Adaptive Characteristics of Urea Cycle Enzymes in the Rat

ROBERT T. SCHIMKE

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda 14, Maryland

(Received for publication, May 8, 1961)

The response of the mammal to varying dietary protein intake under conditions in which amino acid and energy requirements are adequately satisfied is a form of adaptation involving the utilization of varying proportions of carbohydrate and amino acids as sources of energy, and is therefore analogous to the adaptation seen in bacteria on change in form of energy source (1). The amino nitrogen resulting from the utilization of amino acids for energy requirements is excreted largely as urea. This paper deals with investigations into the mechanisms by means of which the rate of urea synthesis is altered in response to differences in dietary protein consumption.

Previous studies of the effects of dietary protein on rat liver arginase have demonstrated correlations between the levels of enzyme activity and dietary protein (2-4). This suggests that an increase in enzyme activity may be one factor in adaptation to varying dietary protein intake. With the recent characterization of the enzymatic steps involved in urea synthesis, largely by Ratner, Petrack, and Anslow (5-7), and by Cohen et al. (8, 9), specific assays have become available for the other enzymes involved in the synthesis of urea. Therefore, the relationships of urea cycle enzymes to dietary protein have been re-evaluated by studying all enzymes of the pathway leading from ammonia to urea, i.e. carbamyl phosphate synthetase, ornithine transcarbamylase, argininosuccinate synthetase, argininosuccinate cleavage enzyme, and arginase.

This report will present studies on (a) relationships of dietary protein to urea cycle enzymes, glutamic-aspartic transaminase, glutamic-alanine transaminase, and lactate, glutamate, malate, and glucose 6-phosphate dehydrogenases; (b) time courses of change in urea cycle enzymes after abrupt changes in levels of protein consumption; and (c) purification and characterization of arginase and ornithine transcarbamylase from animals with differing enzyme activities. The results indicate that adaptation to differing consumption of protein involves changes in activities of all urea cycle enzymes in the liver. The quantitative relationships between dietary protein and enzyme levels suggest that adaptation can be accounted for largely on the basis of differences in the levels of enzyme activity. Purified preparations of arginase and ornithine transcarbamylase from animals with differing enzyme activities have similar physiochemical and kinetic characteristics, indicating that adaptation is associated with differences in the actual content of specific enzyme protein.

EXPERIMENTAL PROCEDURE

Materials and Methods

Animals-Litter mate, male Osborne-Mendel rats were used in all experiments. They were routinely maintained on a diet of Purina Rat Chow (crude protein content 23%) ad libitum until 2 weeks before use. At this time, they were placed on the special diet that was to be the base line diet for the given experiment for another 14 days. In this way, all animals were uniformly adapted to the specific diet. This procedure was found to decrease the variation in analyses considerably. Animals were placed one or two to a cage for the duration of the experiment, where they were never without food or water. Food consumption and weight differences were determined every 2 days. Animals were selected for assay in a random manner, both as to specific animals in a given dietary group, and with regard to order of killing and assay of enzyme activities. For the collection of urine, animals were placed in individual metabolic cages, and three daily samples were collected under toluene. Urinary urea was determined on aliquots of urine by the method of Archibald (10), modified by Ratner (11).

Dietes—Vitamin-free casein (Nutritional Biochemicals Corporation) was used as the protein source in all diets. The diets were made up to contain ingredients in the amounts specified in Protein Depletion Diet, U. S. P. XV (tentative), with the substitution of the desired amount of casein for a similar amount of dextrin. Thus, the difference in diets involved essentially the substitution of protein for carbohydrate.

Enzyme Assays

General—After exsanguination of the rat by decapitation, the liver was rapidly removed and weighed. Exactly 1 g of liver was immediately homogenized in 19 ml of ice-cold distilled water in a standardized manner for 60 seconds. The homogenate was used for all enzyme assays. It was found that a 1:20 water homogenate was the most convenient preparation and that it in no case resulted in activities that were significantly lower than those obtained by extraction procedures with various buffers and detergents. In some cases, assays were not completed until 3 to 4 hours after preparation of the homogenates. Of all the enzymes studied, only argininosuccinate synthetase and arginase showed losses of activity during this time, and therefore these enzymes were assayed first. The conditions for assay of all enzymes gave satisfactorily linear relationships between enzyme activity and homogenate protein concentration over a 4 to 5-fold range in all cases. All assays were performed in duplicate.

Urea Cycle Enzymes—The assays are based on the colorimetric determination of urea and citrulline by the method of Archibald (10), as modified by Ratner (11), with 1-phenyl-1,2-propanedione-2-oxime for urea and 2,3-butanedione-2-oxime for citrulline determinations. The methods used were patterned

1 The protein depletion diet contains dextrin, 84%; corn oil, 9%; salt mixture (12), 4%; agar, 2%; cod liver oil, 1%; and vitamin fortification mixture (Nutritional Biochemical Corporation), 2%.
after those of Brown and Cohen (13) for the assay of urea cycle enzymes in tadpole liver. However, the use of homogenates instead of extracts, and change in species, have required certain alterations in specific conditions for the attainment of maximal enzyme activities. All incubations were performed at 37° in 12-ml thick walled centrifuge tubes, and the reactions were stopped by the addition of 15% perchloric acid. The protein precipitates were removed by centrifugation, and aliquots of the supernatant fluid were added to 2.5 ml of the sulfuric acid-phosphoric acid solution used for the development of color. Blanks routinely included a substrate blank, in which the assay medium minus homogenate was incubated for the appropriate time, and a tissue blank, in which the perchloric acid was added to the entire incubation mixture plus homogenate at zero time. Urea or citrulline standards were added to the incubation media and were included with each set of assays. The unit of activity of the urea cycle enzymes is expressed as micromoles of product formed per hour at 37°. Specific activity for homogenates is expressed as units per g (wet weight) of tissue.

