ABSTRACT

The gluconeogenic capacity of mammary tissue of lactating cow was investigated by incubating mammary tissue slices with alanine, glutamate, lactate, pyruvate, or glycerol in conjunction with acetate and glucose (10 mM or 1 mM). In no case was any substrate incorporated into glucose per se. In lactose synthesis, glucose was the major source of carbon although glycerol also was incorporated into lactose. Alanine, glutamate, lactate, or pyruvate were not incorporated into lactose at optimum (10 mM) or suboptimum (1 mM) concentrations of glucose. Activity of glucose-6-phosphatase was negligible in mammary tissue, less than 1% of the activity in liver or kidney tissue from the same cows. Pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and fructose-1,6-diphosphatase were in cow mammary tissue, but the activities were lower than in liver. Gluconeogenic substrates were not converted to glucose regardless of whether the incubation contained an optimum (10 mM) or a suboptimum (1 mM) glucose concentration. Consistent with the inability of cow mammary tissue to convert gluconeogenic metabolites to glucose is the virtual absence of glucose-6-phosphatase and the lack of excess gluconeogenic substrates available to the intact mammary gland of lactating cow.

INTRODUCTION

Gluconeogenesis, the conversion of noncarbohydrate precursors to glucose, is an extremely important process in ruminant animals. The rumen fermentation of dietary carbohydrate to volatile fatty acids (1, 14) results in only small amounts of glucose being absorbed from the gastrointestinal tract of ruminants (4, 17, 27, 32). In contrast to nonruminants, gluconeogenesis is the major source of glucose for ruminant animals.

Gluconeogenesis in nonlactating ruminants occurs predominantly in liver and kidney tissues (9, 10, 18, 19). In reviewing glucose metabolism in the ruminant, other investigators have suggested that the mammary gland may have the ability to synthesize glucose from noncarbohydrate precursors (25, 29). This premise is based on the fact that glucose is an extremely important substrate for mammary gland metabolism with the mammary tissue of a lactating cow requiring up to 60 to 85% of the total glucose available to the animal (13). Indeed, studies by Mepham and Linzell (25) have suggested that glutamate was converted to glucose by mammary tissue of goat.

Our studies were to assess pathways and importance of gluconeogenesis in mammary tissue of cow. The gluconeogenic capacity of cow mammary tissue was investigated by incubating mammary tissue slices with alanine, glutamate, lactate, pyruvate, and glycerol. The incorporation of these substrates into lactose also was examined since gluconeogenesis in the mammary gland probably would not produce glucose per se but rather glucose-6-phosphate which then would be utilized for the synthesis of the galactose moiety of lactose. Fatty acid synthesis and CO₂ production from the above substrates as well as from acetate and glucose also were examined for a more complete picture of metabolism of mammary gland. In addition, activities of certain key gluconeogenic enzymes were determined in cow mammary tissue.

MATERIALS AND METHODS

Chemicals

Cofactors and enzymes in the enzyme assays were obtained from Sigma Chemical Co., St.

Animals

Lactating Holstein cows were the source of mammary tissue. Cows were in mid-lactation and were producing in excess of 20 kg of milk per day at the time of slaughter. They were fed roughage ad libitum plus 1 kg of a 14% crude protein concentrate mix for every 3 kg of milk produced. The roughage portion of the diet was approximately 70% corn silage and 30% mixed alfalfa-grass hay. The basal concentrate mix was high-moisture shelled corn and soybean meal plus a mineral and vitamin (A and D) supplement.

Tissue Samples

Cows were stunned with a blow to the head and jugular veins were severed. A sample of mammary tissue was removed and placed immediately in a beaker of ice cold tris-sucrose buffer (pH 7.3, .3 M sucrose, 23 mM Trizma-HCl, 6.8 mM Trizma base, 1 mM glutathione, and 1 mM ethylenediaminetetraacetate (EDTA)). The tissue remained in the ice cold buffer until it was sliced for carbon flux incubations or homogenized for enzyme analysis (approximately 5 to 10 min).

