Purine Nucleotide Cycle

EVIDENCE FOR THE OCCURRENCE OF THE CYCLE IN BRAIN*

(Received for publication, June 16, 1974)

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Work carried out 50 years ago showed that slices of brain produce ammonia when incubated under suitable conditions (1). This observation has been confirmed on many occasions both in vivo and in vitro. For example, ammonia appears during ischemia of the brain and electrical stimulation of peripheral nerves. Ammonia disappears from the brain during sleep and anesthesia (2, 3). The explanation commonly given to account for ammonia formation involves transamination of amino acids with a-ketoglutarate to form glutamate, and the subsequent deamination of glutamate by glutamate dehydrogenase. Objections to this mechanism have been raised on experimental grounds (2, 4) and on theoretical grounds (5).

Evidence is presented below which shows that the reactions of the purine nucleotide cycle occur in extracts of rat brain, and that their activities are such that they can account for the rates of ammonia production observed in vitro and in vivo.

EXPERIMENTAL PROCEDURE

Preparation of Brain Extract

Male rats weighing 350 to 500 g were decapitated. The whole brains (1.6 to 1.8 g each) were removed, rinsed free of blood, and homogenized with a TenBroeck homogenizer (an all glass hand-operated homogenizer), in 3 ml/g of brain of Tris-citrate buffer, pH 7.3 (20 mM Tris base and 6.4 mM citric acid), containing 1 mM dithiothreitol. The extract was centrifuged first at 31,000 × g for 20 min in a Sorvall RC2-B centrifuge, and then at 85,000 × g for 30 min in head number 30 of a Spinco model L preparative centrifuge. The high speed supernatant was freed of endogenous metabolites by passage through a column packed with Sephadex G-25 (coarse grade) which was pre-equilibrated with a solution containing Tris-citrate buffer, pH 7.3 (20 mM Tris base and 6.4 mM citric acid), 15 mM potassium phosphate, 150 mM KCl, and 1 mM dithiothreitol. The pooled protein solution was concentrated by ultrafiltration using an Amicon diaflo membrane (type PM-30). The concentrated brain extract, which contained 29 mg of protein/ml, was used in the cycling experiments.

Brains of three rats were pooled for the determination of enzyme activities. The brains were treated as described above, except that additional extractions were carried out. The residue from the low speed centrifugation was homogenized again in 2 ml of buffer/g of brain, and centrifuged at 31,000 × g as above. The process of re-extraction was repeated up to six times, and a separate high speed supernatant was prepared from each low speed supernatant.

Cycling Experiment

The complete reaction mixture contained 0.24 mM GTP, 0.50 mM IMP, 4 mM aspartate, 27 mM imidazole-HCl buffer, pH 7.0, 8.3 mM MgCl₂, 8.4 mM phosphoenolpyruvate (trycyclohexylammonium salt), and brain extract, 9.7 mg of protein/ml. The brain extract, which...
accounted for one-third of the total volume, contributed 6.7 mM Tris, 2.1 mM citrate, 0.33 mM dichloroethanol, 50 mM KCl, and 5 mM potassium phosphate. The control mixture was identical except that it lacked aspartate. All of the above constituents, with the exception of aspartate, were mixed in a glass-stoppered test tube. Phosphoenolpyruvate was added last (zero time). The mixture was incubated in a water bath at 30°C for 12 min. This temperature was chosen because at the physiological temperature, 38°C, protein precipitation occurs at a significant rate. Amination was then initiated by addition of aspartate to the test mixture. An equivalent volume of water was added to the control. The progress of the reactions was followed by removing 0.6-mI samples for spectral analysis at 5-min intervals. The samples were added to a quartz cuvette and the light path was reduced to 1 mm by the insertion of a quartz spacer 3-mm thick. Spectral scans were made from 310 nm to 240 nm on a Perkin Elmer model 356 spectrophotometer. The changes in total adenine nucleotide were calculated using the following equations (9):

$$\Delta \text{Adenine nucleotide (mm)} = (\Delta A_{340} - 1.14 \Delta A_{293})/0.88$$

$$\Delta \text{Adenine nucleotide (mm)} = (\Delta A_{293} - 1.5 \Delta A_{260})/0.6$$

The spectral scans showed that amination came to a stop after 1.5 hours, and that deamination began spontaneously. The spectral scans were continued for another 1.5 hours until deamination stopped.

Other samples (1.0 ml) were removed from the reaction mixture at various times and deproteinized by addition of an equal volume of 12% perchloric acid. After centrifugation, these samples were neutralized with a mixture of 2 N KOH and 0.5 M triethanolamine, and allowed to stand on ice for 20 min. Insoluble potassium perchlorate was then removed by centrifugation, and the samples were frozen rapidly and stored frozen for later analysis of metabolites.

Enzymes of the purine nucleotide cycle and of various side reactions were assayed before the start of the cycling experiment and after they had been in the reaction mixture for 4 hours at 30°C.