**Carbamyl Phosphate Synthetase** Activity of this enzyme, which converts ammonia and bicarbonate to carbamyl phosphate, is assayed as the rate of citrulline formation in a coupled system containing excess ornithine and ornithine transcarbamylase. Ornithine transcarbamylase was isolated from beef liver by the method of Burnett and Cohen (14), or from rat liver by the modifications described herein. The conditions were those of Brown and Cohen (13), except for the inclusion of an ATP-generating system. The constituents of the assay medium were as follows: ammonium bicarbonate, 0.05 M, ATP, 0.005 M, adjusted to pH 7.0 with KOH before addition; N-acetyl-L-glutamate, 0.005 M, adjusted to pH 7.0 with KOH before addition; magnesium sulfate, 0.005 M, L-ornithine, 0.010 M, and phosphoenolpyruvate, 0.010 M, adjusted to pH 7.0 with KOH before addition. To each milliliter of the medium were added 200 units of ornithine transcarbamylase and 5 μg of pyruvic kinase (Boehringer and Soehne, Mannheim, Germany). Immediately before use, the solution was saturated with carbon dioxide to a pH of 7.0. Incubations were performed for 15 minutes in 250 μl of the medium, to which were added 25 μl of tissue homogenate with Lang-Levy constriction pipettes. The reaction was stopped by the addition of 1.0 ml of 15% perchloric acid. A 1.0-ml aliquot was used for color development.

**Ornithine Transcarbamylase**—The activity of this enzyme was measured directly as the rate of citrulline formation. The assay medium contained glycylglycine, 0.05 M, pH 8.0; L-ornithine, 0.015 M; and carbamyl phosphate, diithion salt (California Corporation for Biochemical Research, assayed as 76% pure), 0.020 M. The carbamyl phosphate was added just before use because of instability in solution. Incubations were undertaken for 15 minutes in 1.0 ml of the medium containing 25 μl of tissue homogenate. The reaction was stopped by the addition of 2.5 ml of 15% perchloric acid. A 100-μl aliquot was used for color development.

**Argininosuccinate Synthetase and Arginine Synthesis (Over-all Reaction)**—Argininosuccinate synthetase activity was measured as the rate of urea formation in a coupled system containing an excess of argininosuccinate cleavage enzyme and arginase. In the absence of added cleavage enzyme, the assay simply measures the over-all ability of the homogenate to convert citrulline to arginine. The conditions are modified from those of Ratner (11). In the absence of added cleavage enzyme, the rate of urea formation in the system as described below is 50 to 60% of that in its presence. Argininosuccinate cleavage enzyme was purified from rat liver by a modification of the method of Ratner (11), resulting in preparations with specific activities of 125 to 150 units per mg of protein. The arginase (Worthington Biochemical Corporation) was free of other urea cycle enzyme activities and contained an activity of 21 amoles per hour per mg of protein. The assay medium contained potassium phosphate, 0.05 M, pH 7.8; L-aspartate, 0.005 M, adjusted to pH 7.8 with KOH before addition; L-citrulline, 0.005 M; ATP, 0.002 M, adjusted to pH 7.8 with KOH before addition; phosphoenolpyruvate, 0.010 M, and magnesium sulfate, 0.003 M. To each milliliter of the medium were added 40 units of argininosuccinate cleavage enzyme, 1 mg of arginase, and 10 μg of pyruvate kinase. Incubations were performed for 30 minutes in 250 μl of medium, to which were added 50 μl of tissue homogenate. The reaction was stopped by the addition of 1.0 ml of 15% perchloric acid. A 1.0-ml aliquot was used for color development.

**Argininosuccinate Cleavage Enzyme**—Activity was measured as the rate of urea synthesis in the presence of excess arginase. The medium consisted of: sodium phosphate, 0.05 M, pH 7.4, and argininosuccinate, 0.015 M (California Corporation for Biochemical Research, assayed as 89% enzymatically active material), adjusted to pH 7.4 with NaOH before addition; 1 mg of arginase was added per ml of medium. Incubations were performed for 15 minutes in 250 μl of medium, to which were added 25 μl of tissue homogenate. The reaction was stopped by the addition of 1.0 ml of 15% perchloric acid. A 1.0-ml aliquot was used for color development.

**Arginase**—The conditions of assay were modified from those described by Greenberg (15). Activity was increased 110% by the preinculcation of homogenate or purified preparations at a final concentration of 0.05 M MnSO₄ for 5 minutes at 55°. The medium contained l-arginine, 0.250 M, pH 9.7, and manganese sulfate, 0.001 M. Incubations were undertaken for 10 minutes in 1.0 ml, containing 20 μl of tissue homogenate. The reaction was stopped by the addition of 2.5 ml of 15% perchloric acid. A 100-μl aliquot was used for color development.

**Transaminases**—The conditions used for the assay of glutamic-aspartic and glutamic-alanine transaminases were those of Zuchlewski and Gaebler (16). α-Ketoglutarate and pyruvate were measured as their phenylhydrazones by differential spectrophotometry (17). Oxalacetate was measured as pyruvate after decarboxylation with anilino citrate (18). Pyridoxal phosphate was not found to increase transaminase activity under these conditions.

**Dehydrogenases**—Malic dehydrogenase (DPN-dependent) was determined by the method of Ochoa (19) as modified by Fitch and Chaikoff (20). Lactic dehydrogenase was assayed by the method of Kubowitz and Ott (21). Glucose 6-phosphate dehydrogenase was measured by the method of Fitch, Hill, and Chaikoff (22). Glutamic dehydrogenase was assayed by the method of Lowry, Roberts, and Lewis (23), with the concentration of α-ketoglutarate increased 3-fold.

