Metabolic Activity in Calcified Tissues: Aconitase and Isocitric Dehydrogenase Activities in Rabbit and Dog Femurs*

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(Received for publication, March 13, 1959)

Although citrate occupies a central position in the metabolic oxidation of carbohydrates, it is of considerable interest that at any one instant the largest proportion of total body citrate is found in the calcified tissues (1). Levels of citrate as high as 5.25 per cent of the dry, fat-free herring spine have been reported (2). The bone citrate is firmly bound, not being readily extracted with water even after steam treatment, and apparently withstands the ravages of time, since it has been detected in medieval and prehistoric samples (3).

Until recently it was generally accepted that citrate accumulation in calcified tissues was a result of an imbalance in the enzyme pattern found in the area of growth (4). Thus, an extremely low level of isocitric dehydrogenase activity in the head of the rabbit femur was considered as a metabolic block which, by preventing the metabolism of isocitric acid, allowed an equilibrium to be reached among isocitric acid, cis-aconitic acid, and citric acid, an equilibrium which by far favors the latter compound. In preliminary reports, however, it has been suggested that the activity of isocitric dehydrogenase in the epiphyseal area of the rabbit femur is considerably higher than previously reported (5,6). This communication expands on these suggestions, and data are presented on the activities of aconitase and isocitric dehydrogenase in various areas of the femurs from dogs and rabbits.

EXPERIMENTAL

Preparation of Tissues—Rabbits of the New Zealand White strain were taken from the stock colony for use in this experiment. The growing rabbits were 8 to 9 weeks old when killed and showed normal epiphyseal cartilage. Adult rabbits, taken from the breeding stock, were 3 to 4 years old. The dogs used in the experiment were mongrels; the growing animals were approximately 9 months old and the adults, 4 years old. The animals were exsanguinated by means of jugular drainage; the femurs were removed rapidly, partially cleaned of adhering tissue, and then frozen in solid CO₂. While frozen, the femurs were cleaned of muscle, connective tissue, and periosteum, and then were sawed into several sections. In the case of young animals, the sections consisted of epiphysis, epiphysis-metaphysis, and cortex. The marrow was removed from the shaft marrow space and assayed separately. In the case of old animals, the same relative areas were employed, although an epiphyseal plate no longer existed. The calcified sections were crushed in solid CO₂ to permit passage through an 8 mesh sieve. Extracts of the sections were made by treating 1 gm.-samples with 2.0 ml. of 0.1 M Tris buffer, pH 7.4, at 0°, and then by centrifuging them for 15 minutes at 1100 X g.

Methods of Analysis—Aconitase activity was estimated by the spectrophotometric method of Racker (7) in which 0.2 ml. of tissue extract is added to 2.8 ml. of 0.05 M phosphate buffer, pH 7.4, containing 0.03 M citric acid, and the increase in absorbancy at 240 μ is measured for several minutes. To estimate the quantity of cis-aconitic acid formed, an extinction coefficient of 3.62 cm.² per μmole was employed.

Isocitric dehydrogenase activity was determined by the spectrophotometric method of Grafflin and Ochoa (8) with the addition of KCN to prevent the reoxidation of reduced TPN, and nicotinamide to inhibit TPNase activity. The complete system consisted of 2.3 ml. 0.05 M Tris buffer, pH 7.4; 0.1 ml. of 0.018 M MnCl₂; 0.1 ml. of 0.00135 M TPN⁺; 0.1 ml. of 0.006 M DL-isocitrate; 0.1 ml. of 0.03 M KCN; 0.1 ml. of 0.66 M nicotinamide; and 0.2 ml. of tissue extract. The increase in absorbancy was observed for 1 to 2 minutes at 15-second intervals and the formation of TPNH calculated with the use of an extinction coefficient of 6.22 cm.² per μmole.

The protein content of the extracts was determined by the method of Lowry et al. (9).

RESULTS AND DISCUSSION

Preliminary Experiments—When the metabolic activity of the rabbit femurs was first investigated, it was apparent that considerable isocitric dehydrogenase activity could be demonstrated in the various sections of the bone. Since these observations were in striking contrast to the values in the literature, a more thorough study was indicated. Fig. 1 shows the rapid reduction of TPN⁺ which was obtained in the presence of epiphyseal extracts and the complete assay system. If isocitrate and TPN⁺ were omitted from the system, no change in absorbancy was observed. The addition of isocitrate did not elicit a response until TPN⁺ was also introduced. A reversal of the order of addition produced no reaction until both reactants were added. Although not shown in the figure, a lack of manganese ions resulted in reduced activity, and DPN⁺ could not substitute for TPN⁺. These findings suggest that the previous reports in the literature (4) may have been minimal activities attributable to a lack of cofactors necessary for optimal values.

* The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or as reflecting the views of the Navy Department or the United States naval service at large.