**Metabolite Assays**

Ammonia was measured using glutamate dehydrogenase following the procedure of Tabor (10). The complete reaction mixture contained 80 mM potassium phosphate buffer, pH 7.6, 0.4 mM EDTA, 0.84 mM ADP, 0.08 mg/ml of DPNH, 4 mM a-ketoglutarate, and 0.04 mg/ml of glutamate dehydrogenase in 50% glycerol (specific activity 45 units/mg). The mixture was incubated at room temperature for 30 min, and the reaction was then initiated by addition of the deproteinized sample. 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. Each determination was done in duplicate. One nanomole of ammonia could be measured with a precision of about 10%.

**Enzymatic Determination of Purines and Purine Nucleosides**

Assays contained 0.1 mM potassium phosphate buffer, pH 7.4, and deproteinized, neutralized extract in a final volume of 3.0 ml. The purines and purine nucleosides were assayed by their conversion to uric acid using the sequential addition of the following enzymes: xanthine oxidase for the assay of hypoxanthine plus xanthine; followed by nucleoside deaminase for the assay of inosine; followed by adenylosuccinase for the assay of adenine; followed by guanine deaminase (guanase) for the assay of guanine plus guanosine. The formation of uric acid was measured on the double beam spectrophotometer set to read absorbance at 293 nm minus absorbance at 250 nm.

**Enzymatic Determination of IMP, Adenine Nucleotide, and Guanine Nucleotide**

The following were preincubated at room temperature: 0.05 ml of 1 M glycine-NaOH buffer, pH 9.6, 0.05 ml of deproteinized extract, and 20 μg of snake venom 5'-nucleotidase (specific activity 20 units/mg). Under these conditions, the preparation of snake venom used by us hydrolyzed IMP to inosine; AMP, ADP, and ATP to adenosine; and GMP, GDP, and GTP to guanosine. After 45 min the volume was adjusted to 3 ml with 0.1 mM potassium phosphate buffer, pH 7.4, and inosine, adenosine, and guanosine were assayed as described above. This assay yields the sum of nucleotides and nucleosides. The difference between this assay and the assay for nucleosides described above yields IMP and the sums of both AMP plus ADP plus ATP, and GMP plus GDP plus GTP.

AMP was determined according to Williamson and Corkey (11), except that we used the double beam spectrophotometer set to read absorbance at 400 nm minus absorbance at 540 nm. Protein was determined by the method of Lowry et al. (12).

**Enzyme Assays**

Each assay was based on the reference cited, but was modified to yield optimum conditions with extracts of rat brain. The enzyme rates were linear as a function of protein concentration over the range quoted.

**Adenylate Deaminase**

The deamination of AMP to IMP was measured by following the decrease in absorbance at 262.5 nm minus 310 nm. The reaction mixture contained 27 mM imidazole-HCl buffer, pH 7.0, 50 mM LiCl, 2 mM AMP, 6 mM ATP, and high speed supernatant of rat brain, 0.05 to 0.2 mg of protein, in a volume of 0.6 ml. The light path was 0.05 mm (13).

**Adenylosuccinate Synthetase**

The formation of adenylosuccinate from IMP was measured by following the change in absorbance at 259 nm minus 310 nm. The complete reaction mixture contained 27 mM imidazole-HCl buffer, pH 7.0, 50 mM KCl, 8.3 mM MgCl, 4 mM aspartate, 0.5 mM IMP, 0.3 mM GTP, 1.1 mM phosphoenolpyruvate (tricyclohexylammonium salt), and high speed supernatant of rat brain, 1 to 2 mg of protein in a total volume of 0.6 ml. The light path was 1 mm. The presence of adenylosuccinase does not interfere with this assay since adenine nucleotide and adenylosuccinate have the same absorbance at 259 nm.

**Adenylosuccinate Oxidase**

The conversion of adenylosuccinate to AMP was measured by following the change in absorbance at 282 nm minus 310 nm. The complete reaction mixture contained 15 mM potassium phosphate buffer, pH 7.4, 0.2 mM adenylosuccinate (ammonium salt), and high speed supernatant of rat brain, 0.5 to 1.0 mg of protein, in a total volume of 0.6 ml. The light path was 1 mm. The presence of adenylosuccinate deaminase does not interfere with this assay since AMP and IMP have the same absorbance at 282 nm.

**Adenine Deaminase**

The deamination of adenosine to inosine was measured by following the decrease in absorbance at 262.5 nm minus 310 nm. The reaction mixture contained 0.1 mM potassium phosphate buffer, pH 7.4, 0.5 mM adenosine, and high speed supernatant of brain, 0.05 to 0.1 mg of protein, in a volume of 0.6 ml. The light path was 1 mm (14).

**Nucleoside Phosphorylase**

The rate of conversion of inosine to hypoxanthine was measured in a coupled assay by following the rate of formation of uric acid as described below. The reaction mixture contained 0.1 mM potassium phosphate buffer, pH 7.4, 0.5 mM inosine, xanthine oxidase, 0.02 unit/ml, and high speed supernatant of rat brain, 0.05 to 0.1 mg of protein, in a total volume of 5 ml (15).