**Protein**—Protein was determined by the method of Lowry et al. (24).

The conditions for assay of the above enzymes have been found to yield maximal values for enzyme activities in the author's hands. Furthermore, during purifications of arginase and ornithine transcarbamylase (see below) and argininosuccinate cleavage enzyme by a modification of the method of Ratner (11),
Total yields of enzyme activity at no point increased above that present in the homogenate as measured under the conditions described. Such findings suggest that no enzyme activity is present in the liver which is not available for assay in the homogenate state in the case of these three enzymes. Nevertheless, some caution may well be necessary in regard to the absolute values for enzyme activities as measured in crude homogenates and with coupled enzyme systems for assay. However, concern in this study has not been primarily with absolute values, but rather with the relative changes produced by specific dietary alterations.

RESULTS

Relationships of Urea Cycle Enzymes to Dietary Protein—Between dietary protein concentrations of 15 and 60%, rats grow at essentially similar rates. Above or below this protein concentration, the rate of growth is markedly decreased. Therefore, it can be inferred that the utilization of amino acids for protein synthesis is not altered by changes in dietary protein within this range. On a 15% protein diet the energy requirements of the animal are satisfied largely by carbohydrates, whereas energy requirements are increasingly met by the utilization of amino acids as the protein content of the diet is raised, so that on a diet containing 60% protein, approximately 50% of energy requirements are met by amino acids. The utilization of dietary protein for energy requirements involves transamination of certain amino acids to keto acid intermediates and the excretion of the resulting amino nitrogen in the form of urea. Thus, the extent of use of metabolic pathways involved in the synthesis of urea and transamination can be roughly estimated on the basis of dietary protein intake under the specific conditions in which the excess dietary protein is largely used for satisfying energy needs of the animal.

The experimental design used to evaluate the effects of varying the protein content of the diet on hepatic levels of the enzymes under consideration consisted of the following procedure: two groups of 18 animals, weighing 50 to 60 g and 140 to 150 g each, were placed in three subgroups in such a manner that six animals of each weight were maintained on diets containing 15, 30, and 60% protein. Food consumption, weight gain, and urea excretion were determined as outlined under “Materials and Methods.” At the end of 14 days, the animals were killed in three groups of 12 animals each within a 48-hour period, and livers were assayed for enzymes as described under “Enzyme Assays.” A separate group of 18 rats weighing 140 to 150 g each initially was used for the assays of argininosuccinate synthetase activity.

Table I shows the effects of varying protein intake on total body and liver weights, food consumption, and urea excretion of rats in the two age groups. The animals were healthy regardless of the diet, and the only difference noted on casual observation was the marked increase in water consumption of the animals on the 60% protein diet. An analysis of variance was performed for each enzyme, body and liver weight, and liver protein concentration for effects of diet and age. Significance of difference between individual groups was determined by a standard t test, and was accepted only at the p = 0.01 level. Differences in dietary protein content were associated with significant differences in the size and protein content of the liver; animals on the high protein diet had larger livers that contained more protein. Although the protein intake of the animals varied from 1.8 to 8.5 g daily, the caloric intakes were not significantly different. Urea excretion varied from 0.78 g on the 15% protein diet to 3.60 g per day on the 60% protein diet. However, the ratio of urea excretion to protein intake was not significantly altered within the dietary protein range of 15 to 60%.

Table II shows the effects of varying proportions of protein on the levels of urea cycle enzymes. The concentration of urea cycle enzymes (activity in units per g of liver) is roughly proportional to the percentage of dietary protein. The relationship of each of the urea cycle enzymes to dietary protein intake is depicted in Fig. 1. It is evident that there exists a remarkably close correlation between the total content of each of the urea cycle enzymes per liver and the amount of protein consumed daily or, as demonstrated by the constant ratio of urea excretion to protein intake, the daily excretion of urea. This relationship is seen to hold for each of the enzymes involved in urea synthesis and for animals of differing ages.

Table III shows the results for the hepatic levels of dehydrogenases and transaminases. The levels of glutamic-alanine and glutamic-aspartic transaminases showed variations in levels comparable to those found for the urea cycle enzymes. How-

TABLE I

Effects of dietary protein on body weight, liver weight, liver protein content, and urea excretion of rats

<table>
<thead>
<tr>
<th></th>
<th>15%</th>
<th>30%</th>
<th>60%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>81 ± 2</td>
<td>185 ± 4</td>
<td>89 ± 3</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>2.91 ± .42</td>
<td>8.30 ± .65</td>
<td>4.25 ± .43</td>
</tr>
<tr>
<td>Liver protein (mg per g)</td>
<td>204 ± 5</td>
<td>211 ± 3</td>
<td>209 ± 4</td>
</tr>
<tr>
<td>Diet consumption (g per day)</td>
<td>14.2 ± 1.1</td>
<td>13.3 ± 1.1</td>
<td>8.5 ± 0.8</td>
</tr>
<tr>
<td>Protein intake (g per day)</td>
<td>1.90</td>
<td>1.80</td>
<td>2.5</td>
</tr>
<tr>
<td>Urea excretion (g per day)</td>
<td>0.78 ± .12</td>
<td>1.62 ± .21</td>
<td>0.34 ± .15</td>
</tr>
<tr>
<td>Urea excretion (g per day)</td>
<td>0.43 ± .10</td>
<td>0.34 ± .15</td>
<td>0.45 ± .18</td>
</tr>
</tbody>
</table>

* Per cent of dietary protein.
TABLE II
Effects of dietary protein on levels of urea cycle enzymes in rat liver

