The Purine Nucleotide Cycle

THE PRODUCTION OF AMMONIA FROM ASPARTATE BY EXTRACTS OF RAT SKELETAL MUSCLE*

(Received for publication, July 6, 1971)

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SUMMARY

Extracts of rat skeletal muscle convert aspartate to ammonia and fumarate plus malate. The conversion is dependent on the presence of catalytic amounts of IMP, adenylosuccinate, or AMP. In addition, GTP is required as a source of energy. A GTP-regenerating system must be supplied since the accumulation of GDP inhibits the operation of the cycle. It is proposed that the purine nucleotide cycle accounts for the production of ammonia during muscular work.

Ammonia is produced when muscle does work (1-11). The amount of ammonia produced by frog skeletal muscle is proportional to the work done (2). As a rough guide, in frog leg muscle 1 mole of ammonia is produced per 10 to 50 moles of lactate (8). Soon after the discovery of adenylate deaminase (12), it was recognized that the reaction catalyzed by this enzyme (Reaction I) is the major source of ammonia in muscle (4, 7). This conclusion was later confirmed indirectly when it was shown that there is a relatively rapid turnover of the 6-amino group of the adenine mononucleotides of striated muscle in vivo, in contrast to a very slow turnover of the purine ring nitrogen atoms (13). Moreover, it was shown that glutamate dehydrogenase is low or absent from skeletal muscle (14-17).

The deamination of AMP results in the formation of IMP. The regeneration of AMP from IMP occurs in two reactions which are catalyzed by adenylosuccinate synthetase (Reaction II) and adenylosuccinase (Reaction III) (18-22).

\[
\begin{align*}
\text{Reaction I} & : \text{AMP} + \text{H}_2\text{O} \rightarrow \text{IMP} + \text{NH}_3 \\
\text{Reaction II} & : \text{IMP} + \text{aspartate} + \text{GTP} \rightarrow \text{adenylosuccinate} + \text{GDP} + \text{P}_1 \\
\text{Reaction III} & : \text{Adenylosuccinate} \rightarrow \text{AMP} + \text{fumarate}
\end{align*}
\]

Net reaction

Aspartate + GTP + H$_2$O $\rightarrow$ fumarate + GDP + P$_1$ + NH$_3$

EXPERIMENTAL PROCEDURE

Methods

Preparation of Muscle Extract

Muscle was obtained from the hind legs of a rat weighing about 300 g. It was finely chopped using a Molinari chopper (24) and suspended in 3 volumes of a solution containing 90 mM potassium phosphate, pH 6.5, and 180 mM KCl. The suspension was blended for 20 sec in a small, stainless steel Waring Blender and stirred slowly in the cold for 1 hour. The mixture was filtered through cheesecloth, and the filtrate was centrifuged in head 38 of a Sorvall RC-2B centrifuge at 31,000 x g for 10 min, and then in head 30 of a Spinco model L preparative ultracentrifuge at 85,000 x g for 30 min. The high speed supernatant so obtained was adjusted to contain 5 mM EDTA and 0.1 mM dithiothreitol. Portions of 5 ml each were placed on a column (1.8 X 15.5 cm) of Sephadex G-25 and eluted with a solution containing 15 mM potassium phosphate, pH 6.5, 280 mM KCl, 5 mM EDTA, and 0.1 mM dithiothreitol. Fractions of 1 ml were collected. The fractions richest in protein were pooled; the resulting solution contained about 6 mg of protein per ml.

Spectrophotometric Measurements of Adenine Mononucleotide, Adenylosuccinate and IMP Concentrations

Adenylosuccinate, adenine nucleotide, and IMP are substances with strong ultraviolet absorption spectra. Although they overlap somewhat, these spectra can readily be distinguished from one another (23). Changes in the concentrations of the three nucleotides can be followed by obtaining ultraviolet absorption spectra as a function of time. The amounts of IMP, adenylosuccinate, and adenine nucleotide present are then calculated from

\[\text{Aspartate} + \text{GTP} + \text{H}_2\text{O} \rightarrow \text{fumarate} + \text{GDP} + \text{P}_1 + \text{NH}_3\]
appropriate equations involving the extinction coefficients of each substance at specifically chosen wave lengths. The calculation of the concentration of each of the three nucleotides is particularly simple when their total concentration remains constant, as was the case in the experiments to be described.

