The Purine Nucleotide Cycle

STUDIES OF AMMONIA PRODUCTION AND INTERCONVERSIONS OF ADENINE AND HYPOXANTHINE NUCLEOTIDES AND NUCLEOSIDES BY RAT BRAIN IN SITU*

(Received for publication, August 23, 1977)

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Electrical shock treatment produces a rapid loss of high energy phosphates in rat brain. The [ATP]/[ADP] ratio decreases to one-third of its control value within 10 s. The ammonia content increases 3-fold during the first minute after starting the stimulus. The total adenine nucleotide plus adenosine content of brain decreases and an equivalent amount of hypoxanthine-containing compounds appears. Adenosine, inosine, and hypoxanthine accumulate, and there is a transitory accumulation of adenylosuccinate. The adenosine content of brain decreases and an equivalent decrease to one-third of its control value within 10 s. The energy phosphates in rat brain. The [ATP]/[ADP] ratio, are rapidly restored to control values, but other metabolite contents are restored more slowly. The transient rise in adenylosuccinate and IMP provides evidence that the ammonia production is due in part, and possibly in whole, to the operation of the purine nucleotide cycle.

We showed previously that cell-free extracts of rat brain contain the enzymes of the purine nucleotide cycle and that the operation of the cycle can be demonstrated in such extracts under suitable conditions (1). We now present evidence that the purine nucleotide cycle operates in rat brain in vivo. In normal brain, only a small percentage of cells are firing at a given moment, and there is an averaging out of the metabolic transients that occur in individual cells. One way to obviate this is to trigger as many cells as possible at the same time. Experiments are described in which transient changes in tissue extracts are then established by sodium-potassium ATPase. The resulting energy drain is accompanied by changes in the contents of adenine and hypoxanthine nucleotides and nucleosides as well as ammonia and adenylosuccinate.

* This work was supported by National Institutes of Health Grant GM-07261. Publication 1187 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Mass. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported in part by Training Grant GM-212 from the National Institutes of Health.

EXPERIMENTAL PROCEDURES

Electrical Stimulation and Sampling of Rat Brains—Male Sprague-Dawley rats weighing 225 to 250 g were shaven between the ears with small animal clippers and Nair lotion hair remover was applied to the area 24 h before use. The animals were also deprived of food at this time. Immediately before use, the rats were immobilised in a restraining cage which was held in a brain-blowing apparatus (2). The animals were stimulated for a period of 10 s with repeating monophasic pulses of 8.25 ms duration at a pulse repetition rate of 60/s. The voltage was kept constant at 100 V during each pulse. Current was delivered from a Grass square wave stimulator (model S4E) through silver electrodes which were coated with electrode jelly and applied to the shaved foreheads of the rats. The average current delivered under these conditions was in the range of 60 to 95 mA. The current varied from rat to rat, as well as during the course of 10 s of stimulation, due to variations in the resistance. With control rats, electrodes were held in place for 10 s, but no stimulus was applied.

The brains were freeze-blown at various times after starting the stimulation. Samples of the cerebrum, ranging from 0.5 to 1.0 g, were obtained. None of the cerebellum was removed by the freeze-blowing technique. The samples were stored in a liquid nitrogen freezer until they were analyzed. In the case of rats sampled 30 and 60 min after the electric shock treatment, the animals were removed from the restraining cage immediately after the treatment and were returned to it 2 or 3 min prior to sampling. The rats were kept in the restraining cage until sampling for all other time points.