1 The abbreviation used is: Tris, tris(hydroxymethyl)amino- methane.
also suggest a more rapid conversion of cis-aconitic acid to citric acid of bakers' yeast. The experiments of Johnson (11) and Racker (7) have indicated that the rate of formation of cis-aconitic acid from citric acid is about one-seventh as rapid as its formation from isocitric acid by purified heart muscle aconitase. A similar difference in rates was observed for the aconitase of cortical bone. The pH optimum of the enzyme was determined to be 7.0 to 7.5 which corresponds very well with the pH optimum of pig heart isocitric dehydrogenase (10). Further evidence was obtained to substantiate the fact that isocitric dehydrogenase was actually being measured. Three samples of sodium isocitrate from different commercial sources displayed identical activities when substituted in the system. O-0, incomplete assay system with isocitrate and TPN+ added when indicated. O—O, incomplete assay system with isocitrate added as indicated but TPN+ withheld.

The method of extraction of isocitric dehydrogenase from the calcified sections was investigated, and it was found that treatment with Tris buffer with constant agitation with a stirring rod for 10 minutes produced maximal activity in the extracts. Extraction with Tris or phosphate buffer or sodium chloride solution for periods up to 4 hours did not produce greater activity. Furthermore, adsorption of the enzyme by the bone particles was excluded as a problem, since purified pig heart isocitric dehydrogenase could be quantitatively recovered when added to cortical bone chips. The pH optimum of the enzyme was determined to be 7.0 to 7.5 which corresponds very well with the pH optimum of pig heart isocitric dehydrogenase (10). Further evidence was obtained to substantiate the fact that isocitric dehydrogenase was actually being measured. Three samples of sodium isocitrate from different commercial sources displayed identical activities when substituted in the system. Furthermore, the product of the reaction, a-ketoglutarate, was determined as the 2,4-dinitrophenylhydrazone. Within 10 per cent this corresponded to the TPN reduced.

Similar preliminary studies were carried out on the aconitase of femurs, and it was found that extraction of the calcified tissues for 10 minutes resulted in maximal activity in the extracts. Adsorption of aconitase to the bone chips does not appear to occur, since it was possible to recover a sample of rat kidney aconitase quantitatively when it was added to a sample of cortical bone particles. Racker (7) has indicated that the rate of formation of cis-aconitic acid from citric acid is about one-seventh as rapid as its formation from isocitric acid by purified heart muscle aconitase. A similar difference in rates was observed for the aconitase of bakers' yeast. The experiments of Johnson (11) also suggest a more rapid conversion of cis-aconitic acid to citric acid than the reverse reaction under his conditions with the use of a pigeon breast muscle preparation. These differences in rates were also observed in the femur extracts. However, since we were most interested in the rate of citric acid degradation in the femur, this substrate was used in the analyses.

**Assay Results—Data concerning the activities of aconitase and isocitric dehydrogenase of various sections of the young rabbit femur are presented in Table I. Aconitase activities, reported as μmoles of cis-aconitic acid formed per hour per mg. of protein, were similar in the epiphysis, epiphysis-metaphysis, and in the bone marrow. Cortex had somewhat less activity on the basis of protein and markedly less activity per gm., wet weight, of tissue. These values for aconitase are less than those reported by Dixon and Perkins (4), who measured the appearance of citric acid from cis-aconitic acid. Values approximating those of Dixon and Perkins (4) were obtained, however, when either isocitric acid or cis-aconitic acid was utilized as the substrate. The aconitase activity in various sections of femurs from old rabbits was also determined and found to be considerably lower than in the young animal. The head, neck, cortex, and marrow had values of 1.24, 0.74, 0.07, and 4.46 pmoles per hour per gm. of tissue, respectively.

Isocitric dehydrogenase activity in the femur sections from young rabbits was many times higher than that reported by Dixon and Perkins (4). The highest activity per gm. of tissue was found in the marrow, with slightly less in the epiphysial area, and little activity in the cortical area. The values presented were slightly higher than those presented in a preliminary note (5) and are probably a reflection of the improved assay system utilized. In the first studies neither KCN nor nicotinamide was added to the assay system; therefore some reoxidation of the reduced TPN or cleavage of TPN may have occurred, resulting in lower activity values.

The low level of isocitric dehydrogenase activity in the cortical area of bone could be the result of either the presence of an inhibitor of the enzyme or low enzyme content. The latter suggestion seems most probable in view of our inability to demonstrate any indication of an inhibitor. It was found that the

![Fig. 1. Time course of isocitric dehydrogenase activity of epiphysial rabbit femur. △—△, complete system as described in text. O—O, incomplete assay system with isocitrate and TPN+ added when indicated. ●—●, incomplete assay system with isocitrate added as indicated but TPN+ withheld.](http://www.jbc.org/)