The wave lengths chosen were 282, 270, and 262.5 mp. The extinction coefficients of the nucleotides at those wave lengths are given in Table I. The equations derived for a 1-mm light path are

\[
\Delta \text{Adenylosuccinate} = \Delta A_{282}/1.0 \quad (1)
\]

\[
\Delta \text{Adenine nucleotide} = (\Delta A_{262.5} - 1.14 \Delta A_{282})/0.88 \quad (2)
\]

\[
\Delta \text{Adenine nucleotide} = (\Delta A_{270} - 1.5 \Delta A_{282})/0.60 \quad (3)
\]

At 282 mp, IMP and AMP (adenine nucleotide) absorb equally. Hence, any change in absorbance at 282 mp is due only to changes in adenylosuccinate concentration, resulting in the simple form of Equation 1. At 262.5 and 270 mp the contribution of adenylosuccinate is first subtracted out by using an appropriate multiple of \(\Delta A_{282}\). The remainder represents interconversion of IMP and adenine nucleotide, and is divided by the respective difference extinction coefficient to give the change in adenine nucleotide. The maximum difference between IMP and AMP occurs at 262.5 mp, and there is less of a correction for adenylosuccinate at 262.5 mp than at 270 mp. Equation 2 is thus more precise than Equation 3; the latter is used merely to check the adenine nucleotide values from the former to insure that there was no gross distortion of the spectra. It was assumed that

\[
\Delta \text{IMP} = - (\Delta \text{Adenylosuccinate} + \Delta \text{Adenine nucleotide}) \quad (4)
\]

The question of the conservation of the total nucleotide level is raised in the next section.

The spectral scans were run at 2 mp per sec on a Cary model 15 split beam recording spectrophotometer using cuvettes with a light path of 10 mm which was shortened to 1 mm by inserting quartz spacers 9 mm thick. The reference cuvette contained an amount of IMP equivalent to the initial concentration of IMP, adenylosuccinate, or adenine nucleotide in the complete reaction mixtures, but lacked aspartate. The recorder was zeroed at 310 mp before starting each scan.

**Calculations of \(\epsilon_{\text{max}}\) for Adenylosuccinate**

When adenylosuccinate was converted to adenine nucleotide in the reaction mixture described below, a point of constant absorbance was located at 259 mp. Both compounds must have the same extinction coefficient at this wave length. \(\epsilon_{\text{max}}\) for adenosine inosinate is 15.4 (Table I). The ratio \(A_{282}/A_{282}\) for adenylosuccinate was determined to be 1.25. It follows that \(\epsilon_{\text{max}}\) is 1.25 \(\times\) 15.4 = 19.3. No correction for the appearance of fumarate was made in this calculation. The accumulation of significant amounts of fumarate did not occur in our extracts since little cumulative change was noted even at 259 mp on recycling, and the isobestic point at 282 mp was observed consistently both for the transformation of IMP to adenylosuccinate and for adenine nucleotide to IMP. A simple explanation is that fumarase present in the extract catalyzed the conversion of most of the fumarate to malate. This explanation is supported by assays of malate and fumarate which we report below.

When IMP was converted to adenylosuccinate by preparations in which the adenylosuccinate activity had been destroyed by freezing and thawing, an isobestic point at 275 mp was observed.