Groups of rats initially weighing 50 to 60 g each (Group A) or 140 to 150 g each (Group B) were grown on diets containing 15, 30, and 60% casein for 14 days as described in the text. Each value represents the mean of 6 animals ± standard error. Enzyme activities are expressed as micromoles of product formed per g of wet weight liver at 37°. Values for activities of argininosuccinate synthetase are based on a separate series of rats weighing 140 to 150 g at the start of the 14-day experimental period.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>15%</th>
<th>30%</th>
<th>60%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A (µmoles)</td>
<td>B (µmoles)</td>
<td>A (µmoles)</td>
</tr>
<tr>
<td>Carbamyl phosphate synthetase</td>
<td>828 ± 18</td>
<td>520 ± 27</td>
<td>849 ± 20</td>
</tr>
<tr>
<td>Ornithine transcarbamylase</td>
<td>18,200 ± 240</td>
<td>11,680 ± 200</td>
<td>18,500 ± 260</td>
</tr>
<tr>
<td>Argininosuccinate synthetase</td>
<td>121 ± 12</td>
<td>121 ± 12</td>
<td>207 ± 20</td>
</tr>
<tr>
<td>Argininosuccinate cleavage enzyme</td>
<td>426 ± 10</td>
<td>270 ± 8</td>
<td>555 ± 6</td>
</tr>
<tr>
<td>Arginase</td>
<td>38,300 ± 1,500</td>
<td>29,700 ± 1,300</td>
<td>40,400 ± 900</td>
</tr>
</tbody>
</table>

*Per cent of dietary protein.

Fig. 1. Relationships of total liver content of urea cycle enzymes to daily protein consumption. Protein intake in grams per day is plotted against enzyme content in units per liver. Brackets indicate ± 4 standard errors. The circles represent animals initially weighing 50 to 60 g each, and the squares indicate animals weighing 140 to 150 g each at the start of the experiment. Triangles indicate a separate group of rats weighing 140 to 150 g each, which were initially assayed only for argininosuccinate synthetase.

However, the dehydrogenases showed a variety of patterns. Glutamic and malic dehydrogenases underwent no significant changes with varying dietary protein intake. Lactic dehydrogenase was found to have an inverse relationship to dietary protein, or a direct relationship to dietary carbohydrate. Most striking was the 5- to 6-fold increase in glucose 6-phosphate dehydrogenase in animals on the 60% protein diet, when there was no change in the animals on the 30% protein diet.

The enzyme activities of urea cycle enzymes from animals on 15 and 60% protein diets were additive when incubated together, indicating that no freely soluble activator or inhibitor accounted for the differences in activities observed. Fractionation of liver homogenates into subcellular compartments by the method of Schneider and Hogeboom (25) revealed that there was no effect of dietary protein on the distribution of carbamyl phosphate synthetase and ornithine transcarbamylase, both of which are present only in mitochondria. Study of the distribution of arginase as outlined by Rosenthal et al. (26) showed that activity was present only in the supernatant fraction regardless of the dietary protein intake.

Time Course of Adaptation of Urea Cycle Enzymes—A total of 62 rats weighing 50 to 60 g each was placed on a 30% protein diet for 7 days. At the end of this time, 28 and 16 animals were placed on diets containing 60 and 15% casein, respectively, and
TABLE III

Effects of dietary protein on levels of hepatic dehydrogenases and transaminases

A separate group of 36 animals was used in this experiment. Groups of rats initially weighing 50 to 60 g each (Group A) or 140 to 150 g each (Group B) were grown on diets containing 15, 30, and 60% casein for 14 days. Weight gains, liver weight, and hepatic protein contents were not statistically different from those shown in Table I. Enzyme activities are expressed as micromoles of product formed per minute per g of wet weight tissue. Each value represents the mean of 6 animals ± standard error.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>15% *</th>
<th>30%</th>
<th>60%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A µmoles</td>
<td>B µmoles</td>
<td>A µmoles</td>
</tr>
<tr>
<td>Lactic dehydrogenase</td>
<td>152 ± 8</td>
<td>117 ± 6</td>
<td>92 ± 6</td>
</tr>
<tr>
<td>Malic dehydrogenase</td>
<td>230 ± 12</td>
<td>136 ± 10</td>
<td>246 ± 11</td>
</tr>
<tr>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>1.12 ± .07</td>
<td>1.24 ± .09</td>
<td>1.40 ± .08</td>
</tr>
<tr>
<td>Glutamic dehydrogenase</td>
<td>20.8 ± 3.1</td>
<td>17.6 ± 3.1</td>
<td>22.2 ± 2.6</td>
</tr>
<tr>
<td>Glutamic-aspartic transaminase</td>
<td>102 ± 4</td>
<td>110 ± 4</td>
<td>138 ± 6</td>
</tr>
<tr>
<td>Glutamic-alanine transaminase</td>
<td>39.6 ± 2.3</td>
<td>34.0 ± 1.9</td>
<td>63.4 ± 8.2</td>
</tr>
</tbody>
</table>

* Per cent of dietary protein.

18 were maintained on the 30% casein diet. At specified time intervals thereafter, livers were removed and assayed for the hepatic content of urea cycle enzymes, and lactic, malic, glutamic, and glucose 6-phosphate dehydrogenases.

Fig. 2 shows the rate of increase of total body weight and liver weight after change from a 30% casein diet to one containing either 15 or 60% casein. On change of diet to either a higher or a lower protein content, the animals initially decreased their rate of growth. After 3 to 4 days, the rate of growth increased to that found in the animals maintained on the 30% protein diet. Similarly, the size of the liver remained essentially constant for 4 days, and thereafter increased most rapidly in the animals on the 60% protein diet. On the other hand, the concentration of liver protein attained maximal levels within 4 days of change of diet.