**Table I**

<table>
<thead>
<tr>
<th>Section and No. of assays</th>
<th>Aconitase activity</th>
<th>Isocitric dehydrogenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per mg. protein</td>
<td>Per mg. wet weight</td>
</tr>
<tr>
<td>Epiphysis (8)</td>
<td>0.395 ± 0.050</td>
<td>6.60 ± 0.70</td>
</tr>
<tr>
<td>Epiphysis-metaphysis (8)</td>
<td>0.391 ± 0.060</td>
<td>7.09 ± 1.02</td>
</tr>
<tr>
<td>Cortex (8)</td>
<td>0.228 ± 0.060</td>
<td>0.41 ± 0.12</td>
</tr>
<tr>
<td>Marrow (9)</td>
<td>0.387 ± 0.060</td>
<td>12.55 ± 1.50</td>
</tr>
</tbody>
</table>

* Aconitase activity expressed as μmoles of cis-aconitic acid formed per hour. Isocitric dehydrogenase activity expressed as μmoles of isocitric acid oxidized per hour. Values are followed by the standard error of the mean.
cortical extract when mixed with the epiphysial extract did not inhibit the activity of the latter. Also, a heat-denatured preparation of the cortical extract did not inhibit the epiphysial enzyme.

Assays were also performed on femurs from old rabbits and the activities for the head, neck, cortex, and marrow were 15.9, 18.5, 4.3, and 57.8 μmoles of isocitrate oxidized per hour per gm. of tissue, respectively. Thus, both aconitase and isocitric dehydrogenase decrease markedly in activity during the aging process.

The aconitase and isocitric dehydrogenase activities found in various sections of femurs from young dogs are presented in Table II. Aconitase activity, reported as μmoles of cis-aconitic acid formed per hour per gm. of wet tissue, was approximately the same in the epiphysial and epiphysial-metaphysial areas, with the lowest activity in the cortical bone. The mid-shaft marrow showed the greatest activity on this basis of reporting. The measurement of aconitase of mature dogs was attempted. However, the values obtained were low and variable, thus they are not reported.

Isocitric dehydrogenase activities, reported as μmoles of isocitric acid oxidized per hour per gm. of wet tissue, also were approximately the same in the epiphysial and epiphysial-metaphysial areas with the lowest activity in the cortical area. As in the case of aconitase, the mid-shaft marrow demonstrated the greatest isocitric dehydrogenase activity on the basis of weight of tissue. The dehydrogenase activity of the head, neck, and marrow of femurs from adult dogs was found to be 17.7, 17.4, 1.2, and 8.0 μmoles of isocitric acid oxidized per hour per gm. of tissue, respectively. The observations on the dog femur are qualitatively the same as for the rabbit, that is, the isocitric dehydrogenase activity exceeds the aconitase activity and the activities of both enzymes are considerably lower in the adult animal.

General Discussion

The source of the aconitase and isocitric dehydrogenase found in the head of the femurs from dogs and rabbits is believed to be the cellular elements which pervade the trabeculated areas. A variety of studies has been reported on the metabolic activity of bone marrow, and the findings in this area of research have been reviewed recently (12). Whereas it is to be expected that the reactions occurring in the mid-shaft marrow are qualitatively the same as the marrow elements of the trabeculated bone of the head of the femur, it does not follow that the quantitative aspects will be similar. The present work compares the rates of reaction in the various sections of femurs from young and adult animals and thus adds to our knowledge of the metabolic capacity of the femur.

The findings of considerable isocitric dehydrogenase in the head of femurs from rabbits and dogs is in disagreement with previous reports and necessitates a re-evaluation of hypotheses as to the mechanism of citrate accumulation in calcified areas. One possibility presently under consideration is that there is a regulation of the level of TPN+ in the marrow elements of the trabeculated bone and, consequently, a regulation of isocitric dehydrogenase activity.

The findings reported here might also be of importance in the interpretation of the mechanism of action of parathyroid hormone. The administration of this hormone causes a rapid increase in the level of citric acid of the blood (13). The calcified tissues have been implicated as the source of this citric acid, since the spongiosal citric acid increases more rapidly than arterial or venous citric acid. These results have been discussed in terms of the capacity of bone to synthesize citric acid but inability of it to metabolize citrate because of a lack of isocitric dehydrogenase (14, 15). In view of the present findings, the possibility of parathyroid hormone affecting the metabolism of citrate by bone tissue must be considered. In this regard, Neuman et al. (14) have reported on the ability of parathyroid hormone to destroy the chromophoric group of reduced TPN. A subsequent report (15) that this destruction is mediated by a fraction other than the calcium-mobilizing fraction of parathyroid hormone suggests a complex relationship, the elucidation of which must await further experimentation.

SUMMARY

1. The activities of aconitase and isocitric dehydrogenase in various areas of the femurs from rabbits and dogs were investigated.
2. In both animals the isocitric dehydrogenase activity exceeded the aconitase activity of the epiphysis, epiphysis-metaphysis, cortex, and mid-shaft marrow.
3. Young rabbits and young dogs demonstrated considerably greater enzyme activity in the various sections of the femurs than mature animals.
4. The results are discussed in terms of citric acid accumulation in calcified tissues and the mechanism of parathyroid hormone action.

REFERENCES

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