### Table I

**Millimolar extinction coefficients at various wave lengths**

<table>
<thead>
<tr>
<th>Substance</th>
<th>(\lambda_{\text{max}})</th>
<th>(\epsilon_{\text{max}})</th>
<th>(\epsilon_{282})</th>
<th>(\epsilon_{270})</th>
<th>(\epsilon_{262.5})</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMP</td>
<td>248.5</td>
<td>12.2</td>
<td>1.5</td>
<td>4.1</td>
<td>6.2</td>
</tr>
<tr>
<td>AMP</td>
<td>259</td>
<td>15.4</td>
<td>1.5</td>
<td>10.1</td>
<td>15.0</td>
</tr>
<tr>
<td>SAMP</td>
<td>268</td>
<td>19.3</td>
<td>11.5</td>
<td>19.1</td>
<td>17.6</td>
</tr>
<tr>
<td>AMP minus IMP</td>
<td>0</td>
<td>6.0</td>
<td>15.0</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td>SAMP minus IMP</td>
<td>0</td>
<td>6.0</td>
<td>15.0</td>
<td>8.8</td>
<td></td>
</tr>
</tbody>
</table>

Calculations similar to those above gave an \(\epsilon_{\text{max}}\) for adenylosuccinate of 19.2. The \(\epsilon_{\text{max}}\) used for IMP was 12.2 (Table I). The values of 19.3 and 10.3 compare very well with the extinction coefficient of 19.2 calculated by Carter and Cohen (25) on the basis of the phosphorus content of adenylosuccinate, and with their value of 19.9 calculated from an isobestic point of 295.5 mp for conversion of adenylosuccinate to an equimolar mixture of AMP and fumarate.

The loss of nucleotides by dephosphorylation does not interfere with the spectral analysis, since it does not alter the spectra. For example, the spectrum of adenosine is virtually identical with that of AMP, ADP, and ATP. Any adenosine formed would thus remain in the calculation as "adenine nucleotide." Inosine, whether formed by dephosphorylation of IMP or deamination of adenosine, would remain as part of "IMP." Under our experimental conditions some loss of nucleotides into nucleosides may have occurred, which could account for the gradual lowering of efficiency during repeated operation of the cycle. However, most of the nucleotide must have been preserved intact, since the conversion of IMP to adenine nucleotide still proceeded to the extent of 70% of the added nucleotide even on the last cycle. Unlike deamination, analogous steps for reamination are not known for the nucleosides.

**Assays**

Ammonia determinations were performed according to the diffusion procedure of Seligson and Seligson (26) on 0.6-ml samples that were deproteinized by the addition of 0.5 ml of 10% trichloroacetic acid. Protein was assayed by the method of Lowry et al. (27). Malate and fumarate were assayed by enzymatic methods (28).

**Demonstration of Cycle**

1. **Starting with IMP**—The complete reaction mixture contained 0.52 mm IMP, 0.3 mm GTP, 4 mm aspartate, 27 mm imidazole-Cl buffer, pH 6.7, 8.3 mm MgCl₂, 1.67 mm creatine phosphate, 1.2 units per ml of crystalline yeast hexokinase (specific activity 70 units per mg), and muscle extract, 1.0 mg of protein per ml. The muscle protein extract, which accounted for \(\frac{1}{4}\)th of the total volume, contributed 2.5 mm orthophosphate, 47 mm KCl, 0.83 mm EDTA, and 17 mm dithiothreitol to the complete reaction mixture. The reaction was started by adding the extract. Control mixtures lacked either aspartate or IMP.

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\(1\) The abbreviation used is: SAMP, adenylosuccinate.
Relatively large volumes of these mixtures were prepared because of the large number of analyses which were performed in each experiment. Creatine phosphate and 2-deoxyglucose were added to these reaction mixtures in amounts and at times shown in the results. Samples of the reaction mixtures were withdrawn for following the progress of the reactions in the spectrophotometer and for analyzing the amounts of ammonia, fumarate, and malate produced. The schedule for making additions and withdrawing samples for analysis is described under "Results and Discussion." Alternate additions of creatine phosphate and 2-deoxyglucose were made until four cycles of amimation and deamination had been completed. The reaction mixtures were kept in stoppered tubes in a water bath set at the same temperature as that maintained in the spectrophotometer, namely 31°C.