**Guanase**

The rate of conversion of guanine to xanthine was measured in a coupled assay by following the rate of formation of uric acid as described below. The complete reaction mixture contained 0.1 mM Tris-HCl buffer, pH 8.8, 0.07 mM guanine, xanthine oxidase, 0.04 unit/ml, and high speed supernatant of rat brain, 0.05 to 0.1 mg of protein, in a total volume of 5 ml (16).

**Xanthine Oxidase**

The formation of uric acid from hypoxanthine was measured by following the change in absorbance at 293 nm minus 350 nm. The complete reaction mixture contained 0.16 mM hypoxanthine, 0.1 mM potassium phosphate buffer, pH 7.4, and 1 to 2 mg of high speed supernatant of rat brain, in a total volume of 3 ml (17).

These conditions are based on routine assays carried out in our laboratory.
Ammonia Production in Brain

**Pyruvate Kinase**

The complete reaction mixture contained 42 mM triethanolamine buffer, pH 7.4, 4.2 mM EDTA, 8.3 mM MgCl₂, 10 mM KCl, 0.53 mM phosphoenolpyruvate (tricyclohexylammonium salt), 9.07 mg/ml of DPNH, 1.5 mM ADP, 17 μg/ml of lactate dehydrogenase, and high speed supernatant of rat brain. 5 to 10 μg of protein, in a total volume of 3 ml. The change in absorbance was measured at 400 nm minus 340 nm (18).

**Adenylate Kinase**

The reaction mixture contained 40 mM triethanolamine buffer, pH 7.4, 4.4 mM EDTA, 8 mM MgCl₂, 10 mM KCl, 0.08 mg/ml of DPNH, 1.5 mM AMP, 1 mM ATP, 0.6 mM phosphoenolpyruvate (tricyclohexylammonium salt), 40 μg/ml of lactate dehydrogenase, 10 μg/ml of pyruvate kinase, and high speed supernatant of rat brain, 5 to 10 μg of protein, in a total volume of 2.5 ml. The change in absorbance was measured at 400 nm minus 340 nm (19).

**5′-Nucleotidase**

The complete reaction mixture contained 1 mM AMP, 24 mM imidazole-HCl buffer, pH 7.0, 10 mM MgCl₂, and high speed supernatant of rat brain, 1 to 2 mg of protein, in a total volume of 1 ml. The mixture was incubated at 30° for 20 min. Inorganic phosphate was measured according to the method of Mozersky et al. (20). The assays were performed in the presence and in the absence of 1 mM adenosine α,β-methylene diphosphonate, a potent inhibitor of 5′-nucleotidase (21). The difference between the rates of hydrolysis of AMP in the presence and absence of adenosine α,β-methylene diphosphonate is quoted as the 5′-nucleotidase activity.

**p-Nitrophenyl Phosphatase**

The reaction mixture contained 5 mM p-nitrophenyl phosphate, 27 mM imidazole-HCl buffer, pH 7.0, 8.3 mM MgCl₂, and high speed supernatant of rat brain, 0.3 to 0.6 mg of protein, in a total volume of 2 ml. The control lacked substrate. Aliquots of 0.1 ml were withdrawn at 3-min intervals and added to 0.3 ml of 1 M borate buffer, pH 9.5, and the absorbance was measured at 400 nm using a Zeiss PMQ II spectrophotometer (22).

**Purine Nucleotide Cycle Enzymes in Rat Muscle**

Rat muscle was extracted in 90 mM potassium phosphate buffer, pH 6.5, 180 mM KCl, and a high speed supernatant was prepared as described previously (9).

**Adenylate Deaminase**—The incubation mixture contained 27 mM imidazole-HCl buffer, pH 6.2, 2 mM AMP, 100 mM KCl, and muscle extract in a total volume of 0.6 ml. The extract contributed 0.15 to 0.3 mM potassium phosphate, 0.3 to 0.6 mM KCl, and 15 to 30 μg of protein. The decrease in absorbance at 282.5 nm was measured on a Zeiss recording spectrophotometer. The light path was 0.5 mm.

**Adenylosuccinate Synthetase**—The complete reaction mixture for the assay of muscle adenylosuccinate synthetase contained 27 mM imidazole-HCl buffer, pH 7.0, 8.3 mM MgCl₂, 0.6 mM AMP, 0.3 mM GTP, 4 mM aspartate, 0.56 mM phosphoenolpyruvate (tricyclohexylammonium salt), 50 mM KCl, and muscle extract, 0.7 to 1.4 mg of protein, in a total volume of 0.6 ml. The extract contributed 7.5 to 15 mM potassium phosphate and 15 to 30 mM KCl. The increase in absorbance was measured on a Perkin Elmer model 356 spectrophotometer set to read 260 nm minus 340 nm. The light path was 1 mm.

