Aerobic Metabolism of Glucose by Bone*

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Osseous tissue is capable of producing net amounts of lactic acid. This has been demonstrated in vivo by the measurement of arteriovenous differences (2), in vitro during incubation of slices (3), and in tissue culture (4). Since parathyroid hormone is closely involved in this problem, the recent findings (2, 9) that it may alter citrate and lactate formation (5) might indicate that the pentose cycle is also operative.

As part of a larger study on osseous tissue, we have been interested in factors involved in bone demineralization. Since the Embden-Meyerhof pathway is the route of lactate formation (3), and the fact that lactate thus formed seems to arise entirely from glucose; that the pentose cycle contains many of the glycolytic enzymes (6), on the other hand, the finding of glucose 6-phosphate dehydrogenase activity in the epiphyseal plate (5) might indicate that the pentose cycle is also operative.

The demonstration of certain enzymatic activities of the citric acid cycle, including citrogenase, aconitase and isocitric dehydrogenase (7), malic dehydrogenase (5), and succinic dehydrogenase (8), suggests that the Krebs cycle is available for further metabolism.

As part of a larger study on osseous tissue, we have been interested in factors involved in bone demineralization. Since parathyroid hormone is closely involved in this problem, the recent findings (2, 9) that it may alter citrate and lactate formation, or both, by bone cells have been of interest. In order to approach the problem of how parathyroid hormone might produce such changes, it seemed essential to establish first the actual participation of known pathways in the metabolism of bone. The present study was developed to assess the relative importance of the Embden-Meyerhof, pentose, and Krebs' cycle routes to the over-all metabolism of osseous tissue.

The results described below demonstrate that in epiphyseal-metaphyseal bone slices incubated aerobically, lactic acid is the major metabolite end product of glucose metabolism; that the lactate formed seems to arise entirely from glucose; that Krebs' cycle activity is low; and that the largest part of the lactate is formed via the Embden-Meyerhof pathway with a small contribution of the pentose cycle.

**Experimental Procedure**

Metabolism was studied by incubation in vitro of bone slices of the distal end of femurs from 6-week-old rabbits. Animals were deprived of food for 18 hours and were then killed by exsanguination. All subsequent steps in the preparation of the slices, which required 2 to 3 hours, were carried out in the cold. Adherent muscle and tendon were removed from the bone, and slices 0.1 to 0.3 mm thick were cut parallel to the axis of the shaft. The outer layer of cartilage was trimmed from each slice, leaving a section containing epiphyseal and metaphyseal regions separated by the growth line.

Incubations were conducted in 250-ml Erlenmeyer flasks, each modified with a ground glass stopper, a center well to contain a 20-ml scintillation-counting vial, and a single stoppered side arm. Each flask was charged with bone slices and 2.0 ml of 0.24 m Tris buffer at pH 7.4 (gas phase, air) or 8.0 ml of Ca++-free Krebs' bicarbonate buffer at pH 7.4 (gas phase, 5% CO₂ in air). A variety of C¹^-labeled substrates was employed, as described below. The final volume was 9.0 ml. After gassing and addition of substrate, the flasks were shaken at 37°C for various periods of time. In all experiments, reactions were terminated by tipping in 0.5 ml of 60% perchloric acid. In those experiments in which Tris buffer was used, a vial containing 2 ml of Hyamine hydroxide 10-X was placed in the center well at the start of the incubation. When Krebs' bicarbonate buffer was used, the flasks were chilled in ice at the end of the incubation period, and the vials containing the Hyamine were rapidly inserted before the acid was tipped in. An additional incubation for 60 to 90 minutes at 37°C after addition of perchloric acid served to demineralize the slices and allowed complete diffusion of the CO₂ into the Hyamine.

C¹4O₂ trapped in the Hyamine was measured in a liquid scintillation counter after 15 ml of a toluene-phosphor solution¹ were added to each vial. The incubation medium was centrifuged, and the supernatant solution was adjusted for 60 to 90 minutes at 37°C after addition of perchloric acid served to demineralize the slices and allowed complete diffusion of the CO₂ into the Hyamine.

¹ The phosphor solutions were prepared as follows: The toluene-phosphor solution contained 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2-(phenyloxazole)-benzene per liter of toluene. The toluene-ethanol-phosphor solution contained 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2-(phenyloxazole)-benzene per liter of 20% ethanol in toluene (volume for volume). Under our conditions, C¹⁵O was detected in the toluene-phosphor system at an efficiency of 45%, and in the toluene-ethanol-phosphor system, at an efficiency of 30%. Unless otherwise stated, all counts in the latter system were corrected to that which would be obtained in the former system.
Hyamine hydroxide 10-X, 2,5-diphenyloxazole, and 1,4-bis-2-(phenyloxazole)-benzene were obtained from the Packard Instrument Company, Inc., and silicic acid, from Bio-Rad Laboratories. The chloroform used to elute the silicic acid columns was reagent grade and was redistilled. All other chemicals used were reagent grade.