2. Starting with Adenine Nucleotide (ATP)—The reaction mixtures were as described in 1 except that the IMP was replaced with 0.48 mM ATP, and that creatine phosphate was omitted at the start. Creatine phosphate was added later as indicated. The complete reaction mixture also contained muscle extract, 0.9 mg of protein per ml, and 1.0 unit per ml of yeast hexokinase (specific activity 83 units per mg). The temperature was 27°C.

3. Starting with Adenylosuccinate—The reaction mixtures were as described in 1 except that the IMP was replaced with 0.29 mM adenylosuccinate, and the creatine phosphate concentration was 0.74 mM. The complete reaction mixture also contained muscle extract, 0.7 mg protein per ml, and 0.7 units per ml of yeast hexokinase (specific activity 30 units per mg). The temperature was 28°C.

Materials

Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratory. Creatine phosphate was obtained from Boehringer; L-aspartic acid and 2-deoxyglucose from Sigma; and imidazole from Eastman Organic Chemicals. Dithiothreitol was obtained from Calbiochem and from P-L Laboratories.

Except for AMP and adenylosuccinate, nucleotides were purchased from P-L Laboratories. AMP was purchased from Boehringer. The ammonium salt of adenylosuccinate was prepared by Mr. Peter Frick of this laboratory according to the procedure of Carter and Cohen (95). Portions (1 ml) of an 18 mM solution of this preparation were placed on a column (1.8 × 15 cm) of Sephadex G-10, and were eluted with 20 mM KCl. Fractions of 1 ml were collected. There was some overlap of the tubes containing adenylosuccinate and adenine. Because of this, only the fractions which lacked ammonia were combined; they accounted for 80% of the adenylosuccinate placed on the column.

A suspension of crystalline yeast hexokinase in ammonium sulfate was purchased from Boehringer. The enzyme (2 mg in 0.2 ml) was centrifuged down and dissolved in 0.5 ml of 20 mM imidazole-HCl buffer, pH 7.2 or 6.7. The solution was placed on a column (1.8 × 15.5 cm) of Sephadex G-25 and eluted with the imidazole-HCl buffer. The pooled peak fractions contained 0.2 to 0.3 mg of protein per ml at a specific activity of 40 to 80 units per mg.

RESULTS AND DISCUSSION

The reaction sequence IMP → adenylosuccinate → AMP → IMP → etc. can be demonstrated in particle-free extracts prepared from muscle provided that certain precautions are taken. First, endogenous substrates must be removed if it is desired to demonstrate the dependence of the reaction sequence on substrates such as purine nucleotides or aspartate. This can be achieved by gel filtration of the extract on a column of Sephadex G-25. Secondly, a GTP-regenerating system must be provided to counter the inhibitory effects of GDP on the adenylosuccinate synthetase reaction. Thirdly, complete conversion of AMP to ATP must be avoided, or if it occurs, must be reversed, otherwise no AMP will be available for the adenylate deaminase reaction. In theory the second and third conditions could be met by providing a specific GTP-regenerating system without providing an ATP-regenerating system. At present a specific GTP-regenerating system is not readily available. Because of this, a relatively unspecific NTP-regenerating system was employed which consisted of creatine phosphate and endogenous creatine phosphokinase, in conjunction with hexokinase. The latter served to convert ATP back to ADP without affecting the GTP. Endogenous myokinase then generated an adequate level of AMP.