Preparation of Tissue Extracts—Frozen rat brains were extracted by a modification of the technique of Vecht et al. (2). The frozen tissues were ground to a powder in a stainless steel mortar cooled with liquid nitrogen. The powder was then warmed to ~80 °C by transferring it to a 10 ml glass homogenizing tube standing in powdered dry ice. Cold 0.1 N HCl in 99% methanol was added (2 ml/g of brain powder), the tube was placed in an acetone-dry ice bath, and the tissue was dispersed with a glass stirring rod to ensure thorough mixing with the acid. The temperature was then raised to 0 °C by transferring the tube to crushed ice, and 4 ml of 1.23 N perchloric acid containing 5 mM EGTA were added per g of brain. The sample was homogenized for 1.5 min with a motor-driven Teflon glass homogenizer and centrifuged at 31,000 x g for 10 min. The supernatant was decanted and the pellet was homogenized again in 3 ml/g of tissue of 1.23 N perchloric acid containing 5 mM EGTA. The supernatant from the first centrifugation was adjusted to the second supernatant, and this mixture was centrifuged at 31,000 x g for 10 min. The supernatant from the second centrifugation was adjusted to pH 6.5 to 7.0 with a mixture of 6 N KOH and 0.5 M triethanolamine and allowed to stand on ice for 30 min. The mixture was then centrifuged. Pyruvate and a-ketoglutarate were assayed immediately after the preparation of the extract. The remaining extract was stored frozen.

Metabolic Assays—Ammonia was measured in a reaction mixture

* The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether) N,N'-tetraacetic acid.

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which contained 40 mM Tris HCl buffer, pH 7.5, 0.6 mM ADP, 0.06
mM DPNH, 4 mM α-ketoglutarate, and 0.08 mg/ml of glutamate
dehydrogenase in 50% glycerol (specific activity 45 units/mg). The
mixture was incubated at room temperature for 15 min and
the reaction was then initiated by addition of the deproteinized
extracts. The decrease in absorbance at 340 nm was measured on a
Perkin-Elmer model 356 double beam spectrophotometer set to read
absorbance at 400 nm minus absorbance at 340 nm. Ammonia was
measured both in the tissue extracts and in blanks composed of the
reagents used to prepare the extracts combined in the usual propor-
tions.

Purines and purine nucleosides were assayed as described previ-
ously (1), except that the reaction mixture contained 50 mM potas-
sium phosphate buffer, pH 7.4. IMP was assayed by hydrolysis to
inosine in a reaction mixture which contained 0.06 ml of 1 M
NaOH buffer, pH 9.0, containing 10 mM MgCl2, 0.10 ml of deprotein-
ized extract, and 0.01 ml (40 μg) of 5'-nucleotidase from snake
venom (Crotalus atrox). The mixture was incubated at room temper-
ature for 15 min. The volume was then adjusted to 3 ml with 50 mM
potassium phosphate buffer, pH 7.4, and inosine was assayed as
described previously (1). The assay yielded the sum of inosine and
IMP. The difference between this assay and the assay for inosine
yielded IMP.

Adenylosuccinate was measured with adenylosuccinase. The re-
ation mixture contained 25 mM triethanolamine-HCl buffer, pH 7.1,
and 1.0 ml of tissue extract in a total volume of 2 ml, was
preincubated at room temperature for 60 min in order to achieve a
stable baseline. The reaction was then initiated by addition of
adenylosuccinase. The decrease in absorbance at 232 nm was mea-
sured on a Perkin-Elmer model 356 double beam spectrophotometer
set to read absorbance at 310 nm minus absorbance at 282 nm. The
light path was 1 cm, and the instrument was set to 0.01 absorbance
unit full scale. When assays of samples containing adenylosuccinate
were performed in the presence of 6 mM β-phosphonate analogue of
adenylosuccinate, no change in absorbance was observed for 90 min.
The analogue is a competitive inhibitor of adenylosuccinase with a
K<sub>i</sub> for the rat muscle enzyme of 7.0 × 10<sup>-8</sup> M (3). In model experiments,
it completely inhibited the activity of 0.8 nmol of authentic adenos-
ylsuccinate. Extracts from control brains maintained a flat baseline
for 30 min following addition of adenylosuccinate. The noise level
was such that a change of 0.00695 absorbance unit was significant;
this is equivalent to 0.05 nmol of adenylosuccinate, or 0.5 nmol/g of
brain. Most duplicate assays agreed to within 10% or better.