**Adenylosuccinate**—The assay for muscle adenylosuccinate was identical with that of the brain enzyme except for the presence in the muscle assay of 15 to 30 mM KCl and 7.5 to 15 mM potassium phosphate contributed by the muscle extract.

**Materials**

Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratory. GTP, AMP, ADP, guanine, hypoxanthine, inosine, and pyridine nucleotides were purchased from P-L Laboratories. Adenosine was purchased from Calbiochem. Phosphoenolpyruvate (tricyclohexylammonium salt) and AMP were obtained from Boehringer, while ATP came from both Boehringer and P-L Biochemicals.

1-Aspatic acid, α-ketoglutarate, dihydroxyacetone, 5-phospho3-hydroxy-1-pyrophosphate, ribose 1-phosphate, ribose 5-phosphate, and p-nitrophenyl phosphate were obtained from Sigma. GTP was obtained from Boehringer. Snake venom 5′-nucleotidase was obtained from Sigma. This preparation was contaminated with other phosphatases and was capable of hydrolyzing ADP, ATP, GDP, and GTP.

**RESULTS**

As is shown below, preparations of brain cytoplasm catalyze the conversion of IMP to AMP and of AMP to IMP. The demonstration of these reactions is complicated by the occurrence of various side reactions.

**Conversion of IMP to AMP**—The formation of adenine nucleotide from IMP begins immediately after the addition of aspartate. Fig. 1 shows that it is accompanied by a concomitant decrease in hypoxanthine compounds. No accumulation of adenylosuccinate was observed. This is not unexpected since in rat brain the specific activity of adenylosuccinate is about five times that of adenylosuccinate synthetase. Nucleotide analyses show that all of the adenine nucleotide formed was converted to ATP during this phase of the cycle. This indicates that AMP reacted with endogenous ATP to produce ADP via endogenous adenylosuccinate kinase. The ADP was in turn phosphorylated to ATP by the action of endogenous pyruvate kinase and added phosphoenolpyruvate.

The nucleoside triphosphate regenerating system is required for the adenylosuccinate synthetase step, insofar as it regenerates GTP from GDP and thereby counters the powerful inhibitory effect of GDP on the enzyme. In addition the regenerating system prevents the deamination of AMP by causing the conversion of AMP to ATP. As soon as phosphoenolpyruvate becomes depleted the regenerating system stops functioning, accumulation of IMP ceases, and deamination begins spontaneously. For each molecule of IMP aminated to AMP, one molecule of GTP is converted to GDP. The conversion of the AMP and GDP to their respective triphosphates consumes 3 molecules of phosphoenolpyruvate. In the absence of side reactions, a 3-fold excess of phosphoenolpyruvate over IMP should therefore be sufficient to bring about complete conversion of IMP to ATP. In practice a much larger excess of phosphoenolpyruvate has to be added, because the high phosphatase activity of the extract, which continually hydrolyzes GTP, ATP, and IMP. In the experiment shown in Fig. 1, the amination came to a halt at 80% of completion and the amount of phosphoenolpyruvate used was 7 times the amount expected from the amount of IMP consumed.

The IMP added to the reaction mixture is also hydrolyzed by phosphatases (reaction I) and the resulting inosine is acted upon by nucleoside phosphorylase (reaction II). Both enzymes are present in the brain extract. During the preincubation period, inosine and hypoxanthine accumulate. The hypoxanthine

\[ \text{IMP} + \text{H}_2\text{O} \rightarrow \text{inosine} + \text{Pi} \]  

(1)

\[ \text{inosine} + \text{Pi} \rightarrow \text{hypoxanthine} + \text{ribose} 1\text{-phosphate} \]  

(II)

Thine formed in reaction II is converted back to AMP by hypoxanthine-guanine phosphoribosyltransferase. This is discussed later. The conversion of IMP to inosine might also be partially reversed by inosine kinase (reaction III) (24), although such activity has not been demonstrated in brain. When the amination

\[ \text{IMP} \rightarrow \text{AMP} \]  

(3)

\[ \text{AMP} \rightarrow \text{ADP} \]  

(4)

\[ \text{ADP} \rightarrow \text{ATP} \]  

(5)

An amount of ATP equivalent to 2 μM in the final reaction mixture escaped separation by the gel filtration procedure.
decrease. This occurs by the following sequence of reactions:

1. The reaction mixture for the first time when the phosphoenolpyruvate was added at zero time. Amination was initiated by the addition of aspartate. The reactions were run at 30°C. At times indicated by the experimental points, samples were withdrawn for spectral and enzymatic analyses. Spectral scans were made from 310 nm to 240 nm on a Perkin Elmer model 356 spectrophotometer. O, adenine nucleotide measured spectrally; □, adenine nucleotide measured enzymatically; ◊, hypoxanthine compounds, i.e. IMP plus inosine plus hypoxanthine plus xanthine; Δ, AMP; ▽, guanine nucleotides; ×, ammonia. A base-line value of 0.08 mM ammonia was subtracted from all points plotted in the figure.