Conversion of IMP to Adenylosuccinate and AMP—An experimental demonstration of the sequential operation of adenylosuccinate synthetase and adenylosuccinase in an extract from rat skeletal muscle is shown in Fig. 1A. Sixteen spectral scans from 310 to 245 nm were run at appropriate time intervals after starting the reaction. The concentrations of IMP, adenylosuccinate, and adenine nucleotide were calculated from these scans. Note that the figure shows the appearance of adenylosuccinate and adenine nucleotide, and the disappearance of IMP. Adenylosuccinate builds up rapidly at first, then levels off and declines. After an initial lag, adenine nucleotide accumulates at a steady rate. Much if not all of the AMP formed was probably converted to ATP by the regenerating system, leaving at most very low levels of AMP. Little or none of the AMP formed was deaminated during this phase, as was indicated by ammonia analyses. The high level of GTP which was present (0.3 mM) inhibited adenylic deaminase. Orthophosphate also inhibits this enzyme. The liberation of orthophosphate by the adenylosuccinate synthetase reaction therefore would tend to increase the inhibition of adenylic deaminase in the reaction framework under consideration. Orthophosphate liberation by myokinase is probably minimum because of the absence of calcium ions and the presence of 8.3 mM MgCl2.

Conversion of AMP Back to IMP—If an amount of 2-deoxyglucose approximately equivalent to the amount of creatine phosphate originally present is now added, there is a rapid conversion of most of the adenine nucleotide to IMP. (Hexokinase was present throughout the experiment.) Eight spectral scans from 310 to 245 nm were run at appropriate time intervals after adding 2-deoxyglucose. The concentrations of intermediates calculated from this series of scans are shown in Fig. 1B. There is little or no difference between the rate of IMP appearance and adenine nucleotide disappearance. A very small but significant amount of adenylosuccinate is formed and dissipated while the system is running towards IMP. Under these conditions the NTP regenerating system is exhausted, and GDP accumulates. As has already been mentioned, adenylosuccinate synthetase is strongly inhibited by GDP. Hence the activity of adenylosuccinate synthetase becomes rapidly inhibited by the accumulation of the reaction product, GDP.

Ammonia Production and Purine Nucleotide Cycle—The experiments described above show that if an excess of nucleoside...
Fig. 1. A, conversion of IMP to SAMP and AMP. The complete reaction mixture contained 0.52 mM IMP, 0.3 mM GTP, 4 mM aspartate, 27 mM imidazole hydrochloride buffer (pH 6.7), 8.3 mM MgCl₂, 1.67 mM creatine phosphate, 1.2 units per ml of yeast hexokinase (specific activity 70 units per mg), and rat muscle extract equivalent to 1.0 mg of protein per ml. The protein extract contributed 2.5 mM orthophosphate, 47 mM KCl, 0.83 mM EDTA, and 17 μM dithiothreitol to the reaction mixture. The reactions were started by adding the protein extract and were run at 31°C. The progress of the reactions was followed by repeated spectral scans between 310 and 250 nm on a Cary model 15 recording spectrophotometer. The reference cuvette contained the same reaction mixture except that it lacked aspartate. The light path was 1 mm. The spectral scans were started at the times indicated by the experimental points. The first scan, which was started 1 min after adding the protein extract, served as the baseline for Part A; the last scan, which was started at 178 min, served as the baseline for Part B. B, conversion of AMP back to IMP. When the formation of adenine nucleotides had ceased after 127 min, 8.3 μl of 0.2 M 2-deoxyglucose per ml of reaction mixture was added to give a final concentration of 1.67 mM. Spectral scans were then continued as described above.

TABLE II
Amounts of creatine phosphate and 2-deoxyglucose added during cycling experiments

<table>
<thead>
<tr>
<th>Experiment starting with</th>
<th>1st Cycle</th>
<th>2nd Cycle</th>
<th>3rd Cycle</th>
<th>4th Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMP</td>
<td>(1.7)</td>
<td>1.7</td>
<td>2.2</td>
<td>2.7</td>
</tr>
<tr>
<td>ATP</td>
<td>1.5</td>
<td>2.1</td>
<td>2.0</td>
<td>2.7</td>
</tr>
<tr>
<td>SAMP</td>
<td>(0.7)</td>
<td>1.2</td>
<td>1.5</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Species of parentheses are the initial levels of creatine phosphate present in the experiments starting with IMP or adenylosuccinate.