Tissue Incubations

Mammary slices were prepared with a Stadie-Riggs hand microtome. A tissue slice weighing between 125 and 150 mg was placed in each incubation flask (25 ml Erlenmeyer). Incubations were in Krebs-Ringer bicarbonate buffer solution (pH 7.35 to 7.45). All incubations contained 10 mM acetate concentration, .1 unit/ml of insulin, and glucose (either 1 mM or 10 mM concentration). In some cases, a 5 mM concentration of glycerol, lactate, pyruvate, glutamate, or alanine also was included in the incubation media. The appropriate radioactive substrate also was included (approximately 1 µCi per incubation), and the incubation volume was 3 ml. Mammary slices were placed in the incubation media, gassed with an atmosphere of O2 and CO2 (95:5), stoppered with rubber serum caps, and incubated for 3 h at 37 C in a shaking incubator.

The incorporation of radioactive substrate into glucose, lactose, and fatty acids as well as the oxidation to CO2 was determined with mammary tissue slice incubations. The methods to determine CO2 production and fatty acid synthesis were as described by Bauman et al. (5). Similar to previous studies (5, 7, 8, 24), we found that at the end of the 3-h incubation the radioactive fatty acids were almost exclusively within the tissue slice while over 95% of the lactose synthesized from radioactive substrates was in the media.

The amount of radioactive substrates incorporated into glucose and lactose by mammary tissue incubations was determined by descending paper chromatography with ethyl acetate-acetic acid-formic acid-water (18:3:1:4) as the solvent system (22, 24). Twenty microliters of the incubation media was spotted on Whatman DE-81 paper strips (2 cm x 57 cm) and the strips were developed for 24 h. Reference standards of [1-14C] lactose and [U-14C] glucose were run concurrently. Strips were air-dried, cut into 1 cm bands and placed in scintillation vials with 1.5 ml water. After 30 min, 10 ml of triton-toluene cocktail were added, and the total radioactivity in the glucose and lactose peaks was determined.

Tissue Homogenates

An additional portion of the fresh tissue was used in the preparation of homogenates for assays of enzyme activity. All steps of homogenization were at 4 C. The tissue was minced into small pieces and mixed with two volumes of the tris-sucrose buffer previously described. The tissue was then homogenized with a Sorvall Omni-Mixer followed by five times through a Potter-Elvehjem teflon-glass homogenizer as described by Mellenberger et al. (24). The cell debris was removed by centrifuging for 8 min at 1000 x g in a Sorvall Superspeed RC2-B automatic refrigerated centrifuge. The centrifuged homogenate was filtered through two layers of cheese cloth. The cell debris was discarded, and the supernatant was centrifuged for 20 min at 10,000 x g to precipitate the mitochondria. The mitochondria and the supernatant from this step were saved.
The mitochondria were resuspended in 10 ml of the tris-sucrose buffer by the use of a ground glass hand homogenizer and centrifuged for 20 min at 10,000 × g. The supernatant was discarded and the procedure repeated to wash the mitochondria twice. After the second wash, the mitochondria were resuspended in 10 ml of tris-sucrose buffer and sonicated with two 45 s bursts from a Bronson Sonifer Cell Disruptor (Model W140). This mitochondria fraction was stored on ice until enzyme assays later the same day.

The supernatant from the original precipitation of the mitochondria was centrifuged at 105,000 × g for 60 min in a Beckman L2-65B ultracentrifuge to separate the microsomes from the cytosol. The microsomes were resuspended and sonicated the same way as the mitochondria. Both the cytosol and microsomal fractions were stored on ice until enzyme assays.