2. Conversion of AMP to IMP—Fig. 1 shows that amination occurs by the following sequence of reactions:

- **Guanine + H₂O → guanosine + P** (reaction IV)
- **inosine + ATP → IMP + ADP** (reaction III)
- **GMP is hydrolyzed to guanosine (reaction IV), and this in turn is converted to guanine by nucleoside phosphorylase (reaction V). The final step is the irreversible deamination of guanine to xanthine by guanase (reaction VI). Guanase is specific for guanine and does not react with either guanosine or GMP. The decrease in guanine nucleotides lags behind the decrease in adenine nucleotides because of the relatively slow rates of reactions IV and V. Xanthine does not react further due to the absence in the brain extract of xanthine oxidase (Table I). The increase in hypoxanthine compounds agrees well with the decrease in adenine plus guanine nucleotides during the deamination half of the cycle.

In some of the 12 similar experiments that were run the spectra obtained became distorted during the deamination phase. The characteristic peak at 262.5 nm (the wavelength of the difference maximum between AMP and IMP) shifted, sometimes to shorter and other times to longer wavelengths, and the isosbestic point at 250 nm disappeared. As a result, the calculations based on changes at 262.5 nm and 270 nm became increasingly divergent. Thus during deamination, the spectral method for following the interconversion of purine nucleotides was, in some experiments, less accurate than the enzymatic analysis.

The spectral distortions that occur during the deamination phase of some experiments are probably due to side reactions involving guanine nucleotides. These side reactions do not necessarily occur at the same rate in both the complete reaction mixture and the control which lacks aspartate, since phosphoenolpyruvate may not be completely exhausted at the same time in the two systems. Although guanine breakdown is always observed during the deamination phase of the cycle, the occurrence of spectral shifts seems to depend on a difference in the reaction rates of the control and the test cuvettes.

3. Brain tissue contains hypoxanthine-guanine phosphoribosyltransferase in high activity (25). The enzyme converts the purine bases guanine and hypoxanthine into their respective monophosphates, while the other product, hypophosphosphate, is hydrolyzed to orthophosphate by pyrophosphatase (reactions VII and VIII). 5-Phosphoribosyl 1-pyrophosphate (PP-ribose-P) is synthesized in the brain from ribose 5-phosphate and ATP (reaction IX). Ribose 5-phosphate is generated from ribose 1-phosphate by the enzyme phosphoribomutase (reaction X) (26). This sequence of reactions together with the reaction catalyzed by nucleoside phosphorylase constitutes the salvage pathway whereby purine bases and nucleosides are reconverted to nucleotides.

The brain extracts used by us contained all of these enzymes as is demonstrated in the experiment shown in Table II. Hypoxanthine is converted to IMP in the presence of either PP-ribose-P or ribose 5-phosphate plus ATP. Ribose 1-phosphate is also effective in generating IMP, but it reacts more slowly. The concentrations of the substrates used in the experiment are similar to those found during the amination phase of the cycling experiment, but the protein concentration was only half. Phosphoenolpyruvate was added to prevent the breakdown of ATP to AMP by endogenous ATPases, which

\[\text{guanine} + \text{PP-ribose-P} \rightarrow \text{GMP} + \text{PP}_1 \rightarrow 2 \text{P}_1 \]  
\[\text{hypoxanthine} + \text{PP-ribose-P} \rightarrow \text{IMP} + \text{PP}_1 \rightarrow 2 \text{P}_1 \]  
\[\text{ATP} + \text{ribose 5-phosphate} \rightarrow \text{PP-ribose-P} + \text{AMP} \]  
\[\text{ribose 1-phosphate} \rightarrow \text{ribose 5-phosphate} \]
Assays were performed at 30°C. Wet weight. p-Nitrophenyl phosphatase was assayed at 25°C. All other high speed supernatant protein extracted from rat brain was 38.7 mg/g of tissue/min and represent the total extractable activity. The total parentheses. Enzyme activities are quoted as micromoles/g wet weight min observed as described under “Experimental Procedure” and are shown in parentheses. Enzyme activities are quoted as micromoles/g wet weight of tissue/min and represent the total extractable activity. The total high speed supernatant protein extracted from rat brain was 38.7 mg/g wet weight. p-Nitrophenyl phosphatase was assayed at 25°C. All other assays were performed at 30°C.