triphosphate-regenerating substrate is employed, the adenine nucleotides become "trapped" in the form of ATP; this state of affairs mimics resting muscle. If, on the other hand, a limited amount of a nucleoside triphosphate-regenerating substrate is employed in conjunction with a strong ATP drain, then, when the energy-regenerating substrate becomes exhausted, the adenine nucleotides are rapidly converted to IMP. This situation mimics muscle in maximum tetani. In the experiments with muscle extract described above all of the adenine nucleotides are converted to IMP; this differs from the normal, physiological tetani in which only small amounts of IMP are formed. The experiments reported here were designed to demonstrate speci-
Dilution of the reaction mixtures caused by the additions to IMP. The values given in the table are corrected for the small amounts of creatine phosphate and 2-deoxyglucose in the course of the experiments.

Ammonia at the end of each half-cycle, which was observed in the complete reaction mixture. Figs. 2 to 4 show that ammonia was released only during the conversion of AMP (adenine nucleotide) to IMP. The values given in the table are corrected for the small dilution of the reaction mixtures caused by the additions of creatine phosphate and 2-deoxyglucose in the course of the experiments.

The experimental conditions were as described in Figs. 2 to 4. The amounts of IMP, ATP, or SAMP present in the complete reaction mixture are shown in the first column. Details of the experiments are given in Table II. Aliquots of the complete reaction mixture were analyzed for ammonia at the end of each half-cycle, which was observed in the complete reaction mixture. Figs. 2 to 4 show that ammonia was released only during the conversion of AMP (adenine nucleotide) to IMP. The values given in the table are corrected for the small dilution of the reaction mixtures caused by the additions of creatine phosphate and 2-deoxyglucose in the course of the experiments.

<table>
<thead>
<tr>
<th>Purine nucleotide present at start</th>
<th>Conditions</th>
<th>Start of 1st cycle</th>
<th>End of 1st cycle</th>
<th>End of 2nd cycle</th>
<th>End of 3rd cycle</th>
<th>End of 4th cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment A, 0.52 mm IMP</td>
<td>Complete</td>
<td>0.04</td>
<td>0.49</td>
<td>0.96</td>
<td>1.34</td>
<td>1.88</td>
</tr>
<tr>
<td>IMP</td>
<td>IMP omitted</td>
<td>0.04</td>
<td>0.03</td>
<td>0.05</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td>Aspartate</td>
<td>Aspartate</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Experiment B, 0.48 mm ATP</td>
<td>Complete</td>
<td>0.02</td>
<td>0.57</td>
<td>1.01</td>
<td>1.56</td>
<td>1.90</td>
</tr>
<tr>
<td>ATP</td>
<td>ATP omitted</td>
<td>0.01</td>
<td>0.03</td>
<td>0.05</td>
<td>0.07</td>
<td>0.10</td>
</tr>
<tr>
<td>Aspartate</td>
<td>Aspartate</td>
<td>0.00</td>
<td>0.49</td>
<td>0.45</td>
<td>0.50</td>
<td>0.52</td>
</tr>
<tr>
<td>Experiment C, 0.99 mm SAMP</td>
<td>Complete</td>
<td>0.06</td>
<td>0.36</td>
<td>0.50</td>
<td>0.73</td>
<td>0.97</td>
</tr>
<tr>
<td>SAMP</td>
<td>SAMP omitted</td>
<td>0.02</td>
<td>0.07</td>
<td>0.05</td>
<td>0.06</td>
<td>0.10</td>
</tr>
<tr>
<td>Aspartate</td>
<td>Aspartate</td>
<td>0.02</td>
<td>0.30</td>
<td>0.25</td>
<td>0.28</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Ammonia production by the reactions of the purine nucleotide cycle. Conditions were similar to those described for Fig. 2, except that the first cycle was started with 0.48 mM ATP (instead of IMP), followed by the first portion of 2-deoxyglucose. Details are given under "Methods." A, complete reaction mixture; ○, ATP omitted from reaction mixture; Δ, aspartate omitted from reaction mixture. DG, 2-deoxyglucose; CP, creatine phosphate.

