were observed in incubated slices of subcortical white matter and in cortical slices from cats treated with methionine sulfoximine (6). We suggest that the glutamic dehydrogenase and glutamine synthesizing reactions may function in ammonia toxicity not in a detrimental fashion but as a protective mechanism to minimize ammonia toxicity comparable decreases of oxygen consumption were observed in incubated slices of subcortical white matter and in cortical slices from methionine sulfoximine-intoxicated cats (6). We suggest that the glutamic dehydrogenase and glutamine synthesizing reactions may function in ammonia toxicity not in a detrimental fashion but as a protective mechanism to maintain lower levels of free, intracellular ammonia and preserve oxidative metabolism.

The absence of a pronounced effect of ammonia on the utilization of glutamic acid and γ-aminobutyric acid by cortical mitochondria is of interest. These two compounds participate in an alternate pathway between the α-ketoglutarate to succinate stage of the citric acid cycle which is present only in central nervous system gray areas (34), located primarily in neuronal mitochondria, and capable of independently supporting oxidative metabolism (15, 35). The invulnerability of this pathway to ammonia toxicity may provide the brain with a ‘safety valve’ mechanism for maintenance of metabolism around any block of α-ketoglutarate oxidative decarboxylation. Studies of the relationships between these

### Table 3. Oxidative Phosphorylation by Cat Cerebral Cortex Mitochondria

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Oxygen Uptake μM/min</th>
<th>Phosphate Esterification μM/min</th>
<th>P/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate 10 mM</td>
<td>3.25</td>
<td>6.85</td>
<td>2.1</td>
</tr>
<tr>
<td>Succinate 0.5 mM</td>
<td>2.4</td>
<td>6.05</td>
<td>2.5</td>
</tr>
<tr>
<td>NH₄Cl 15 mM</td>
<td>2.4</td>
<td>6.05</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Each value is the mean of 2 determinations.
two alternate pathways of α-ketoglutarate metabolism in brain are reported elsewhere (19). No analogous situation obtains for the metabolism of pyruvate. On the basis of the findings reported here, it is proposed that a primary toxic effect of ammonia on the brain may be a direct interference with the oxidative decarboxylation of pyruvic and α-ketoglutaric acids. The exact mechanism of this toxic effect of ammonia and proper approaches to its therapeutic reversal remain to be elucidated.

REFERENCES

24. FAZEKAS, J. F. AND A. N. BERSMAN. Cited by BERSMAN (9).