It was established by Shaw and Knodt (1) that the normal lactating udder (in situ) of the cow utilizes almost 2 mg. of \( \beta \)-hydroxybutyric acid per 100 ml. of blood traversing the udder; it was calculated that this was sufficient to account for the short chain fatty acids of milk fat or for about 40 per cent of the oxygen consumption of the secreting gland if it were oxidized for energy purposes. Later work on ketotic cows (2) and perfused udders (3) showed that the utilization of this substrate by the lactating udder is greatly increased when the arterial levels are high. However, the ultimate fate of this substance within the mammary gland tissue remained uncertain.

The importance of the lower aliphatic acids in ruminant metabolism has come to be recognized as a result of current interest in the metabolic activities of rumen microorganisms. In ruminants, acetate, propionate and butyrate are primary products of carbohydrate digestion and very little glucose, as such, is absorbed from the gut. The diurnal variations in the arterial levels of acetate are appreciable and the utilization of acetate by the udder is dependent on the arterial levels (4). Acetate is primarily incorporated by the perfused lactating udder into the volatile fatty acids characteristic of the ruminant milk fat (5). Butyrate, when injected intravenously, is incorporated to a lesser extent into milk fat, and participates in a net synthesis of carbohydrate in the ruminant (6, 7).

This is a report on the incorporation of carboxyl-labeled \( \beta \)-hydroxybutyrate into milk components by a perfused udder, and on the effect of acetate on the utilization of \( \beta \)-hydroxybutyrate by the perfused udder. A summary of some of the results of this study has been reported.1

**EXPERIMENTAL**

Lactating bovine mammary glands were isolated and perfused by the procedure described earlier (8, 9). One-half of the udder was dissected out and perfused with heparinized blood, while the other half was used as a control.

The volatile fatty acid content of the blood was determined by steam distillation according to Scarisbrick (10) and calculated as acetate. The \( \beta \)-hydroxybutyrate in the blood was determined by the method of Weichselbaum and Somogyi (11). The volatile fatty acid content of the mammary tissue was measured by rendering the tissue macerate slightly alkaline and extracting it with boiling water. The extract was analyzed by the Scarisbrick procedure. It was our experience that the blood samples for acetate determination could not be kept beyond 6 hours, even in ice; the use of preservatives, such as thymol or fluoride, interfered with the analysis.

The utilization of acetate by the perfused lactating mammary gland was first studied by adding known amounts of acetate to the perfusate, initially, and determining blood and tissue acetate at the end of the perfusion. The difference in acetate content between the perfused tissue and the control tissue, if positive, was assumed to be acetate taken up by the gland but not metabolized. Usually, this amount was very small.

In order to study the possible competition between the two substrates, arteriovenous differences, rather than total utilization, were used as the criteria. \( \beta \)-Hydroxybutyrate was added to the blood to give a concentration of 30 to 50 mg./100 ml. Perfusion of the half udder was begun, and one passage of the entire quantity of the blood was allowed in order to bring about equilibrium between blood and tissue. Three sets of arterial and venous samples were drawn simultaneously in the next three successive passages of blood for the determination of the uptake (arteriovenous difference) of \( \beta \)-hydroxybutyrate. Acetate was then added to the blood to obtain a concentration of 10 to 20 mg./100 ml. One complete passage of the blood was allowed for equilibrium and then three sets of arterial and venous samples were drawn for analysis in as many passages of blood. Usually, however, only two sets of samples could be analyzed for acetate since the third set could not be analyzed within the desired 6-hour time period in most cases.

Five perfusions were conducted to form the preliminary work for experiments on possible competition. Since they may be of interest to students of perfusion, the observations made in the course of this work may be summarized without a detailed discussion. Unless an adequate perfusion rate was maintained, equilibrium between blood and tissue was difficult to establish. In perfusions with a good rate of blood flow, one passage of blood sufficed to establish equilibrium. Nonlactating udders showed no uptake of acetate once equilibrium was established. This is similar to observations on the utilization of \( \beta \)-hydroxybutyrate by the perfused udder (3). When acetate was added to the blood, approximately 1 hour after the beginning of perfusion,
acetate was utilized in essentially a normal manner, the arterio-
venous difference being usually 5 to 0 mg./100 ml. of blood per
passage.

**Tracer Perfusion**—The lactating mammary gland of a cow of
known history was perfused in the usual manner. At the
beginning of perfusion, 2.33 gm. of β-hydroxybutyrate labeled
with C14 in the carboxyl group (racemic mixture synthesized by
Tracerlab, Inc., Boston) was added to the blood. The total
activity added was 1 mc. and the specific activity in the counting
system was 3.5 x 104 c.p.m. per μmole. The gland was perfused
for 2 hours and 360 ml. of milk was recovered from the udder at the end of perfusion with the aid of an injection of oxtoxin
(10 i.u.) in the arterial side of the apparatus. The milk was
fractionated essentially according to the procedure of Dimant
*et al.* (12). The lactose was recrystallized five times from
methanol until constant specific activity was reached.