The experiment shown in Fig. 1 was continued as follows. When the spectrophotometric scans indicated that the first cycle of the sequence IMP → adenylosuccinate → AMP → IMP was completed, a second portion of creatine phosphate was added to initiate a second cycle. When the spectrophotometric scans on a fresh aliquot of the complete reaction mixture indicated that the resynthesis step of the second cycle had been completed, 2-deoxyglucose was again added to initiate the deamination portion of the second cycle. The successive additions of creatine phosphate and 2-deoxyglucose were repeated twice more; in each case the times of the additions were judged on the basis of spectrophotometric scans. In this manner the reaction sequence IMP → adenylosuccinate → AMP → IMP was cycled four times in succession. The amount of ammonia which can be expected to form during the deamination portion of the cycle (Fig. 1B).
Recycling Starting with Adenine Nucleotide (ATP)—The reaction mixtures were made up as described in “Methods.” After the first set of aliquots for ammonia analysis was taken, 2-deoxyglucose was added to initiate the deamination. Samples of the complete reaction mixture were monitored in the Cary spectrophotometer against an appropriate IMP-containing mixture. The successive additions of creatine phosphate and 2-deoxyglucose were continued as in the previous experiment through three and one-half cycles: four deamination and three reamination steps.

The ammonia analyses of the recycling experiment starting with ATP are shown in Fig. 3. In the control lacking ATP, very little ammonia was produced. At the first deamination step the control lacking aspartate released an amount of ammonia equal to the added ATP, and no further ammonia was released thereafter. The complete reaction mixture showed a stepwise ammonia release after each of the four additions of 2-deoxyglucose. Correcting for the 9% volume increment caused by the creatine phosphate and 2-deoxyglucose additions, 4.1 moles of ammonia were liberated per mole of ATP originally present (Table III, Experiment B).

The results show a small decrease in the adenine nucleotide level at the end of the first reamination step. The decrease probably occurred after the added creatine phosphate had been used up. This may account for the barely significant increase in ammonia in the complete reaction mixture during the first reamination step.

Recycling Starting with Adenylosuccinate—The reaction mixtures were made up as described under “Methods.” The first set of aliquots for ammonia analysis was taken immediately after adding the extract to start the reaction. The conversion of adenylosuccinate to adenine nucleotide in the complete reaction mixture was followed in the Cary spectrophotometer by using an appropriate IMP-containing mixture in the reference cuvette. After the second set of aliquots were taken for ammonia analysis, 2-deoxyglucose was added to initiate the first deamination step. The successive additions of creatine phosphate and 2-deoxyglucose were continued as in the previous experiments through four deamination steps.

The ammonia analyses of the recycling experiment starting with adenylosuccinate are shown in Fig. 4. In the control lacking adenylosuccinate, only a little ammonia was released. The control lacking aspartate produced nearly a stoichiometric amount of ammonia at the first deamination step, and the ammonia level remained roughly constant thereafter, as in the experiment starting with ATP. The complete reaction mixture showed the expected ammonia release at all four deamination steps. Correcting for the 6% increase in volume caused by the additions of creatine phosphate and 2-deoxyglucose, 3.1 moles of ammonia were produced per mole of adenylosuccinate originally present (Table III, Experiment C).

These experiments show that the catalytic requirement for ammonia release from aspartate can be met by adenine nucleotide (ATP) or adenylosuccinate, as well as by IMP.

Initiation of Purine Nucleotide Cycle with Glucose—In the
The requirement of GTP for the operation of adenylosuccinate synthetase

Unless otherwise indicated, the reaction mixture was as described in Fig. 1, except that it contained 4.2 mM creatine phosphate, and 0.65 mg of muscle protein per ml and lacked hexokinase. The spectrophotometric assay employed is described under "Methods."