Enzyme Assays

All enzyme assays were at 37 C in the linear range of activity with regard to time and protein concentration. Fructose-1,6-diphosphatase activity was measured by following NADP+ reduction at 340 nm in a Beckman DU spectrophotometer coupled with a Gilford multiple sample absorbence recorder (Model 2000). Pyruvate carboxylase and phosphoenolpyruvate carboxykinase activities were based on the incorporation of 14CO2 into product. Glucose-6-phosphatase activity was determined by measuring the amount of inorganic phosphate liberated from glucose-6-phosphate. All enzyme activities were expressed per milligram of protein with the subcellular protein concentrations determined by the method of Lowry et al. (23).

Fructose-1,6-diphosphatase (EC 3.1.3.11) was assayed via a coupled reaction according to Sillero et al. (28). The reaction volume was 3 ml and contained the following concentrations of reactants: 50 mM imidazole buffer (pH 7.0); 100 mM KCl; 10 mM MnCl2; 1.0 mM glucose-6-phosphate; .25 mM NADP+; .17 units/ml glucose-6-phosphate isomerase; and .07 units/ml glucose-6-phosphate dehydrogenase. Fructose-1,6-diphosphatase was omitted for blank reactions.

Pyruvate carboxylase (EC 6.4.1.1) was assayed by the 14CO2-fixation assay described by Ballard and Hanson (3). The reaction volume was 1.5 ml. Blank reactions were carried out by omitting either the enzyme preparation or glucose-6-phosphate. The reaction was preincubated at 37 C for 5 min before the addition of enzyme preparation. A 10-min incubation was terminated by the addition of 1 ml of 10% trichloroacetic acid. Following centrifugation to precipitate protein, inorganic phosphate in the supernatant was determined by the method of Fiske and SubbaRow (15) as modified by Flynn et al. (16).

RESULTS

Results in Table 1 indicate utilization of the various substrates for lactose synthesis, fatty acid synthesis, and CO2 production. All incubations contained 10 mM acetate and 10 mM glucose concentrations which previously have given optimum and physiological rates of fatty
acid and lactose synthesis with ruminant mammary tissue slices (5, 7, 8, 24). In our studies, glucose was the major source of carbon for lactose synthesis although glycerol also was incorporated into lactose by incubations of cow mammary tissue. There was no detectable utilization of alanine, glutamate, lactate, or pyruvate for lactose synthesis (Table 1). Thus, the gluconeogenic substrates which enter the central pathways of gluconeogenesis as pyruvate or tricarboxylic acid (TCA) cycle intermediates were not utilized for lactose synthesis. Additionally, the substrates (excluding glucose) yielded no detectable glucose synthesis (data not shown).

Consistent with previous reports on fatty acid synthesis in ruminant mammary tissue (6), acetate was the major source of carbon for synthesis of fatty acid by incubations of cow mammary tissue (Table 1). The rate of acetate incorporation into fatty acids was approximately 45 times greater than that of lactate (the next most actively incorporated substrate). All metabolites were oxidized to CO₂, but on a carbon basis acetate was the predominant source of CO₂ production by slices of cow mammary tissue (Table 1). In cases of comparable substrates, the rates of lactose synthesis, fatty acid synthesis, and CO₂ production are similar to previous reports (5, 7, 24).

The possibility existed that the high glucose concentration might be masking the ability of the slices of cow mammary tissue to utilize other substrates for the synthesis of glucose or lactose. Therefore, a second study involving mammary tissue from the same animals used a suboptimum glucose concentration (1 mM). In these studies, similar to results with high glucose media, radioactive lactose was produced only from glucose or glycerol (Table 2). Additionally, alanine, glutamate, lactate, pyruvate, and glycerol were not incorporated into glucose per se (data not known). The rate of lactose synthesis from glucose with the 1 mM glucose media represented only 12% of that with the 10 mM glucose media (compare Table 1 with Table 2). Acetate incorporation into fatty acids also was reduced as the rate with the low glucose media was 84% of that with the 10 mM glucose media. Previous studies have reported that with a media containing glucose at 1 mM concentration the glucose incorporation into lactose represented 13% (unpublished) and acetate incorporation into fatty acids represented 85% (7) of the rates with an optimum 10 mM glucose media.