The events involved in the change from 30 to 15% dietary protein suggested that no net degradation of enzyme had occurred. Because of the relatively small change in protein consumption resulting from this dietary change, a further study was undertaken on the course of change in arginase and ornithine transcarbamylase resulting from a change from 60 to 15% dietary protein. Table IV shows the results of this experiment. Indeed, it is demonstrated that there is a net loss of total enzyme activity when rats were abruptly changed from a high to a low protein diet, although the growth of the rats was not altered. The rates of increase in activity of arginase on change from low to high protein diet were similar to the rates of decrease in activity on change from high to low protein diet. However, the increase in activity was twice as rapid as the decrease in the case of ornithine transcarbamylase.

That the changes in enzyme activities were an effect of adrenocortical stimulation in the form of nonspecific stress has been excluded by the demonstration that the daily intraperitoneal
substituted cellulose column chromatography and were highly reproducible. Purified preparations were obtained with a minimum of steps with relatively good yields.

Purification Procedure—Both enzymes were purified from a liver acetone powder. Step 1: 1 g of liver acetone powder was extracted with three 15-ml portions of 0.005 M Tris-Cl, pH 7.2, containing 0.01 M MnSO$_4$. The sediment was removed by centrifugation. Step 2: the extract was passed through a DEAE-cellulose column (27) (Eastman Organic Chemicals) previously equilibrated with the extraction solution. Both enzymes passed through the column at this pH value with a 2- to 3-fold purification. Step 3: the DEAE-cellulose eluate was immediately placed on a carboxymethyl cellulose column (California Corporation for Biochemical Research). The cellulose (80 to 200 mesh) was prepared by washing with 1.0 N NaOH, and thereafter with water until neutral. After equilibration with 0.005 M Tris-acetate, pH 6.2, a column (15 x 1 cm) was packed under 2 pounds of air pressure and was adequate for 150 ml of enzyme solution received from the DEAE-cellulose column. A linear NaCl gradient (200 ml) between 0 and 0.3 M in 0.005 M Tris-acetate, pH 6.2, was used to elute the adsorbed protein. Hemoglobin was eluted before arginase. After the hemoglobin emerged, 0.1 M MnSO$_4$, adjusted to pH 7.0, was added to each fraction to a final concentration of 0.01 M MnSO$_4$. The pH of the column has been found to be critical, since at higher pH values, ornithine transcarbamylase was not adsorbed on the column, whereas at lower pH values, arginase rapidly lost activity. Ornithine transcarbamylase was eluted first, and was completely separated from arginase by this procedure. Steps 4 and 5 (ornithine transcarbamylase): fractions from the CM-cellulose column containing ornithine transcarbamylase activity were subjected to fractionation with solid ammonium sulfate. The precipitate obtained between 2.2 M and 3.0 M ammonium sulfate was taken up in 10% solution was again fractionated with ammonium sulfate, and the precipitate brought down between 2.0 M and 2.5 M was dissolved in 5% of the original extract volume in 0.005 M Tris, pH 7.4. The solution was again fractionated with ammonium sulfate, and the precipitate brought down between 2.0 M and 2.5 M was dissolved in 0.005 M Tris, pH 7.4. Steps 6 and 7 (arginase): arginase was precipitated from the injection of 3 mg per 100 g of body weight of cortisone acetate or hydrocortisone sodium succinate (Solu-Cortef, Upjohn) was without effect on the levels of urea cycle enzymes over a 6-day period when the intake of dietary protein was controlled by pair feeding.

Purification and Characterization of Hepatic Ornithine Transcarbamylase and Arginase from Rats on 15 and 60% Protein Diets—The increase in enzyme activities found on varying protein intakes could be associated with alterations in the kinetic properties of the enzymes, the presence of activators or inhibitors, or an increase in the actual content of specific enzyme protein. In order to determine the basis for the observed increases in enzyme activities, purifications of two of the enzymes of the urea cycle were undertaken from animals exhibiting wide differences in enzyme activity. The methods of purification made use of injection of 3 mg per 100 g of body weight of cortisone acetate or hydrocortisone sodium succinate (Solu-Cortef, Upjohn) was without effect on the levels of urea cycle enzymes over a 6-day period when the intake of dietary protein was controlled by pair feeding.

Purification and Characterization of Hepatic Ornithine Transcarbamylase and Arginase from Rats on 15 and 60% Protein Diets—The increase in enzyme activities found on varying protein intakes could be associated with alterations in the kinetic properties of the enzymes, the presence of activators or inhibitors, or an increase in the actual content of specific enzyme protein. In order to determine the basis for the observed increases in enzyme activities, purifications of two of the enzymes of the urea cycle were undertaken from animals exhibiting wide differences in enzyme activity. The methods of purification made use of
active fractions with ammonium sulfate between 2.0 and 2.5 M. The precipitate was taken up in 5% of the original extract volume in 0.005 M Tris, pH 7.0, containing 0.01 M MnSO₄. Arginase was precipitated with an equal volume of cold acetone. The precipitate was dissolved in 2.5% of the original extract volume in 0.005 M Tris, pH 7.0, containing 0.01 M MnSO₄. Any sediment present was removed by centrifugation.

Table V shows the simultaneous purification of arginase and ornithine transcarbamylase from rats grown 8 days on either a 15 or 60% protein diet. It is seen that the yields are similar at each step for both enzymes from animals on the two levels of dietary protein, although the total amounts of enzyme differ.

The purified enzyme preparations were analyzed by starch gel electrophoresis (28). Arginase from both preparations migrated as single bands without other bands being present. Both preparations of ornithine transcarbamylase contained five distinct similar protein bands. Only one of these bands showed differences in the intensity of staining, suggesting differing amounts of protein. Analysis for enzyme activity in the various protein bands revealed that only the band showing differences in staining intensity contained ornithine transcarbamylase. The possibility that one of the other bands contained a protein species that activated or inhibited ornithine transcarbamylase was excluded by the further demonstration that eluates from the other bands revealed that only the band showing differences in staining, suggesting differing amounts of enzyme.