RESULTS

When radioactive glucose, pyruvate, or lactate was used as substrate, silicic acid chromatography of the incubation medium yielded an elution pattern typified by the curve shown in Fig. 1. As will be seen, pyruvic and lactic acids contained appreciable levels of radioactivity. Only small amounts of radioactivity were ever found in citric acid, or in any other of the Krebs' cycle acids. For example, in the figure a total of fewer than 1000 c.p.m. were found in citrate. When the experiments were performed with pyruvate-2-C\textsuperscript{14} and lactate-2-C\textsuperscript{14} at higher specific activities of approximately 2 \times 10^6 c.p.m. per pmole, the total radioactivity in citrate was less than 3000 c.p.m. An unknown material with a variable amount of radioactivity, i.e. from 5\% to 35\% of the counts in the lactate peak, was always eluted from the column as a discrete band. This has been labeled "LC" in Fig. 1. Our studies suggest that this component is a lactic acid condensation product. It was found to react as lactic acid in the colorimetric analysis, was volatile in acid but not in alkali, was easily extracted from 5\% butanol in chloroform by dilute alkali, and, when rechromatographed after alkaline hydrolysis, was converted to lactic acid (Table I). Moreover, when lactic acid was incubated and acidified in the absence of bone slices, this complex was also found. Therefore, the "LC" band is an artifact, and its radioactivity should properly be included in the amount of lactate formed. In our calculations, the radioactivity of this fraction was added to that of the eluted lactic acid peak to give the total radioactivity in lactate.

In order to establish the level of the metabolic activity of the bone slices which might be easily extractable, 500 mg of slices were shaken for 30 minutes in Tris buffer at 3\°. The extract was removed, and the slices were resuspended in fresh buffer. The extract, the resuspended slices, and control slices, which had been placed in Tris buffer at 3\° 30 minutes earlier, were incubated with uniformly labeled glucose-C\textsuperscript{14} at 37\°. This experiment showed that bone slices retained over 90\% of the C\textsuperscript{14}O\textsubscript{2}-producing capacity and 65\% of the C\textsuperscript{14} lactate producing capacity after extraction. The extract was virtually inactive.

The time course of uniformly labeled glucose-C\textsuperscript{14} metabolism by bone slices in bicarbonate buffer was followed until almost complete disappearance of substrate. These curves are shown in Fig. 2. During the first 4 hours, the rates of disappearance of glucose and formation of lactate were constant. Moreover, after an initial lag period, the rate of evolution of C\textsuperscript{14}O\textsubscript{2} was constant until the experiment was terminated. It is apparent that these tissue preparations were refractory to inactivation during the prolonged period of incubation. Hence, it is likely that the period of 2 to 3 hours which elapsed between death of the rabbits and the start of incubation did not affect adversely the viability of the preparation. The rate of lactate formation was 5.2 \mu moles per hour per g of slice. Approximately 1 \mu mole of lactate appeared for each \mu mole of glucose which disappeared. Since the radioactivity of the lactic acid increased at the same rate as the amount of lactate formed, it is clear that total radio-

![Fig. 1. Distribution of radioactivity eluted from silicic acid column. The initial reaction system contained 1.6 \times 10^6 c.p.m. of uniformly labeled glucose-C\textsuperscript{14} with sufficient carrier glucose to give a total of 10 pmoles. Buffer, Krebs' bicarbonate; incubation time, 2 hours; 500 mg of bone slices. A lyophilized powder equivalent to 5 ml of perchlorate-free filtrate was placed on the column. The eluant was butanol in CHCl\textsubscript{3} as follows: 105 ml of 5\%; 140 ml of 15\%; 105 ml of 25\%; and 160 ml of 35\%. The radioactivity shown is that actually counted and has not been corrected for total isotopes in the flask. In this experiment, the counts in each band multiplied by a factor of 8.1 gives the total counts per minute in the reaction flask. The titration curve for eluted acidity of added carriers is not shown, but the peak of each is indicated. All of the acids of the Krebs' cycle are eluted by this procedure.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Radioactivity</th>
<th>&quot;LC&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting &quot;LC&quot;</td>
<td>10,080 c.p.m.</td>
<td>100</td>
</tr>
<tr>
<td>After hydrolysis:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unchanged</td>
<td>550 c.p.m.</td>
<td>5</td>
</tr>
<tr>
<td>Lactate</td>
<td>9,500 c.p.m.</td>
<td>95</td>
</tr>
<tr>
<td>Other</td>
<td>100 c.p.m.</td>
<td>1</td>
</tr>
</tbody>
</table>