The activities of certain key gluconeogenic enzymes were determined in cow mammary tissue (Table 3). The most dramatic observation is the negligible activity of glucose-6-phosphatase. As a positive control, glucose-6-phosphatase activity also was determined in liver and kidney tissues. The activity of mammary tissue (Table 3) represents less than 1% of that in liver or kidney tissue from these same lactating cows. Similarly, Keenan et al. (20) have reported only a negligible activity of this enzyme in endoplasmic reticulum isolated from bovine mammary tissue. Smith et al. (30) also have reported an absence of glucose-6-phos-
TABLE 2. Substrate utilization for lactose synthesis, fatty acid synthesis, and CO₂ production by lactating cow mammary tissue slices incubated with 1 mM glucose and 10 mM acetate concentrations.

<table>
<thead>
<tr>
<th>Incubation media</th>
<th>Lactose synthesis</th>
<th>Fatty acid synthesis</th>
<th>CO₂ production</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1⁴C-acetate + glucose</td>
<td>&lt; 5</td>
<td>4675 ± 770</td>
<td>3770 ± 760</td>
</tr>
<tr>
<td>U-1⁴C-glucose + acetate</td>
<td>120 ± 20</td>
<td>&lt; 5</td>
<td>280 ± 70</td>
</tr>
<tr>
<td>U-1⁴C-alanine + acetate + glucose</td>
<td>&lt; 5</td>
<td>15 ± 5</td>
<td>360 ± 90</td>
</tr>
<tr>
<td>U-1⁴C-glutamate + acetate + glucose</td>
<td>&lt; 5</td>
<td>20 ± 10</td>
<td>1000 ± 200</td>
</tr>
<tr>
<td>U-1⁴C-lactate + acetate + glucose</td>
<td>&lt; 5</td>
<td>120 ± 25</td>
<td>510 ± 100</td>
</tr>
<tr>
<td>2-1⁴C-pyruvate + acetate + glucose</td>
<td>&lt; 5</td>
<td>40 ± 5</td>
<td>250 ± 90</td>
</tr>
<tr>
<td>2-1⁴C-glycerol + acetate + glucose</td>
<td>60 ± 10</td>
<td>5 ± 0</td>
<td>260 ± 40</td>
</tr>
</tbody>
</table>

a All incubations contained 1 mM glucose, 10 mM acetate, .1 units/ml insulin, and 1 μCi/incubation of radioactive substrate (as indicated). Other substrates were included as indicated at 5 mM concentrations.

b Results are expressed as nmoles radioactive substrate incorporated into product/100 mg wet tissue per 3 h. Values represent means ± SE from four mammary glands.

phatase activity in rabbit mammary tissue. Although the activities of pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and fructose-1,6-diphosphatase generally are lower than in ruminant liver and kidney tissue, these enzymes are in cow mammary tissue. Substantial activities of phosphoenolpyruvate carboxykinase and fructose-1,6-diphosphatase had been reported in cow mammary tissue (2). Rabbit mammary tissue has fructose-1,6-diphosphatase activity similar to the cow, but only slight activities of this enzyme are in the mammary tissue of rat and guinea pig (2).

DISCUSSION

Gluconeogenic substrates are not converted to glucose by incubations of cow mammary tissue regardless of whether the incubations contained an optimum (10 mM) or suboptimum (1 mM) glucose concentration. If cow mammary tissue had the ability to utilize substrates which enter the gluconeogenic pathways as pyruvate or TCA cycle intermediates, certainly gluconeogenesis from these substrates should have occurred with the low glucose media. Consistent with the inability of cow mammary tissue to convert gluconeogenic metabolites to glucose is the virtual absence of the final enzyme in this pathway, glucose-6-phosphatase (Table 3; 12, 20). Furthermore, the uptakes of most gluconeogenic amino acids, especially glutamate and aspartate, are not in excess of their utilization for milk protein synthesis. Therefore, the gluconeogenic amino acids would not be available for glucose synthesis in the cow mammary gland (11). Additional glutamate, proline, and aspartate must be synthesized to meet demands for synthesis of milk protein (11).

In light of these results with cow mammary tissue and the lack of any obvious reason for a difference between cow and goat mammary metabolism, the results of Mepham and Linzell
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(25) are difficult to understand. These studies involved a single goat infused with radioactive glutamate in which the specific activity of glucose was determined in the arterial and venous blood. Since the specific activity of glucose was substantially higher in the venous blood leaving the mammary gland than in the arterial blood entering the gland, these workers concluded the mammary gland was incorporating radioactive glutamate into glucose which subsequently passed from the mammary cell into the venous blood (25). In view of the tremendous mammary gland requirement for glucose in the ruminant (13) and the results of our studies, it does not seem logical that the mammary gland would synthesize glucose from noncarbohydrate precursors for export (via blood circulation).

If gluconeogenesis in cow mammary tissue was occurring, it probably would involve the synthesis of glucose-6-phosphate which would be utilized for the production of lactose. Gluconeogenic substrates (alanine, glutamate, lactate, and pyruvate) were not incorporated into lactose in any detectable amounts (Tables 1 and 2). However, these substrates were being oxidized since radioactivity was recovered in CO2.

Glycerol was incorporated into lactose (Tables 1 and 2), which is consistent with the fact that glycerokinase (21) and fructose-1,6-diphosphatase (Table 3; 2) are in cow mammary tissue. Normally, glycerol would not be available in sufficient quantities to furnish carbon for lactose synthesis in the cow mammary gland with the amount of glycerol absorbed being sufficient to produce only about one-half of the α-glycerol phosphate required for milk lipid synthesis (6).

Fructose-1,6-diphosphatase in cow mammary tissue may be involved predominately in the recycling of triose phosphate (originating from the pentose phosphate cycle) back to glucose-6-phosphate as suggested by Smith (29) and Bauman and Davis (6) and demonstrated by the in vivo tracer experiments of Wood et al. (31). Presumably, in ruminant mammary tissue the only triose phosphate which would not be recycled would be that utilized for α-glycerol phosphate formation (necessary for fatty acid esterification) and that metabolized via the Embden-Meyerhoff pathway for the production of oxaloacetate (necessary as a primer for the TCA cycle) (6, 29). The extensive recycling of triose phosphate in ruminant mammary tissue is yet another example of ruminant tissue adaptation to spare glucose.

Pyruvate carboxylase, an anapleotic enzyme, presumably would function in cow mammary tissue to synthesize the oxaloacetate primer required for the normal operation of the TCA cycle. Our results substantiate the work of Baird (2) that phosphoenolpyruvate carboxykinase is in cow mammary tissue (Table 3). However, in view of the lack of gluconeogenesis from TCA cycle metabolites in cow mammary tissue, the physiological role of phosphoenolpyruvate carboxykinase is not clear. Phosphoenolpyruvate carboxykinase activity is not detectable in rabbit mammary tissue (2) and apparently has not been determined in the mammary tissue of other species. Perhaps phosphoenolpyruvate carboxylase also functions to generate oxaloacetate primer in cow mammary tissue. The conversion of phosphoenolpyruvate to oxaloacetate via phosphoenolpyruvate kinase would generate a GTP whereas a similar conversion via pyruvate kinase and pyruvate carboxylase yields no net energy.

The results of substrate utilization for fatty acid synthesis in cow mammary tissue (Tables 1 and 2) are consistent with the pathways of fatty acid synthesis in ruminant mammary tissue. In ruminant mammary tissue, acetate is the major source of carbon for lipogenesis. Substrates which are converted to acetyl CoA in the mitochondria are not utilized to any significant extent because ruminant mammary tissue lacks two key enzymes (ATP-citrate lyase and NADP-malate dehydrogenase) which are involved in the translocation of mitochondria acetyl CoA to the cytosol (see review by Bauman and Davis (6)).

REFERENCES

4 Bartley, J. C., and A. L. Black. 1966. Effect of