Further characterization of the enzymes was made with a density gradient centrifugation between 5 and 20% sucrose. The gradients were made, and fractions collected, as described in the text. Arginase and ornithine transcarbamylase from rats grown 8 days on either a 15 or 60% protein diet. It is seen that the yields are similar at each step for both enzymes from animals on the two levels of dietary protein, although the total amounts of enzyme differ.

Furthermore, the use of this technique for the estimation of molecular weights of proteins in complex mixtures. Thus, by comparing distance traveled in the gradient of a protein of known molecular weight with that of the unknown, an estimate of molecular weight can be obtained. Crystalline yeast alcohol dehydrogenase (Worthington Biochemical Corporation), with a molecular weight of 153,000 (30), was used as the reference protein.

Fig. 5, A and B, shows the results of the sucrose gradient studies. The purity of the arginase preparations is further confirmed by the discrete peak of protein associated with enzyme activity. The ornithine transcarbamylase gradients likewise had a discrete peak of protein associated with enzyme activity. It is noted that other discrete protein peaks were present, but in

<table>
<thead>
<tr>
<th>Day after change in diet</th>
<th>Body weight</th>
<th>Liver weight</th>
<th>Liver protein</th>
<th>Arginase</th>
<th>Ornithine transcarbamylase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>g</td>
<td>mg/g liver</td>
<td>units/g</td>
<td>Total units/liver</td>
</tr>
<tr>
<td>0</td>
<td>154 ± 4</td>
<td>8.75 ± 0.7</td>
<td>225 ± 6</td>
<td>50,000 ± 2000</td>
<td>496,000 ± 9000</td>
</tr>
<tr>
<td>4</td>
<td>176 ± 5</td>
<td>8.60 ± 0.5</td>
<td>221 ± 7</td>
<td>44,500 ± 5900</td>
<td>384,000 ± 6900</td>
</tr>
<tr>
<td>8</td>
<td>194 ± 6</td>
<td>8.25 ± 0.5</td>
<td>220 ± 6</td>
<td>38,700 ± 5400</td>
<td>320,000 ± 9400</td>
</tr>
<tr>
<td>18</td>
<td>265 ± 7</td>
<td>10.4 ± 0.6</td>
<td>217 ± 4</td>
<td>29,000 ± 1200</td>
<td>315,000 ± 8300</td>
</tr>
<tr>
<td>18 (60% casein)</td>
<td>240 ± 7</td>
<td>12.7 ± 0.7</td>
<td>230 ± 6</td>
<td>47,500 ± 1300</td>
<td>500,000 ± 9800</td>
</tr>
</tbody>
</table>

TABLE V

Purification of arginase and ornithine transcarbamylase from rats on 15 and 60% dietary protein

A total of eight rats weighing 70 to 80 g were placed on a 15% casein diet for 2 weeks. Four animals were thereafter placed on a 60% casein diet, and after 8 days the livers were subjected to purification of the two enzymes as described in the text. Equal samples of liver totaling 1 g were pooled from each of the four animals in each diet group, and were made into 1:20 water homogenates. The remaining liver was made into acetone powder by homogenizing with 10 volumes of cold acetone. Total liver weight of the four pooled livers was 16.3 and 24.9 g in the groups on 15 and 60% casein, respectively. The percentages of acetone powder per initial liver weight were similar in the two groups (21.8 and 22.1%, respectively). Equal amounts of acetone powder (2.4 g) were used for isolation in both cases, giving initial extract volumes of 110 cc each. Parallel purification procedures were performed for the two preparations. Specific activity is expressed as units per mg protein. The sediment obtained after the third extraction of the acetone powders was resuspended. Both extracts were then found to contain 5 to 7% of the total arginase activity, and no ornithine transcarbamylase activity.

Preliminary experiments on the effects of arginine and ornithine on extraction of arginase and ornithine transcarbamylase from acetone powder or on activity in homogenates revealed that no stimulation or inhibition was found when arginine or ornithine was injected into the animals 4 hours before death (50 mg per 100 g of body weight) or added to homogenates or during extraction of liver acetone powder.

<table>
<thead>
<tr>
<th>Step</th>
<th>Arginase</th>
<th>Ornithine transcarbamylase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>Yield</td>
</tr>
<tr>
<td>15%*</td>
<td>60%*</td>
<td>15%</td>
</tr>
<tr>
<td>Homogenate</td>
<td>190</td>
<td>291</td>
</tr>
<tr>
<td>Extract</td>
<td>398</td>
<td>600</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>1,070</td>
<td>1,580</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>12,200</td>
<td>18,300</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (Step 4)</td>
<td>53,200</td>
<td>61,100</td>
</tr>
<tr>
<td>Acetone</td>
<td>88,900</td>
<td>87,700</td>
</tr>
</tbody>
</table>

* Preparations from animals on 15 and 60% dietary protein.
SUCROSE GRADIENT ANALYSIS

FIG. 5. Density gradient centrifugation studies of preparations of arginase and ornithine transcarbamylase from livers of rats on 15 and 60% dietary protein. Enzyme preparations used for sucrose gradients were dialyzed overnight against the buffers used in the gradients to remove Tris, which would interfere with protein determinations. All gradients were between 5 and 20% sucrose. The arginase preparations (75 and 125 µg of protein, 100 µl) were placed in glycylglycine gradients, 0.02 M, pH 7.4, containing 0.001 M MnSO<sub>4</sub>. The ornithine transcarbamylase preparations (410 and 500 µg protein, 100 µl), were placed in glycylglycine gradients, 0.02 M, pH 7.6. An alcohol dehydrogenase standard was included with each run. The gradient tubes were centrifuged at 39,000 r.p.m. for 12 hours in an SW-39 head in a Spinco model L ultracentrifuge. The fractions were collected in 44 tubes. Aliquots of 10 µl were used for enzyme assays. Protein was determined on 60.0 µl aliquots in a final volume of 1.0 ml. Base line arrows indicate the peaks of alcohol dehydrogenase activity. Solid lines represent enzyme activity, and dotted lines indicate protein. A, Arginase; B, Ornithine transcarbamylase.