<table>
<thead>
<tr>
<th>Remarks</th>
<th>Rate of IMP disappearance</th>
<th>( \mu M ) per min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0.3 mM GTP present)</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>GTP omitted</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>GTP omitted, 0.3 mM ATP added</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>GTP omitted, 0.3 mM ITP added</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>GTP omitted, 0.6 mM ITP added</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

Table IV

**Requirement for GTP for operation of adenylosuccinate synthetase**

Until otherwise indicated, the reaction mixture was as described in Fig. 1, except that it contained 4.2 mM creatine phosphate, and 0.65 mg of muscle protein per ml and lacked hexokinase. The spectrophotometric assay employed is described under "Methods."

![Image of a table showing the requirement for GTP for the operation of adenylosuccinate synthetase.](image)

The requirements for GTP are as follows:

- **Control (0.3 mM GTP present)**: 5.0 \( \mu M \) per min
- **GTP omitted**: 0.1 \( \mu M \) per min
- **GTP omitted, 0.3 mM ATP added**: 0.1 \( \mu M \) per min
- **GTP omitted, 0.3 mM ITP added**: 0.1 \( \mu M \) per min
- **GTP omitted, 0.6 mM ITP added**: 0.1 \( \mu M \) per min

Table V

**Stoichiometry of (fumarate plus malate) and ammonia produced during three turns of cycle**

The experimental conditions were as described in Fig. 3. The first cycle was started with 0.45 mM ATP. The analyses show the differences in the amounts of ammonia, fumarate, and malate present after the first and the fourth cycle. The results have been corrected for the small dilution of the reaction mixtures caused by the addition of creatine phosphate and 2-deoxyglucose.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>( \Delta\text{Malate} )</th>
<th>( \Delta\text{Fumarate} )</th>
<th>( \Delta(\text{Fumarate} + \text{Malate}) )</th>
<th>( \Delta\text{NH}_3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>1.15</td>
<td>0.14</td>
<td>1.29</td>
<td>1.42</td>
</tr>
<tr>
<td>ATP omitted</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>Aspartate omitted</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Experiments reported so far, creatine phosphate and 2-deoxyglucose were used in an effort to dissect the reactions of the purine nucleotide cycle into phases corresponding to "energy drain" and "energy excess." If the experiment starting with adenine nucleotide (ATP) is repeated, and the deamination is initiated with glucose instead of 2-deoxyglucose, there is again a rapid conversion of adenine nucleotide to IMP. However, after an interval the IMP is converted back to adenine nucleotide (ATP). The reactions of the purine nucleotide cycle can account for the production of ammonia by skeletal muscle. This conclusion is strengthened by reports that glutamate dehydrogenase activity is very low in skeletal muscle (15, 17), whereas adenylate deaminate activity is very high in this tissue (29). Moreover, ammonia production from AMP occurs under conditions of ATP utilization, whereas resynthesis of AMP occurs under conditions of ATP regeneration. In other words, ammonia production occurs under conditions which mimic muscle doing work.

Conclusion—The evidence presented here indicates that the reactions of the purine nucleotide cycle can account for the production of ammonia by skeletal muscle. However, after an interval the IMP is converted back to adenine nucleotide (ATP). The reactions of the purine nucleotide cycle can account for the production of ammonia by skeletal muscle. This conclusion is strengthened by reports that glutamate dehydrogenase activity is very low in skeletal muscle (15, 17), whereas adenylate deaminate activity is very high in this tissue (29). Moreover, ammonia production from AMP occurs under conditions of ATP utilization, whereas resynthesis of AMP occurs under conditions of ATP regeneration. In other words, ammonia production occurs under conditions which mimic muscle doing work.

It is proposed elsewhere that the purine nucleotide cycle may be the pathway for the deamination of amino acids in most tissues (30).

References:
