Pyruvate and Propionate Metabolism in Lactating Cows

EFFECT OF BUTYRATE ON PYRUVATE METABOLISM*

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SUMMARY

The glucogenic behavior of butyrate in ruminants has been reported frequently, but the metabolic nature of this anomalous relationship has remained obscure. To test the possibility that the metabolism of butyrate affects gluconeogenesis, lactating cows were given intravenous injections of tracer amounts of pyruvate-2-14C and, in parallel experiments, of pyruvate-2-14C plus substrate amounts of sodium butyrate (0.7 mm per kg of body weight). When butyrate was injected, less 14C appeared in expired CO2 and more 14C in plasma glucose. The intramolecular labeling pattern in glutamate also changed with butyrate injection, with the result that more of the 14C was located in carbon derived from oxalacetate compared to carbon derived from acetyl-CoA.

These results indicate that butyrate metabolism affects gluconeogenesis by sparing pyruvate oxidation, thereby conserving a glucogenic precursor, and, in addition, by enhancing the conversion of pyruvate to oxalacetate.

Gluconeogenesis is of special importance in ruminants in that alimentary glucose is scant because the rumen microorganisms ferment dietary carbohydrate largely to short chain fatty acids. Thus ruminants depend largely on endogenous synthesis for their glucose, and it has often been suggested that these animals may be able to utilize fatty acids, other than propionate, as glucose precursors (2-9). For several years, recurrent reports have suggested a glucogenic role for butyrate in ruminants (2-8), but this seemingly paradoxical behavior has remained unexplained. For example, Potter (2) showed that butyrate was more effective than propionate for increasing blood sugar levels and relieving hypoglycemic convulsions in lambs and sheep treated with insulin. Kleiber et al. (3) showed that, unlike acetate, butyrate was markedly glucogenic in lactating cows; 14C from butyrate was utilized more for synthesis of lactose than for synthesis of milk fat, even though the latter contains nearly 10 mole % butyrate as part of the triglyceride molecules. It was found that, in terms of fractions of the injected 14C recovered in milk sugar, butyrate-2-14C (3) was equal to propionate-2-14C (10) as a precursor of lactose. 14C was transferred to plasma glucose more rapidly and in much greater amounts from butyrate than from acetate in lactating cows (8, 11).

Efforts to demonstrate a glucogenic pathway for the utilization of butyrate in ruminants have been singularly unsuccessful both in lactating cows (11) and in sheep (12, 13), confirming earlier findings with the rat (14).

A recent report from our laboratory (15) presented evidence for a glucagonolytic action of butyrate in intact lambs, which accounts, at least in part, for the increased blood sugar levels that have been observed after intravenous injection of butyrate in sheep. Reported here is another effect of butyrate which throws additional light on its glucogenic behavior and helps to explain its metabolic role in ruminants.

Lactating cows have been given injections of pyruvate-2-14C and propionate-2-14C, and the intramolecular labeling patterns in glutamic acid have been used to assess the pathway for metabolism of these two glucogenic compounds. Results presented here show that when butyrate was injected together with pyruvate-2-14C it caused preferential utilization of pyruvate to form oxalacetate; less pyruvate was decarboxylated to form acetyl-CoA. Since the conversion of pyruvate to oxalacetate is one of the rate-limiting steps in gluconeogenesis (16, 17), metabolic changes that enhance oxalacetate formation could lead to increased glucose formation.

EXPERIMENTAL PROCEDURE

The 14C-labeled compounds used in these studies were purchased from commercial sources (pyruvate-2-14C) or synthesized in our laboratory by standard methods (propionate-2-14C) (18). The propionate was shown to be radiochemically pure by paper chromatography with the use of butanol-1NH4Cl-ammonia-water (2:1:4) as solvent. The pyruvate, chromatographed in butanol-glacial acetic acid-water (3:1:4), had a small contaminant amounting to 2% or less of the total 14C. This was tentatively identified from the Rf value as parapyruvate.

The experimental cows were normal lactating animals receiving a standard dairy ration. On the day preceding the trial, a veterinarian placed a plastic cannula, approximately ½ inch
in diameter, in each jugular vein. These cannulas remain open for prolonged periods if they are flushed daily with sterile 0.9% sodium chloride solution containing a trace of heparin. They provide a means of rapidly injecting solutions directly into the blood or withdrawing frequent samples of blood for analysis, a sampling procedure which is painless for the cow. This is extremely important in metabolic studies, particularly those involving gluconeogenesis, since any emotional disturbance might obscure responses to experimental treatment.

Respired CO₂ from the cows was collected continuously during a 3-hour period following injection of the ¹⁴C compounds, and samples were assayed for radioactivity by methods described earlier (19). Milk was collected from the cows 3, 10, and 24 hours after the experiment began, and the milk components were separated by standard procedures. The isolation of glutamic acid from milk casein and the procedure used for its stepwise degradation to establish the intramolecular labeling pattern have been described elsewhere (20).

Glucose was isolated as the osazone derivative from plasma samples collected at frequent intervals, and its specific activity was measured by a method developed by Phillips and Mia (21). All ¹⁴C assays were made on a low background, end window, gas flow Geiger counter.

A solution of sodium butyrate (2.3 M) was adjusted to pH 7.4 and sterilized in an autoclave. Sixty milliliters of this solution were injected at a constant rate over a period of about 10 min into one jugular vein. The pyruvate-2-¹⁴C (approximately 1 mC) was then injected as a single dose, followed by a second infusion of 60 ml of sodium butyrate over a 10-min period. Each cow received a total of 280 mmoles of sodium butyrate intravenously over a period of about 25 min, which corresponds to about 2 times the rate of rumen butyrate production estimated for lactating cows ingesting our standard dairy ration (22).

Each cow received injections of pyruvate-2-¹⁴C twice, in trials that were spaced 1 week apart. The trials were identical, except that no butyrate was injected in one of the trials with each cow.

Two trials are reported in which cows were given intravenous injections of propionate-2-¹⁴C given as a single dose at the beginning of the experiments. No butyrate was injected into these cows, but the sampling procedure was otherwise the same as that described for experiments with pyruvate-2-¹⁴C.

**Table I**

<table>
<thead>
<tr>
<th>Cow</th>
<th>Weight</th>
<th>Compound injected</th>
<th>Total activity injected</th>
<th>Butyrate injection</th>
<th>¹⁴CO₂ exhaled in 3 hrs</th>
<th>Total specific activity</th>
<th>% of total ¹⁴C in molecule</th>
<th>¹⁴C distribution</th>
<th>Recovery of ¹⁴C in degradation</th>
<th>¹⁴C vi oxalacetate</th>
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</thead>
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<tr>
<td>II-28</td>
<td>421</td>
<td>Pyruvate-2-¹⁴C</td>
<td>1.39</td>
<td>-</td>
<td>39.0</td>
<td>8.6</td>
<td>21</td>
<td>15.6</td>
<td>24.8</td>
<td>2.8</td>
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<td>H-28</td>
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<td>-</td>
<td>33.3</td>
<td>4.4</td>
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<td>4.6</td>
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<td>-</td>
<td>33.8</td>
<td>10.1</td>
<td>19.2</td>
<td>22.4</td>
<td>36.7</td>
<td>1.9</td>
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<td>24.8</td>
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<td>-</td>
<td>39.7</td>
<td>2.2</td>
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<td>13.4</td>
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<td>+</td>
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<td>4.1</td>
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<td>1.50</td>
<td>-</td>
<td>30.7</td>
<td>4.1</td>
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<td>40</td>
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<tr>
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<td>-</td>
<td>2.8</td>
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<td>32.8</td>
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<tr>
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<td>-</td>
<td>0.78</td>
<td>17.9</td>
<td>30</td>
<td>35.1</td>
<td>5.0</td>
<td>4.2</td>
</tr>
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</table>

a Specific activity of glutamic acid recovered from casein 3 hours after injection of ¹⁴C-labeled compound. Specific activity unit is microcuries of ¹⁴C per g atom of carbon/microcuries of ¹⁴C injected per kg of body weight.

b See Footnote 2 for method used to estimate these values.

c Sample collected 10 hours after injection of propionate-2-¹⁴C.

d Sample collected 24 hours after injection of propionate-2-¹⁴C.

in the experiments. No butyrate was injected into these cows, but the sampling procedure was otherwise the same as that described for experiments with pyruvate-2-¹⁴C.
the fumarate molecule. It may reflect an isotope effect favoring preferential addition of 14C in the C-4 position of glutamate compared to C-2 may be an artifact of the degradation procedure, and C-4 positions reflects the relative amount of 14C that had equilibrated with fumarate (as explained above for propionate). This route would account for the smaller amounts of 14C, distributed nearly equally between the C-4 and C-5 positions of glutamate, that were found after propionate 2 14C was injected.

Since C-2 and C-3 of oxalacetate serve as common precursors of C-2 and C-3, as well as C-4 and C-5, of glutamate, the specific activity of these carbon atoms would be equal if oxalacetate (via decarboxylation) were the sole source of acetyl-CoA entering the cycle. The average specific activities for C-4 and C-5 relative to those for C-2 and C-3 provide an index of the amount of unlabeled acetyl-CoA that mixes with the 14C-acetyl moiety derived from oxalacetate. This ratio varied from 0.11 to 0.14 for the cows given injections of propionate-2-14C, which indicates that, in these animals, about 11 to 14% of the acetyl-CoA that entered the tricarboxylic acid cycle had been derived from the pathway involving decarboxylation of oxalacetate. It is of interest that the calculated value was nearly the same for milk samples collected 3, 10, and 24 hours (see data on cow 65, Table I) after propionate-2-14C was injected. Such a result suggests that oxalacetate may be converted to acetyl-CoA at a fairly constant rate in the lactating cow. It is not apparent what metabolic processes may be responsible for the flux of carbon from oxalacetate to acetyl-CoA, but those processes would increase the requirement for oxalacetate precursors in the same fashion in which synthesis of glucose (gluconeogenesis) and amino acids does. The oxalacetate withdrawn for any of these processes must be replenished to avoid impairing the critically important oxidative functions of the cycle.

After pyruvate-2-14C was injected there was less equilibration of 14C between the C-2 and C-5 positions of glutamate than was the case with propionate, and a much greater part of the 14C entered the cycle as acetyl-CoA. Decarboxylation of pyruvate-2-14C produces acetyl-1,2-14C-CoA, which enters the tricarboxylic acid cycle and labels the C-5 position of the glutamate. Carboxylation of the pyruvate would lead to the formation of oxalacetate-2,3-14C, and, to the extent that this equilibrates with fumarate prior to its conversion to citrate in the normal turnover of cycle intermediates, the 14C would be distributed between the C-2 and C-3 positions of glutamate. In contrast to the situation in propionate metabolism, fumarate is not an obligatory intermediate in the metabolism of pyruvate, but this presumably accounts for the lesser equilibration between C-2 and C-3 in the glutamate after pyruvate-2-14C was injected. The appearance of small amounts of 14C in the C-4 position of glutamate can be attributed to doubly labeled acetyl-CoA derived from oxalacetate that had equilibrated with fumarate (as explained above for propionate metabolism). Thus the difference in 14C between the C-5 and C-4 positions reflects the relative amount of 14C that had entered the cycle by direct decarboxylation of pyruvate-2-14C compared to that introduced as acetyl-CoA following conversion to oxalacetate and equilibration with fumarate.

The 14C distribution provides a basis for estimating the relative amounts of pyruvate-2-14C that entered the cycle as acetyl-CoA compared to that which entered as oxalacetate (24). The flow of carbon from oxalacetate is reflected in the amount of 14C present in the C-2 and C-3 positions, while that via acetyl-CoA is related to the 14C level in the C-5 position. An assessment based on this approach has been made for each sample in Column 10 of Table I. In each case the data indicate that 50% or more of the isotope from pyruvate-2-14C had entered the tricarboxylic acid cycle as oxalacetate. With the use of the same type of estimate, studies with normal fed rats (24, 25) indicated that a greater part of the pyruvate entered the cycle as acetyl-CoA than as oxalacetate. The greater utilization of glutamate for synthesis of oxalacetate in the cow presumably reflects the higher level of gluconeogenesis in ruminants necessitated by their very low amounts of alimentary glucose.

When butyrate was injected together with pyruvate, the amount of 14C in C-5 decreased relative to that in C-2 plus C-3 in each cow. These results indicate that the injection of butyrate had caused a shift in pyruvate utilization favoring oxalacetate formation, a change suggesting increased activity of pyruvate carboxylase. The experimental data indicate that the presence of butyrate enhanced the flow of carbon from pyruvate to oxalacetate by 19 to 75%.

The 14C level in C-5 might also decrease because of increased dilution in a larger acetyl-CoA pool during metabolism of butyrate. It is important to distinguish between this effect of butyrate and that reflecting preferential conversion of pyruvate to oxalacetate. If the results were due only to dilution in a larger pool of acetyl-CoA, then the isotope level in C-4 should be decreased by the same amount as that in C-5, since both of these carbons are derived from acetyl-CoA. In contrast to this possibility, the ratio of 14C in C-4 to that in C-5 increased 1.5 to 3 times. In each cow injected with butyrate, it is only by means of increased metabolism of pyruvate via oxalacetate, discussed above, that the 14C ratio, C-4:C-5, would increase as oxalacetate equilibrated with fumarate and was then converted to doubly labeled acetyl-CoA. This explanation does not exclude an increase in the level of acetyl-CoA during butyrate metabolism, but rather argues that the increased 14C in the C-2, C-3, and C-4 positions, relative to that in C-5, cannot be accounted for solely on a basis of a larger acetyl-CoA pool. In addition, butyrate injection must have caused a preferential utilization of pyruvate to form oxalacetate.

### Discussion

Several factors have been reported to affect the conversion of pyruvate to oxalacetate, a reaction catalysed by the enzyme pyruvate carboxylase (26). In the intact rat, changes in the intramolecular labeling pattern in glutamate have indicated that fasting (24, 25) and diabetes (25) enhanced the utilization of 14C in (C-2 + C-3 + C-4),

\[
\frac{14C}{14C} = \frac{C-2 + C-3 + C-4}{C-2 + C-3 + C-4 + C-5}
\]

An additional amount of 14C that originally entered the cycle as oxalacetate and then appeared in the C-4 (and C-5) positions must be added to that present in the C-2 and C-3 positions to obtain a quantitative estimate of the relative pyruvate flux via oxalacetate, as follows:

\[
\frac{14C}{14C} = \frac{C-2 + C-3 + C-4 + C-5}{C-2 + C-3 + C-4 + C-5} - 100
\]
pyruvate for synthesis of oxaloacetate. This effect of fasting and diabetes has also been demonstrated by measuring the amount of $^{14}C\text{O}_2$ fixed into organic acids in rat liver (37, 28). Addition of acetoacetate (16) or several short chain fatty acids, including butyrate (29), to rat, rabbit, or sheep kidney cortex accelerated the rate of glucose formation from lactate. It was concluded that the effect of these substances involved not only a sparing action on lactate oxidation but also an activation of pyruvate carboxylase by acetyl-CoA.

Utter and Keasch (30) demonstrated that pyruvate carboxylase is dependent on acetyl-CoA for activity. The latter functions as a catalyst, and it has been suggested that its concentration may control the carboxylation of pyruvate, since the enzyme has a $K_m$ for acetyl-CoA of $2 \times 10^{-5}$ M (31), which is very close to the concentration of acetyl-CoA found in the liver (32) and kidney cortex (29) of normal rats. This relationship would make acetyl-CoA a very effective control agent, physiologically, in the metabolism of pyruvate.