TABLE VI
Summary of characteristics of arginase and ornithine transcarbamylase from rats on 15 and 60% protein diets

The values below are all based on experiments with the purified enzyme preparations of highest specific activity as indicated in Table V. Turnover numbers are expressed as moles of product formed per mole of enzyme at 37° per minute.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pH optima</th>
<th>K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>Q&lt;sub&gt;max&lt;/sub&gt; 25-35°</th>
<th>Molecular weight*</th>
<th>Turnover number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginase</td>
<td></td>
<td>Arginine</td>
<td>Ornithine</td>
<td>Carbamyl phosphate</td>
<td></td>
</tr>
<tr>
<td>15%</td>
<td>10.0</td>
<td>2.4 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>2.5 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>1.8</td>
<td>138,000</td>
</tr>
<tr>
<td>60%</td>
<td>10.0</td>
<td>2.3 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>2.5 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>1.8</td>
<td>142,000</td>
</tr>
<tr>
<td>Ornithine transcarbamylase</td>
<td></td>
<td>Arginine</td>
<td>Ornithine</td>
<td>Carbamyl phosphate</td>
<td></td>
</tr>
<tr>
<td>15%</td>
<td>8.0-8.2</td>
<td>1.0 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>3.0 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>2.1</td>
<td>156,000</td>
</tr>
<tr>
<td>60%</td>
<td>8.0-8.2</td>
<td>1.0 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>2.9 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>2.2</td>
<td>168,000</td>
</tr>
</tbody>
</table>

* Estimated by the sucrose density gradient method as described in the text. In the case of ornithine transcarbamylase, this method achieved further fractionation (see Fig. 5B).
† Preparations from animals on 15 and 60% dietary protein.

similar proportions in the two preparations. Thus, the gradient effected further purification of ornithine transcarbamylase to the point where a discrete protein peak was found proportional to enzyme activity. The homogeneity of the protein peaks associated with ornithine transcarbamylase activity was indicated by the finding of single protein bands present in starch gel electrophoresis of fractions taken at the peak of enzyme activity.

Table VI shows a summary of physical and kinetic data on the two enzymes isolated from animals on 15 and 60% dietary protein. K<sub>m</sub> values were plotted by the method of Lineweaver and Burk (31) and were determined at optimal pH values. The turnover numbers were calculated on the assumption that the discrete protein peaks associated with activity in the sucrose gradients contained only specific enzyme protein, and therefore are minimal values. The values for molecular weight and turnover numbers of arginase are in essential agreement with the values reported by Roholt and Greenberg (32) for horse liver arginase. The similarity of turnover numbers for ornithine transcarbamylase isolated from the two groups of rats has not been conclusively established, however, because of the possi-
bility that the large protein peak associated with enzyme activity in the 60% dietary protein sample (Fig. 5B) may mask the presence of a nonactive protein species. However, the finding in the starch gel electrophoresis that the protein peaks associated with enzyme activity in the sucrose density centrifugation are essentially homogeneous would suggest that a single species of protein is indeed present.

It is demonstrated that there are no differences in the enzyme proteins from the two groups of rats on the basis of all parameters determined, including behavior on column chromatography, during purification, and on starch gel electrophoresis. It is concluded, therefore, that the changes in enzyme activities of these two enzymes of the urea cycle, i.e. arginase and ornithine transcarbamylase, are in fact due to differences in the actual content of enzyme protein, rather than to changes in kinetic properties of protein already present.

**DISCUSSION**

It has been demonstrated that adaptation to varying levels of dietary protein is associated with changes in the levels of enzyme activities, as well as differences in the size and protein content of the liver. Two enzymes directly associated with the conversion of amino acids to keto acids, i.e. glutamic-aspartic and glutamic-alanine transaminases, as well as those enzymes associated with the conversion of ammonia to urea, i.e. urea cycle enzymes, show differences in activities that can be related to the rate of amino acid metabolism as measured by dietary protein intake or urea excretion. The quantitative relationships between the hepatic contents of each of the urea cycle enzymes and the daily intake of protein as demonstrated in Fig. 1 suggest that the rate of urea synthesis is increased or decreased by alterations in the content of enzyme activity, rather than by changes in substrate concentrations. Ashida and Harper (33) have also recently demonstrated a relationship between the hepatic content of arginase and urea excretion in the rat.

The levels of dehydrogenases studied showed patterns that differed from those of the transaminases and urea cycle enzymes. Thus, lactate dehydrogenase showed changes opposite to those of the urea cycle enzymes, suggesting that the level of this enzyme may be affected by the extent to which carbohydrate is utilized by the glycolytic pathway. The striking increase in glucose 6-phosphate dehydrogenase in animals on a high protein diet suggests that there is an increase in oxidative utilization of glucose, perhaps in response to an unknown requirement for TPNH. Such variation in patterns of enzymes as produced by differences in dietary protein intake indicate that there is a certain specificity to such patterns, and that they are not simply a reflection of increased availability of amino acids for protein synthesis, or of the storage of protein in the form of specific enzyme protein.

An increased requirement for urea synthesis presumably could be met most economically to the organism by increasing the activity of only that enzyme present in least abundance, i.e. argininosuccinate synthetase. Nevertheless, it is evident that there are comparable increases in all urea cycle enzymes, suggesting that the mechanism controlling the levels of all these enzymes is similar. This finding resembles that of Brown, Brown, and Cohen (34), in which the onset of urea excretion in the tadpole undergoing metamorphosis is associated with increases of all urea cycle enzymes studied.

The changes in levels of urea cycle enzymes observed on change of diet have been interpreted as being related to varying needs for synthesis of urea. Lightbody and Kleinman (2), Mandelstam and Yudkin (4), and Ashida and Harper (33) have given similar interpretations to the effects of varying dietary protein on the levels of rat liver arginase. Such an interpretation implies that the significant dietary change is the increase in the protein content. However, in the studies of these workers, as well as those described herein, the increase in dietary protein has been accomplished at the expense of dietary carbohydrate. Therefore, an alternative interpretation of the changes in enzyme levels could be that high levels of dietary carbohydrate result in some manner in the maintenance of low levels of enzymes involved in amino acid degradation, and that, as the proportion of carbohydrate in the diet is decreased, the levels of the urea cycle enzymes increase.

The effects of various dietary or pharmacological agents on activities of enzymes as measured in tissue slices, extracts, or homogenates under conditions of substrate saturation (35, 36) can result from various mechanisms, including release of activators or inhibitors and alteration in the kinetic properties of the specific enzyme protein, as well as from changes in the net content of enzyme protein. In the present study, it has been shown that kinetic properties of two of the urea cycle enzymes are similar under dietary conditions resulting in differing enzyme activities. The presence of activators or inhibitors has been excluded by various mixing experiments and by the demonstration that differences in activities of arginase and ornithine transcarbamylase from animals on high and low protein diets are maintained throughout a purification procedure, when an activator or inhibitor would probably be removed. Therefore, it is concluded that the observed differences in enzyme activities are due to differing contents of specific enzyme protein.

The assumption that observed changes in the levels of enzyme activities represent changes in the content of specific enzyme protein is not necessarily valid. The invalidity of this assumption in certain cases is indicated by the demonstration of Peigelson and Greengard (37) that increased activity of tryptophan pyrrolase activity after tryptophan injection is in part due to release of a bound enzyme activator, and by the inability of Kenney (38) to demonstrate an increase of antigenically reactive protein when rat liver glutamine-tirosine transaminase has increased 5- to 10-fold in activity after cortisone administration. Thus, the claim that changes in enzyme activity represent synthesis or degradation of enzyme molecules should be reserved for instances when differences in the content of specific protein can be demonstrated.

The studies of Freedland and Harper (39–43), Fitch, Hill, and Chaikoff (20, 22, 44) and Landau, Hastings, and Zotta (45) have demonstrated significant alterations in enzyme activities and metabolic pathways associated with carbohydrate metabolism after changes in dietary forms of carbohydrate. In general, it can be stated that the results conform with the notion that the levels of enzyme activities are related to the extent to which particular reactions or pathways are utilized, as has been shown quantitatively in the relationships of contents of urea cycle enzymes to protein intake and urea excretion.

The results of the above authors on carbohydrate metabolism, as well as those presented here, suggest that adaptation in the mammal is not limited to a few special instances, but is in fact a continuous process involving major pathways of metabolism. The extent of change in activities of the enzymes studied asso-
associated with dietary alterations of protein content was at most 4-fold. This extent of change, however, may not necessarily be limited by the inherent ability of adaptation to take place, but rather by the relatively small range of concentrations in which different energy and nitrogen sources will sustain life in mammals.

The biochemical events associated with alterations in rates of urea synthesis and the use of amino acids as sources of energy provide a useful system for the study of mammalian adaptation. Studies on factors controlling the levels of urea cycle enzymes and transaminases, and the processes involved in enzyme synthesis and degradation during adaptation, are under current investigation.

SUMMARY

1. The effects of wide variation of dietary protein consumption on enzyme activities of pathways directly associated with amino acid metabolism were studied in rats growing at similar rates. The total liver content of all urea cycle enzymes, i.e., carbamyl phosphate synthetase, ornithine transcarbamylase, argininosuccinate synthetase, argininosuccinate cleavage enzyme, and arginase, was found to be directly proportional to the daily consumption of protein. A similar relationship was found for glutamic-alanine and glutamic-aspartic transaminases. Lactic and glucose-6-phosphate dehydrogenases demonstrated different patterns of change, whereas malic and glutamic dehydrogenases were not affected by changes in dietary protein.

2. After an acute increase in dietary protein concentration of protein, changes in the levels of urea cycle enzymes were completed in 4 to 8 days. A decrease in dietary protein resulted in a decrease of enzyme content that was not maximal until 8 days.

3. Purification and characterization of ornithine transcarbamylase from rat livers with widely differing activities revealed no differences in responses during purification, or in pH optima, Kₘ values, temperature coefficients, molecular weights, or turnover numbers. It is concluded, therefore, that the observed differences in enzyme activities are due to differences in the actual content of specific protein rather than to an effect of activators, inhibitors, or an alteration in the kinetic properties of enzyme molecules.

4. It is proposed that the phenomena observed on alterations in the protein content of the diet are analogous to changes in bacteria on alteration of the nature of the energy or nitrogen source, and that such phenomena represent an instance of adaptation in a mammalian system which involves synthesis and degradation of specific enzyme protein.

Acknowledgments—The author would like to thank Dr. H. Tabor for helpful suggestions during the undertaking of this investigation. He wishes to thank Dr. Carl Robinson of the National Institute of Arthritis and Metabolic Diseases for performing the starch gel electrophoreses, and Drs. R. G. Martin and B. N. Ames of the National Institute of Arthritis and Metabolic Diseases for assistance with the sucrose gradient analyses.

REFERENCES

Adaptive Characteristics of Urea Cycle Enzymes in the Rat
Robert T. Schimke


Access the most updated version of this article at
http://www.jbc.org/content/237/2/459.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/237/2/459.citation.full.html#ref-list-1