| for glucose by either the anthrone method (11) or glucose oxidase method (12), and for lactic acid by the Barker-Summerson method (13). The radioactive substrates were obtained from the following sources: from Volk Radio-Chemical Company, D,L-zinc lactate-2-C\textsuperscript{14}, sodium pyruvate-2-C\textsuperscript{14}, p-glucose-1- and 6-C\textsuperscript{14} and uniformly labeled n-glucose-C\textsuperscript{14}; from New England Nuclear Corporation, p-glucose-1- and 6-C\textsuperscript{14} and uniformly labeled n-glucose-C\textsuperscript{14}. The zinc lactate-C\textsuperscript{14} was converted to the sodium salt by ion exchange. The labeled substrates were checked for purity by paper chromatography, and, in addition, the lactate and pyruvate were chromatographed on silicic acid columns. The latter emerged as a discrete band corresponding to the added authentic carrier. The lactic acid, however, contained contaminant comprising 30\% of the gross radioactivity. Only the material in the authentic lactate band was used.
activity in lactate represents a direct index of lactate formation. Based on the starting radioactivity in glucose of 1.98 \times 10^6 c.p.m., approximately 60% was recovered as lactate and pyruvate, 6% was converted to C^14O_2, approximately 1% of the counts appeared in all of the Krebs' cycle acids combined, and a maximum of 1% was found in the perchloric acid-precipitable fraction. Thus one-third of the glucose carbons were distributed among other cellular constituents which were not eluted from the silicic acid column. This would include phosphorylated intermediates, basic compounds, and other molecules which retained a charge under the acidic conditions of the column. The ratio of lactate to pyruvate remained more or less constant at 15. The specific activity of the starting glucose was 1.7 \times 10^4 c.p.m. per \mu m mole, and the mean of the specific activity of newly formed lactate at each time interval was 0.89 \times 10^4 c.p.m. per \mu m mole. This almost theoretical specific activity indicates that all of the lactate arose from the added glucose. It has been reported (3) that the nature of the buffer has a significant effect on the metabolic activity of bone. When comparisons between Tris buffer and bicarbonate buffer with uniformly labeled glucose-C^14 as substrate were conducted, it was found that slices in bicarbonate buffer produced up to 2.5 times more lactate-C^14 and approximately 13 times more pyruvate-C^14 than in Tris buffer. There was no effect on C^14O_2 production. In Tris buffer, the lactate-pyruvate ratio was approximately 90 from glucose-C^14, as well as in other studies when lactate or pyruvate were used as substrates. Typical data are shown in Table II (Experiments 1 and 2).

In order to establish whether or not the pentose pathway plays a significant role in the production of lactate by bone, slices from several rabbits were pooled, and samples were incubated in the same experiment with uniformly labeled glucose-C^14 and glucose-6-C^14, and in two experiments with glucose-1-C^14, in Tris buffer or in bicarbonate buffer. The determination of the proportion of lactate arising from the pentose cycle was based on the total radioactivity in lactate formed from glucose-6-C^14 and uniformly labeled glucose-C^14 and was calculated according to the method of Katz and Wood (14). The pertinent data are shown in Table II. In one trial with five pairs of flasks in Tris buffer, and in four trials with a total of 15 pairs of flasks in bicarbonate buffer, it was found that between 1 and 22% of the 3-carbon end product arose through the pentose pathway.

Aliquots of the lactic acid which were formed in Tris and bicarbonate buffers (Experiments 1 and 5 in Table II) from glucose-1-C^14 and glucose-6-C^14, and which were isolated by the column procedure, were degraded to acetaldehyde and CO_2 by oxidation with permanganate (15). The C^14O_2 was trapped in Hyamine, and the acetaldehyde, in bisulfitc in an oxidation train. Aliquots of the Hyamine and bisulfite solutions were counted by the liquid scintillation technique with appropriate corrections for quenching. In all samples studied, more than 97% of the total radioactivity of the starting lactate was recovered in the acetaldehyde fragment, and the remainder was found in the CO_2 fragment. This distribution of radioactivity is in agreement with theory for either the Embden-Meyerhof pathway.