| Enzyme                      | Maximum activity | Activity under cycling conditions | Cycling conditions |
|-----------------------------|------------------|-----------------------------------|-------------------|
| Adenylosuccinate synthetase | 0.036 (0.46)     | 95                                | 8 mM phosphoenolpyruvate |
| Adenylosuccinase            | 0.18 (0.38)      | 90                                | 0.06 mm adenylosuccinate |
| Adenosine deaminase         | 0.27             | 10-40                             | 0.01-0.10 mm adenosine |
| Nucleoside phosphorylase    | 1.32             | 12-35                             | 0.02-0.12 mm inosine |
| Guanase                     | 1.11             | 70                                | 0.02 mm guanine     |
| Xanthine oxidase            | 0                |                                    |                   |
| Pyruvate kinase             | 97.3             | 3                                 | 0.01 mm ADP, 0.2 mm phosphoenolpyruvate |
| Adenylate kinase            | 40.1             | 1.6                               | 0.01 mm AMP, 0.4 mm ATP |
| 5'-Nucleotidase             | 0.02             |                                    |                   |
| p-Nitrophenyl-phosphatase   | 0.27             |                                    |                   |

Table I

Ammonia Production in Brain

Enzyme activities in particle-free extracts of rat brain and skeletal muscle

Brains of three rats were pooled, multiple extractions were made, and maximum enzyme activities were assayed as described under “Experimental Procedure.” Activities were also assayed under conditions approximating those of the cycling experiment shown in Fig. 1. These assays were all performed in the presence of 27 mm imidazole-HCl, pH 7.0, 8.3 mm MgCl2, 50 mM KCl, 5 to 10 mm potassium phosphate, and 0.08 to 3.2 mg of protein/ml. For each assay, the substrate concentrations which were different from those used in the optimum assay are listed under “Cycling Conditions.” Activities of the purine nucleotide cycle enzymes in skeletal muscle were determined as described under “Experimental Procedure” and are shown in parentheses. Enzyme activities are quoted as micromoles/g wet weight of tissue/min and represent the total extractable activity. The total high speed supernatant protein extracted from rat brain was 38.7 mg/g wet weight. p-Nitrophenyl phosphatase was assayed at 25°C. All other assays were performed at 30°C.

Table II

Purine nucleotide salvage pathway in rat brain

A high speed supernatant was prepared and passed through a Sephadex G-25 column as described under “Experimental Procedure.” The peak protein fractions were pooled to yield a mixture containing 8.4 mg of protein/ml, which was used without further treatment. The complete reaction mixture contained 26 mm imidazole-HCl buffer, pH 7.0, 8 mm MgCl2, 0.11 mm hypoxanthine, 0.17 mm ATP, 8 mm phosphoenolpyruvate (tricyclohexylammonium salt), 1 mm UMP, 0.18 mm ribose 5-phosphate, and brain extract. 5 mg/ml, in a total volume of 0.6 ml. The protein extract contributed 90 mm KCl, 9 mm potassium phosphate, 12 mm Tris, 3.8 mm citrate, and 0.6 mm diithiothreitol. Where indicated 0.18 mm ribose 1-phosphate replaced ribose 5-phosphate. Samples containing 5-phosphoribosyl 1-pyrophosphate (1 mm) lacked ATP, ribose phosphate, and phosphoenolpyruvate. The reactions were initiated by addition of brain extract, and the incubations were carried out at 30°C for 5 or 15 min. The reactions were terminated by the addition of 0.5 ml of 12% perchloric acid. The samples were neutralized and analyses were performed for IMP, inosine, and hypoxanthine as described under “Experimental Procedure.” Note that the maximum amount of IMP expected to be formed was 110 nM; the slightly greater amount observed in one analysis is probably due to an analytical error.

| Reaction mixture               | 5-min incubation | 15-min incubation |
|--------------------------------|------------------|-------------------|
| [IMP] formed                   | [nM]             | [nM]              |
| Complete                       | 29               | 60                |
| minus hypoxanthine             | 0                |                   |
| minus ribose 5-phosphate       | 0                |                   |
| minus ATP                      | 1                | 3                 |
| minus UMP                      | 25               | 20                |
| plus ribose 1-phosphate        | 2                | 29                |
| plus PP-ribose-P               | 59               | 120               |

Table II

Ammonia Production in Brain

Adenine production

In the experiments reported here, ammonia comes from the deamination not only of adenine but also of guanine nucleotide. Ammonia formation was not observed until the nucleotides began to be deaminated. Therefore, the rate of ammonia production closely followed the rate of disappearance of adenine plus guanine nucleotides, and the rate of appearance of IMP plus inosine plus hypoxanthine plus xanthine (Fig. 1). The stoichiometry between ammonia appearance and nucleotide disappearance showed a discrepancy of about 0.06 μM during the deamination phase. This may be due to a systematic error in the analyses.

In a separate experiment, in which endogenous metabolites were not removed from the brain extract by passage through Sephadex, no significant changes in the concentrations of glutamine and glutamate were detected. These substances were measured by standard enzymatic methods (27, 28), and a change of 0.05 μM could easily have been detected.

Under conditions which are similar to those that occur during the deamination phase of the cycle, adenosine deaminase and adenylyl deaminase activities of the brain extract are similar (Table I). (Under these conditions, adenylyl deaminase activity is only a small fraction of its maximum because the enzyme is only partially activated.) This raises the possibility that the adenosine deaminase reaction may contribute substantially to ammonia production in the experiment shown in Fig. 1. However, hydrolysis of 6' mononucleotides is very slow, and the adenosine concentration was an average of 6 μM during the deamination phase. At this concentration, the activity of adenosine deaminase is sufficient to account for about one-third of the adenine containing compounds deaminated.