AMP and the sum of ADP plus GDP were determined according to
Williamson and Corkey (4), except that we used the double beam
spectrophotometer set to read absorbance at 400 nm minus absorb-
ance at 340 nm. The relative amounts of AMP and GDP were then
determined by high pressure liquid chromatography using a Varian
Aerograph, which contained a 25 cm column (0.9 mm internal diameter, 3 m long) containing a strong anion exchange resin (AS-Pellonex-SAX, Reeve Angel, Clifton, N. J.). The mixing vessel of the apparatus contained low concentration buffer consisting of 5 mM potassium phosphate and 5 mM potassium lactate, pH 3.3. The mixing vessel of the high concentration buffer consisted of 0.2
mM potassium phosphate, 0.3 M KCl, and 5 mM potassium lactate, pH 3.3. A gradient was formed by pumping liquid from the mixing
to the column at a rate of 30 ml/h, and from the high
concentration reservoir into the mixing vessel at a rate of 15 ml/h.
The identities of the nucleoside 3'-phosphates were confirmed by
running the same samples after the AMP<sub>2</sub> and GDP<sub>2</sub> had been
converted to ATP and CTP, respectively, with phosphonopyruvate
and pyruvate kinase. α-Ketoglutarate, ATP, glucose 6-phosphate,
creatine phosphate, and pyruvate were assayed by the methods of
Lowry and Passonneau (5). Lactate was determined by the method
of Hohorst (6).

**Materials**—Male Sprague-Dawley rats were obtained from
Charles River Breeding Laboratory. Cambridge electrode jelly was
purchased from Kent Instrument Co. α-Ketoglutarate and EGTA were
delivered in liquid form in 10 mL vials. Purine nucleosides were
purchased from P-L Biochemicals. The phosphonate analogue of
adenylosuccinate, N<sub>2</sub>-(1-carboxy-2-phosphonomethyl)adenosine 5'-phos-
phate (alternative name N<sub>2</sub>-(β ribofuranosyl)purin 6-y1) or 3
phosphonoalanine 5'-monophosphate) was synthesized in our lab-
atory by Dr. Larry Brand. Adenylosuccinate was purified from
yeast by Dr. M. N. Goodman by a modification of the method of
Miller et al. (Ref. 7, see also Ref. 8). Nucleoside phosphorylase,
xanthine oxidase, and snake venom 5'-nucleotidase (Crotalus atrox)
were obtained from Sigma. Glutamate dehydrogenase in 50% glycer-

<sup>6</sup> Collins et al. (9), King et al. (10), and Ferrendelli and McDougal
(11) multiplied the change in ATP by 2 since ATP contains two
phosphoribosyl analogues.
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Fig. 1. Changes in purine nucleotide cycle metabolites of rat brain following electrical shock. Rats were stimulated for 10 s with monophasic pulses of 8.25 ms duration with a pulse repetition rate of 60/s. The voltage was constant at 100 V during each pulse. Current was delivered through silver electrodes applied to the shaved foreheads of the rats. Electrical stimulation was begun at zero time. Brains were removed and frozen rapidly by the method of Veech et al. (2) at the times indicated by the experimental points. In the case of control values shown at zero time, the electrodes were held in place for 10 s but no stimulus was applied. Each point shows the mean ± S.E. for four to eight rats, except for the 10 s points which show the average for two rats. Where standard error bars overlap, only half error bars are drawn for clarity.

..., ammonia; A, AMP; n, IMP; and A, adenylosuccinate (SAMP).

Fig. 2. Changes in adenine mononucleotides and creatine phosphate contents of rat brain following electrical shock. The experimental conditions were as described in Fig. 1. ○, creatine phosphate (CP); ●, ATP; ▲, ADP; ▼, AMP. The experimental points for IMP (broken curve) are shown in Fig. 1.

Fig. 3. Changes in nucleoside and hypoxanthine contents of rat brain following electrical shock. The experimental conditions were as described in Fig. 1. ○, adenosine; △, inosine; and ●, hypoxanthine. Changes in AMP (broken line) and IMP (dotted line) are reproduced from Fig. 1 to facilitate comparison; these changes are not drawn to scale. Hypoxanthine includes xanthine since hypoxanthine measurements were made with xanthine oxidase.