**Table II**

**Evaluation of pentose pathway for lactate formation**

All flasks contained exactly 1.98 \times 10^6 c.p.m. of the appropriate glucose. Sufficient carrier glucose was added in each case to bring total starting glucose to indicated amount. Incubation time was 2 hours. Within each experiment, the flasks were charged with slices from mixed pool of tissue from four rabbits each in Experiments 1 to 4, and from two rabbits in Experiment 5. The labeled substrates in Experiments 1 to 3 were from one supplier, and in Experiments 4 and 5, from a second supplier. The letter G denotes glucose.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Buffer</th>
<th>Bone</th>
<th>Sets of flasks</th>
<th>Initial glucose</th>
<th>C^14O_2 from</th>
<th>C^14-lactate from</th>
<th>Pentose cycle lactate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>G-1-C^14</td>
<td>G-6-C^14</td>
<td>G-U-C^14</td>
<td>G-1-C^14</td>
</tr>
<tr>
<td>1</td>
<td>Tris</td>
<td>400</td>
<td>5</td>
<td>10.0</td>
<td>22,600</td>
<td>9,800</td>
<td>17,300</td>
</tr>
<tr>
<td>2</td>
<td>HCO_3^-</td>
<td>400</td>
<td>5</td>
<td>10.0</td>
<td>4,840</td>
<td>15,100</td>
<td>440,000</td>
</tr>
<tr>
<td>3</td>
<td>HCO_3^-</td>
<td>400</td>
<td>4</td>
<td>40.0</td>
<td>2,740</td>
<td>4,430</td>
<td>113,000</td>
</tr>
<tr>
<td>4</td>
<td>HCO_3^-</td>
<td>400</td>
<td>4</td>
<td>40.0</td>
<td>2,695</td>
<td>6,420</td>
<td>155,000</td>
</tr>
<tr>
<td>5</td>
<td>HCO_3^-</td>
<td>300</td>
<td>3†</td>
<td>5.0</td>
<td>17,160</td>
<td>3,940</td>
<td>11,000</td>
</tr>
</tbody>
</table>

* Pentose cycle lactate is defined as the proportion of the lactate which was produced via the pentose cycle pathway. This was calculated by the following formula (14):

% Pentose cycle lactate = \( \frac{C^14\text{lactate from G-6-C}^{14}}{C^14\text{lactate from G-U-C}^{14}} - 1 \times 100 \)

† Only two flasks were used for uniformly labeled glucose-C^14 (G-U-C^14).
or pentose pathways and indicates that the Entner-Doudoroff pathway (16) is inoperative, since this latter route of metabolism should lead to appreciable C\(^14\) incorporation in position 1 of lactate formed from glucose-1-C\(^14\).

**DISCUSSION**

This study demonstrates that, under our experimental conditions, lactate acid represented the major end product of glucose metabolism. The almost theoretical specific activity of lactate, compared with the initial specific activity of glucose, is of importance in light of the studies of Borle, Nichols, and Nichols (3) on the anaerobic production of lactate from glucose. Those investigators found that almost twice as much lactate was formed as could be accounted for by glucose disappearance and endogenous production. They interpreted this to mean that in the presence, but not in the absence, of glucose, a precursor in the tissue gave rise to lactate. Although "overproduction" of lactate did not seem to occur aerobically, this possibility could not be ruled out from their data. Our data clarify this point.

That the pentose cycle was operating was shown qualitatively by the differences in formation of C\(^4\)O\(_2\) from glucose-6-C\(^14\) compared with that from glucose-1-C\(^14\). The quantitative evaluation of this pathway, however, is handicapped by a lack of sensitivity such that small experimental errors will cause much larger variations in the calculated values (14). For this reason, the pertinent experiments were repeated several times. These data, together with that from the degradation of lactate, indicate that approximately 15% of the lactate devolved from the pentose cycle, and the remainder, from the Embden-Meyerhof pathway.

The role of the Krebs cycle in the overall metabolism of glucose by the bone slices appeared to be minor, based on the large accumulation of lactate and the relatively small evolution of C\(^4\)O\(_2\). In the light of the data in the rate study (Fig. 2) and the similarity of C\(^4\)O\(_2\) production in Tris and bicarbonate buffers, however, it seems that most of the CO\(_2\) arose from the pentose cycle and the remainder, from the Embden-Meyerhof pathway.

The extraction study shows that at least a partial separation of glycolytic from oxidative activities is possible in bone tissue. The extraction study also demonstrates that the bulk of the lactate-forming capacity was not easily washed away and presumably is not due to the fortuitous entrapment of formed elements of the blood.

The reason for the efficacy of bicarbonate buffer in comparison with Tris buffer in promoting lactate formation was not evaluated. Since somewhat similar results were reported by Borle, Nichols, and Nichols (3) between Krebs' phosphate and Krebs' bicarbonate buffer, it is likely that the results we obtained also can be attributed to a specific effect of HCO\(_3^-\) or CO\(_2\) (17, 18).

**REFERENCES**

Aerobic Metabolism of Glucose by Bone
David V. Cohn and Bernard K. Forscher