Activities of Purine Nucleotide Cycle Enzymes in Brain—Adenylosuccinase, adenylosuccinate synthetase, adenylosuccinase, adenylate kinase, and pyruvate kinase activities in extracts of rat brain are shown in Table I. Activities are quoted both under optimal conditions and under conditions comparable to those found during the cycling experiments. Adenylosuccinate synthetase is the least active enzyme of the
cycle, and under the conditions of the in vitro cycling experiment, it operates at close to its $V_{\text{max}}$. Fig. 1 shows that adenylate deaminase operates at an activity that is comparable to that of the synthetase, in other words far below its maximum activity. This is to be expected from the results shown in Table I for a number of reasons. The maximum concentration of AMP during the deamination phase is only 0.2 mM, which is one-tenth that used for the optimum assay. The ATP concentration drops rapidly from 0.15 mM at the beginning of deamination (80 min) to less than 0.01 mM halfway through the deamination, a concentration that is insufficient to activate the enzyme appreciably. Moreover, enzyme concentration versus activity measurements show that the activity of adenylate deaminase decreases with increasing protein concentration. While the assays quoted in Table I contained a maximum protein concentration of 0.3 mg per ml, the cycling experiment contained 9.7 mg/ml. This 30-fold difference in protein concentration results in a decrease of the specific activity of adenylate deaminase of at least 60%. Another factor in the cycling experiment is the presence of orthophosphate, an inhibitor of the enzyme, which falls from 5 mM at the start to about 13 mM at the onset of deamination.

The rate of deamination is not limited by the adenylate kinase activity of the brain extract. In a separate cycling experiment, addition of exogenous adenylate kinase, in amounts up to four times the endogenous adenylate kinase activity, either at the beginning of the experiment or at the onset of deamination, did not significantly alter the rate of deamination. No loss of adenylate kinase activity occurred when the complete reaction mixture was incubated at 30°C for 4 hours. Adenylosuccinate synthetase activity decreased by 50% after 4 hours at 30°C. The other enzyme activities involved in the purine nucleotide cycle or its side reactions remained unaltered, except that adenylosuccinase and nucleoside phosphorlase decreased by 20% during this period.

Table I also shows the activities in particle-free extracts of brain of nucleoside phosphorylase, guanase, and N-nitrophenyl phosphatase. 5'-Nucleotidase is virtually absent from the particle-free extract used in the cycling experiment (0.02 µmol/g fresh weight/min at 30°C). Assays of the whole homogenate of brain showed the presence of 5'-nucleotidase equivalent to 1.3 µmol/g fresh weight/min at 30°C. No xanthine oxidase activity was detected in the extracts, in harmony with the observation that hypoxanthine and xanthine but not uric acid accumulated during the deamination phase of the cycle.

Activity of Purine Nucleotide Cycle Enzymes in Skeletal Muscle—Activities of the purine nucleotide cycle enzymes in muscle are quoted in Table I for comparison with those in brain. The maximum capacity for the amination of IMP in muscle is about 10 times that found in brain, since in muscle amination is limited by the activity of adenylosuccinase, in contrast to brain where the synthetase is the least active enzyme. The maximum capacity of adenylate deaminase in muscle is 30 times that found in brain.

**DISCUSSION**

Rates of ammonia production by rat brain in vivo are related to the level of cerebral activity. An initial rate of 7.5 µmol/g fresh weight/min was observed in rat brain after application of convulsive agents (7, 29, 30). This rate is within the capacity of adenylate deaminase (Table I) (5.2 µmol/g fresh weight/min at 30°C, which is equivalent to 8.3 µmol/g/min at 38°C), but not of glutamate dehydrogenase (Table III, and see below). Ammonia may also be derived from adenosine, guanine, and guanosine, but at a maximum rate of only 2.3 µmol/g/min at 38°C (30).

Several workers have determined the activity of glutamate dehydrogenase in brain (31–33), but these measurements were all made in the direction of reductive amination of α-ketoglutarate. In order to estimate the maximum rates of ammonia production it is necessary to measure the activity of the enzyme in the direction of oxidative deamination of glutamate. Table III shows the rates of amimation and deamination catalyzed by glutamate dehydrogenase of rat brain at several pH values, with either DPN or TPN, and in the presence and absence of the activator ADP. The maximum rate of deamination of glutamate is 2.2 µmol/g fresh weight/min at 30°C and pH 8.0, which is equivalent to 3.5 µmol/g/min at 38°C. This is insufficient to account for the maximum rates of ammonia production observed in vivo. At lower pH values the maximum rates of glutamate deamination are even lower.