Ammonia accumulation reached a maximum 1 min after initiating the shock treatment, inosine reached a maximum between 3 and 5 min, and hypoxanthine reached a maximum after 10 min (Fig. 3). The sum of the contents of these three metabolites was a little less than 0.1 amol/g of fresh weight 5 to 10 min after the shock treatment.

The content of adenine compounds (defined as the sum of AMP, ADP, ATP, and adenosine) decreased during the first minute following stimulation. The decrease was exactly balanced by the appearance of hypoxanthine compounds (defined as IMP plus inosine plus hypoxanthine). Similarly, during the recovery period, the reappearance of adenine compounds was balanced by the disappearance of hypoxanthine compounds (Fig. 4). The accompanying rise in ammonia content exceeded the loss of adenine compounds into hypoxanthine compounds by only about 50%. However, this near correspondence may be fortuitous since ammonia was probably being converted to glutamine and was also diffusing from the brain during the period covered in Fig. 4.
Ammonia Production by Rat Brain

FIG. 4. Comparison of changes in adenine compounds (ATP + ADP + AMP + adenosine) with hypoxanthine compounds (IMP + inosine + hypoxanthine) and ammonia of rat brain following electrical shock. Note that the scale on the right hand ordinate is displaced by 2.3 but is otherwise the same as the scale on the left hand ordinate. "Hypoxanthine compounds" include xanthine (see legend to Fig. 3).

FIG. 5. Changes in glucose 6-phosphate and α-ketoglutarate contents of rat brain following electrical shock. The experimental conditions were as described in Fig. 1. ○, glucose 6-phosphate; ●, α-ketoglutarate.

The α-ketoglutarate content decreased by 60% during the first 10 s of shock treatment and then recovered slowly (Fig. 5). The glucose 6-phosphate content followed a similar time course, decreasing to 50% of control values after 10 s. It then increased rapidly to reach 1.8 times control values at 3 min. Thereafter, its return to control levels was slow. The rapid decline in glucose 6-phosphate reflects a sudden increase in the rate of operation of phosphofructokinase, in agreement with previous observations in mice and rats (13, 14). This is followed by an increased rate of glucose uptake (10, 14) which allows the hexokinase reaction to make up, and in this case to overshoot, the previous level of glucose 6-phosphate. Glycogen was not measured in this experiment. However, previous studies showed that glycogen decreases during seizures (10, 14) and it has been suggested that this might contribute to the accumulation of glucose 6-phosphate (14).

Large increases in lactate concentrations were observed following the shock treatment. Fig. 6 shows that the [lactate]/[pyruvate] ratio increased almost 4-fold within the first minute. These changes are very similar to those reported for convulsions in mice (13). Recovery to control values was not completed for about 30 min. In contrast, the [ATP]/[ADP] ratio decreased to about one-third of control values within 10 min after shock.

TABLE I

Intramitochondrial (DPNH)/[DPN] calculated from metabolites of glutamate dehydrogenase reaction

The experimental conditions were as described in the legend to Fig. 1. Calculations were made using the equation

\[ \frac{[\text{DPNH}]}{[\text{DPN}]} = \frac{[\text{glutamate}]}{[\text{α-ketoglutarate}][\text{ammonia}]} \]