Butyrate is taken up avidly by the liver of ruminants, in contrast to acetate, which is absorbed only to a very limited extent (5, 33). Thus, the $\beta$ oxidation of butyrate would lead to the formation of acetyl-CoA in the liver, and this could influence gluconeogenesis by its effect on the pyruvate carboxylase enzyme. This sequence of events would account for the observed changes in the labeling pattern of glutamate when cows were given injections of butyrate together with pyruvate-2-$^{14}C$, namely, a decrease in the $^{14}C$ level in the C-5 position relative to that in C-2 plus C-3. A simple interpretation of these changes is not possible since, as discussed above, increased levels of acetyl-CoA derived from butyrate would dilute the $^{14}C$ in route to C-5, giving the observed results. On the other hand, the increase in $^{14}C$ in C-4 relative to C-5 when butyrate was injected eliminates the possibility that this dilution alone would be solely responsible for the changes. Furthermore, since acetyl-CoA functions catalytically on pyruvate carboxylase, the effect of changing levels of acetyl-CoA should be greater on the enzyme than on substrate level dilution.

If the glutamate labeling pattern is to be meaningful as an index of changes in gluconeogenesis, then it is essential that the glutamate sample (from casein) reflect, at least in part, synthetic activity at the site where gluconeogenesis is active, i.e. the liver. The arguments presented above have assumed this to be the case, although conclusive evidence of this relationship is difficult to establish in studies with intact animals. In support of this conclusion is the evidence that as much as 90% of the casein glutamate is derived from blood glutamate (34) and that blood pyruvate is not normally absorbed by the mammary gland (35). Thus it seems likely that a significant part, and perhaps a major part, of the $^{14}C$ transfer from pyruvate to glutamate took place outside the mammary gland. Therefore, the liver (and perhaps also the kidney) would be expected to have a major influence on the results observed.

Fig. 1 shows the change in specific activity of plasma glucose at different times after Cow H-28 was treated by injection of pyruvate-2-$^{14}C$. The specific activity increased rapidly, reaching a maximum about 20 to 30 min after the labeled pyruvate was injected. When the cow received butyrate in addition to the pyruvate-2-$^{14}C$, the specific activity of plasma glucose reached a maximum that was nearly 3 times as great. In addition, the concentration of plasma glucose changed from 70.9 mg per 100 ml prior to the injection of butyrate to a level of 76 mg per 100 ml within 10 min of injection of the butyrate; this higher concentration was maintained for at least 20 min. It is not possible to determine to what extent these changes may reflect a sparing effect of butyrate on pyruvate oxidation (as noted earlier), and what part is due to greater activity of pyruvate carboxylase and increased gluconeogenesis. It seems most likely that both played a role and contributed to the anomalous glucogenic effect of butyrate in the cow.

The increase in specific activity of plasma glucose was less pronounced when the other two cows were given injections of butyrate, but in both cases was greater than it was when the cows received pyruvate-2-$^{14}C$ alone. Butyrate injection caused a small increase in plasma glucose level in Cow 210, but did not cause an increase of plasma glucose concentration in Cow 1060. A variable effect of butyrate injection on plasma glucose concentration has been reported previously in ruminants (36-38). In some animals butyrate injection was followed by increased blood sugar concentrations, whereas in others the glucose concentration remained constant or decreased; ketone bodies generally increased following butyrate injection.

This variable hyperglycemic response to butyrate injection is not inconsistent with an effect of butyrate on gluconeogenesis. Under some circumstances, the supply of gluconeogenic precursors may limit the rate of glucose synthesis, or conditions may favor the formation of glycogen or glycerol, rather than glucose. It is only when enhanced gluconeogenesis coincides with a release of glucose into the blood which exceeds peripheral glucose utilization that a hyperglycemia will be observed.

For purposes of delineating events at the molecular level, these results are necessarily indirect, since it is generally impossible to identify and control each factor that influences metabolic studies in the intact animal. However, given the assumption that the intramolecular $^{14}C$ distribution in glutamate reflects changes in pyruvate metabolism at a site of gluconeogenesis, then the results obtained are in good agreement with the postulate that butyrate enhances gluconeogenesis. The manner in which this occurs presumably involves increased levels of acetyl-CoA during butyrate metabolism in the liver, which enhance the carbon flux toward glucose by increasing pyruvate carboxylase activity. Studies in vitro at the enzyme level (30) and with tissue slices (29)
have provided strong support for this type of interaction in animal tissues. We propose that this also occurs in the cow and that it accounts, at least in part, for the anomalous glucogenic behavior of butyrate in these animals.

REFERENCES

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