Other experimental findings show that the glutamate dehydrogenase reaction can at most account for only 50% of the ammonia produced by brain slices in the absence of added substrates (4, 34). However, these experiments did not prove that any ammonia is actually produced via this reaction. Weil-Malherbe and Gordon (4) found that in the presence of 10 mM 5-bromofuroate, an inhibitor of glutamate dehydrogenase, aerobic ammonia formation by slices of brain cortex continued at 95% of the control rate during the 1st hour of incubation, but that bromofuroate caused an increasing inhibition of ammonia production during the 2nd and 3rd hour. We have found that 5-bromofuroate inhibits brain adenylosuccinate synthetase. Dixon plots (35) show the inhibitor is competitive with respect to IMP, with a $K_i$ of approximately 5 mM, and noncompetitive with respect to aspartate with a $K_i > 15$ mM. Bromofuroate also inhibits glutamate dehydrogenase of rat brain. The inhibition is competitive with respect to glutamate, with a $K_i$ of 0.9 mM. Although 5-bromofuroate is a weaker inhibitor of adenylosuccinate synthetase, 10 mM 5-bromofuroate causes a 40% inhibition of the brain enzyme when it is assayed in the presence of 80 µM IMP and 4 mM aspartate. Thus, the effect of high concentrations of bromofuroate on ammonia production, which was quoted as evidence in favor of the involvement of glutamate dehydrogenase, can also be interpreted in terms of the inhibition of adenylosuccinate synthetase.

Weil-Malherbe and Gordon (4) showed further that D-aspartate and D-glutamate are weak inhibitors of ammonia formation by brain slices after the 1st hour of incubation, and that this inhibition can be reversed by L-aspartate. They interpreted this as an effect on glutamate dehydrogenase, but this observation too is consistent with an inhibition of the adenylosuccinate synthetase step of the purine nucleotide cycle by D-aspartate (36) and possibly of transaminase by high concentrations of D-glutamate.

It has been demonstrated that rat brain dispersions produce

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3Corrected from 30° to 38° by multiplying by 1.6 (this correction assumes that the rate increased by a factor of 1.8/10°).

4 Since the validity of this statement depends on the completeness of our extraction of glutamate dehydrogenase, it is worth pointing out that the maximum rate of reductive amination of α-ketoglutarate observed by us (22 µmol/g fresh weight of brain/min at 30°C Table III) agrees closely with the highest rate reported in the literature by Williamson et al. (31) (21 µmol/g/min at 30°C (corrected from the published value at 25° by assuming a temperature coefficient of 1.8/10°).
ammonia at a steady rate equivalent to 0.037 \mu\text{mol/g/min} (6). This rate compares favorably with our measurements of adenylosuccinate synthetase activity (0.058 \mu\text{mol/g fresh weight/min at 38}^\circ\text{C}) (Table I). The steady rate is preceded by possibly by the action of glutaminase.

Guinea pig slices produce ammonia at a steady state rate of 0.13 \mu\text{mol/g/min} in the first 2 hours. Thereafter, rates of ammonia formation decrease (6). The activity of adenylosuccinate synthetase in extracts of guinea pig brain is the same as in rat brain* which means that in these experiments about one-half of the ammonia produced cannot be accounted for in terms of adenylosuccinate synthetase activity. However, the slices were incubated in the absence of substrate for 5 hours, and it seems possible that degradative processes contributed to ammonia production. For example, amide nitrogen in proteins (38) and nucleic acids (6) of brain have been shown to break down under similar conditions. It is also possible that under these nonphysiological conditions glutamate oxidation by glutamate dehydrogenase occurred to a significant extent.

Inhibition of ammonia formation during anaerobiosis and by inhibitors of electron transport was previously attributed to the requirement by glutamate dehydrogenase of DPN for ammonia production (6). However, under uncoupling conditions, the level of ATP, an activator of adenylate deaminase, is low and the level of orthophosphate, an inhibitor of the enzyme, is high. The exact experimental conditions are critical. Enhancement of ammonia formation by addition of uncouplers has been observed in a liver system (39) and in brain (34); this can be accounted for by an increase in the level of AMP while maintaining a reasonably high level of ATP.

The results presented above demonstrate that the purine nucleotide cycle occurs in brain, and that it may account for at least one-half the ammonia production observed with brain slices in the absence of added substrates. Measurements are in progress of rates of ammonia production by rat brain in vivo.

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\begin{table}
\centering
\caption{Ammonia Production in Brain}
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{pH} & \textbf{Deamination with DPN} & \textbf{Amination with DPN} & \textbf{Deamination with TPN} & \textbf{Amination with TPN} \\
& ADP & ADP & ADP & ADP & ADP & ADP & ADP \\
\hline
7.0 & 0.1 & 0.2 & 3.9 & 11 & 1.9 & 11 \\
7.5 & 0.6 & 1.9 & 9.6 & 20 & 0.9 & 4.5 & 22 \\
8.0 & 1.0 & 2.2 & 6.7 & 22 & 0.3 & 1.9 & 20 \\
\hline
\end{tabular}
\end{table}
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