where \( K \), the equilibrium constant, equals \( 3.87 \times 10^{-6} \) M at pH 7.0 (16). Assumptions concerning metabolite distribution are explained in the text. Results are expressed as means ± S.E. for four to eight rats, except in the case of 0.17 min which shows the mean for two animals.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>(DPNH)/(DPN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.05 ± 0.24</td>
</tr>
<tr>
<td>0.17</td>
<td>1.46</td>
</tr>
<tr>
<td>0.25</td>
<td>1.28 ± 0.06</td>
</tr>
<tr>
<td>0.33</td>
<td>1.22 ± 0.19</td>
</tr>
<tr>
<td>0.50</td>
<td>1.08 ± 0.07</td>
</tr>
<tr>
<td>0.67</td>
<td>1.07 ± 0.07</td>
</tr>
<tr>
<td>0.83</td>
<td>0.92 ± 0.12</td>
</tr>
<tr>
<td>1.00</td>
<td>0.96 ± 0.07</td>
</tr>
<tr>
<td>1.25</td>
<td>0.72 ± 0.05</td>
</tr>
<tr>
<td>1.5</td>
<td>0.74 ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>0.68 ± 0.03</td>
</tr>
<tr>
<td>3</td>
<td>0.60 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>0.53 ± 0.02a</td>
</tr>
<tr>
<td>10</td>
<td>0.63 ± 0.05a</td>
</tr>
<tr>
<td>30</td>
<td>0.94 ± 0.05</td>
</tr>
<tr>
<td>60</td>
<td>0.98 ± 0.05</td>
</tr>
</tbody>
</table>

* Significantly different from controls (\( p < 0.05 \)) by paired t test (17).
Ammonia Production by Rat Brain

The [DPNH]/[DPN] ratio for the intramitochondrial space was calculated from the glutamate, α-ketoglutarate, and ammonia contents. It was assumed that the intracellular water accounts for 50% of the fresh weight, that glutamate and α-ketoglutarate are evenly distributed throughout the intracellular water, and that ammonia is evenly distributed throughout the total brain water. The latter was taken to be 78% of the wet weight (15). The total glutamate content varied very little, and the value used for the calculations was 10.0 μmol/g of fresh weight. The results of these calculations show that there was no change in the intramitochondrial [DPNH]/[DPN] ratio calculated from the metabolites of glutamate dehydrogenase during the first minute after applying electrical shock (Table I). This is the period during which most of the ammonia is liberated. The oxidation-reduction state became somewhat more oxidized during the recovery period, perhaps because additional DPNH was used to remove ammonia as glutamate.

DISCUSSION

Early work describing the effect of convulsions on the contents of various brain metabolites was reviewed by Klein and Olsen (18). It was shown that convulsive electrical activity induced by drugs or electrical stimulation results in a decrease in the contents of creatine phosphate and ATP (18, 19). Energy utilization of brain can increase 3- to 4-fold during electrical stimulation (9, 10). The associated convulsions interfere with respiration, thereby lowering the oxygen supply available to the brain. An increase in the cerebral blood flow occurs as a compensatory response (20, 21). The increases in the rates of glycolysis (13, 14, 22) and the citric acid cycle (13) produced by electrical shock are inadequate to prevent a decrease in high energy phosphates. The (lactate)/(pyruvate) ratio increases 4-fold after starting the shock treatment (Fig. 6), a reflection of a proportional rise in the cytoplasmic [DPNH]/[DPN] ratio. On the other hand, there is no immediate change in the intramitochondrial [DPNH]/[DPN] ratio (calculated from the glutamate dehydrogenase equilibrium), and only a small change towards being more oxidized during the recovery period (Table I).

Following electrical stimulation, the decrease in adenine compounds is exactly balanced by the increase in hypoxanthine compounds, but the amount of ammonia that accumulates (0.31 μmol/g of fresh weight) exceeds the amount of hypoxanthine compounds that accumulates (0.20 μmol/g of fresh weight) by 55% (Fig. 4). As is pointed out under "Results," the maximum rate of operation of adenylosuccinate synthetase is between 0.09 and 0.12 μmol/g of fresh weight/min. If the enzyme is operating at its maximum rate over the minute during which 0.31 μmol of ammonia/g accumulates, then this could account for the IMP "missing" from the stoichiometry (0.20 + 0.09 = 0.29 μmol/g). Thus, all of the observed increase in ammonia content could arise via the adenylate deaminase reaction. However, the question remains if, and to what extent, the glutamate dehydrogenase reaction contributes to ammonia production. Put differently, while our data show clearly that a large portion of the ammonia arises via the purine nucleotide cycle, they do not rule out some contribution by the glutamate dehydrogenase reaction.

Ammonia production is associated not only with a rapid rise in IMP from <0.005 to 0.15 μmol/g of fresh weight (Fig. 1), but also with a rapid fall in α-ketoglutarate from 0.18 to 0.07 μmol/g of fresh weight (Fig. 5). A similar fall has been reported to occur in response to electrically induced convulsions in mice (13). The fall in α-ketoglutarate content can be interpreted in at least two ways. It may be due to the sudden rise in ammonia which leads to a readjustment of the glutamate dehydrogenase equilibrium in favor of glutamate formation. In other words, α-ketoglutarate falls because the ammonia produced by other reactions is partially converted to glutamine. Alternatively, the glutamate dehydrogenase equilibrium may be shifted in the direction of ammonia formation by the removal of α-ketoglutarate by some other reaction, such as the α-ketoglutarate dehydrogenase reaction. The latter alternative has been advanced in support of ammonia production via the glutamate dehydrogenase reaction in kidney (23). A shift in the ratio [reduced pyridine nucleotide]/[oxidized pyridine nucleotide] in the mitochondria would also lead to a readjustment of the equilibrium between glutamate, α-ketoglutarate, and ammonia. Unfortunately, there are currently no suitable substrates that can be used to calculate the [DPNH]/[DPN] ratio in this compartment other than the substrates of glutamate dehydrogenase, and using these would lead to a circular argument. (The β-hydroxybutyrate dehydrogenase reaction equilibrium is used for calculating intramitochondrial [DPNH]/[DPN] in liver, but this is not practical in the case of brain (24).) Assuming that the glutamate dehydrogenase reaction is at or near equilibrium during the experimental period, calculations of the intramitochondrial [DPNH]/[DPN] ratio show that no changes occur in this ratio during the period over which most of the ammonia is liberated (Table I). No change in intramitochondrial [DPNH]/[DPN] was observed in brains of rats in which seizures were induced by single electric shocks (25). Similar results were reported for the first minute of sustained epileptic seizures, the period over which ammonia accumulated; thereafter, the ammonia content stabilized, and calculations showed that the intramitochondrial [DPNH]/[DPN] then became more oxidized (14).

Ammonia formed during electrical activity can be removed by the glutamate dehydrogenase reaction. The activity of the enzyme is 11 μmol/g of fresh weight/min at 30° and pH 7, which is equivalent to 18 μmol/g of fresh weight/min at 38° (1). Ammonia can also be removed by the glutamine synthetase reaction. The activity of this enzyme is 2.3 μmol/g of fresh weight/min at 37° (26). Ammonia can also be lost by diffusion into the blood, but no quantitative estimate of the rate at which this may occur has been reported.

When mice were stimulated electrically, the glutamate content of 13 μmol/g of fresh weight did not change significantly, whereas the glutamine content increased from 4.5 to 5.5 μmol/g of fresh weight (27). This suggests that in our experiments more ammonia may have been formed in response to the shock treatment than was actually measured and that it was partly converted to glutamine.

The adenosine content of brain rises during electrical stimulation (Fig. 3) (28) and ischemia (29). Adenosine causes vasodilation and is probably involved in controlling blood flow through the brain (29, 30) in a manner similar to that described earlier for heart (31) and skeletal muscle (32, 33). Adenosine is released from brain slices in response to electrical stimulation, anoxia, and various chemical agents (34, 35). Adenosine stimulates accumulation of cyclic AMP in brain slices (36), but inhibits adenylyl cyclase of plasma membranes from rat liver (37). Adenosine is produced by the action of 5'-nucleotidase on AMP. The data shown in Fig. 3 suggest that the AMP content may be the primary determinant of...
Ammonia Production by Rat Brain

adenosine production. Adenosine then acts as an extracellular signal indicative of the intracellular level of AMP.

REFERENCES
The purine nucleotide cycle. Studies of ammonia production and interconversions of adenine and hypoxanthine nucleotides and nucleosides by rat brain in situ.
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