**Fractionation of Milk Fat for Butyric Acid**—Approximately 1.0
gm. of the milk fat was used for the C14 assay of the individual
lower fatty acids. Butyric acid was first separated by the
procedure of Keeney (13). The forerun and the butyric acid
band were titrated separately, rendered alkaline and evaporated
to dryness (7.75 mole per cent butyric acid). Four drops of
20 N H2SO4 were added to the sodium butyrate extracted with a
mixture (1:1) of ether and petroleum ether. The solvent was
removed in a vacuum, and the acid dissolved in 1.0 ml. of chloro-
form and put through the silica gel column for lower fatty acids
shown in Table I. It will be noted that the utilization of acetate
was appreciable and relatively uniform. The volatile fatty acids
in Table I represent the minimal quantity of acetate metabolized.
The actual rates obtained during perfusion are expressed as percentages of the estimated rates in
vivo.

The data on acetate utilization by the perfused udder are
shown in Table I. It will be noted that the utilization of acetate
was appreciable and relatively uniform. The volatile fatty acids
measured in the tissue after perfusion, though calculated as
acetate, consisted of higher acids as well; therefore, the figures
in Table I represent the minimal quantity of acetate metabolized.

**Preparation of Higher Volatile Fatty Acids**—The sodium salts
of the acids other than butyric acid were dissolved in 200 ml. of
water to which 100 gm. of magnesium sulfate was added. Two
ml. of 20 N H2SO4 were then added and the solution was distilled
until mass crystallization occurred. The volatile fatty acids
(but soluble and insoluble in water) were titrated, rendered
 alkaline with NaOH, and evaporated. The sodium salts were
then dissolved in a small quantity of water, made acidic with
sulfuric acid, and extracted from liquid with a mixture (1:1) of
ether and petroleum ether. Better yields were obtained by
liquid-liquid extraction than by adding anhydrous sodium sulfate
to the solution followed by extraction. The solvent was evapo-
rated in a vacuum and the acids were fractionated by the Ramsey-
Patterson (14) procedure for higher volatile fatty acids. Seven
gm. of silica were employed, a procedure which was found to
require 7.2 ml. of 98 per cent methanol, 0.3 ml. of the dye solution,
and 4 drops of 1.0 N ammonia for the best separation and re-
covery. The individual acids were rechromatographed to con-
stant specific activity.

**Treatment of Higher Acids**—The nonvolatile acids remaining
in the residue after distillation were extracted with ether and the
solvent evaporated off on a water bath. The acids were
separated into saturated and unsaturated fatty acids by a modifi-
cation of the Twitchell procedure (15). The higher fatty acids
were not further fractionated in view of their low specific activity.

**Assay for C14**—All assays for radiocarbon were made with a
windowless gas flow counter and a standard scaler. The solutions
were prepared for counting by transferring aliquots contain-
ing not more than 200 μg. of materials into aluminum
planchets (1½ inches diameter) and drying under an infrared
lamp. While it was assumed that this would give "infinitely
thin" samples, usually all planchets were made up so as to con-
tain approximately the same weight of material in each. The
fatty acids were counted as the sodium salts, the lactose was
counted as such and casein was dissolved in 1.0 N HCl and
counted in a plastic planchet.

**Rate of Blood Flow**—By use of the data of Shaw *et al.* (16) the
rates of blood flow through the udder in *vivo* were estimated from
the levels of milk production. The actual rates obtained during
perfusion are expressed as percentages of the estimated rates in
vivo.

**RESULTS**

The data on acetate utilization by the perfused udder are
shown in Table I. The data were obtained from the perfusion
of bovine udders as shown in Table I. It will be noted that the utilization of acetate
was appreciable and relatively uniform. The volatile fatty acids
measured in the tissue after perfusion, though calculated as
acetate, consisted of higher acids as well; therefore, the figures
in Table I represent the minimal quantity of acetate metabolized.

The rates of blood flow in the four perfusions reported in
Table I were relatively low (20 to 50 per cent of the estimated
rate in *vivo*), although they compare well with those in the
literature. The data are presented to illustrate the relative
uniformity of acetate uptake by the gland even at such rates of
perfusion.

In the three perfusions in which competition was studied, the
rates of blood flow approached that estimated for intact udders

**TABLE I**

*Utilization of acetate by perfused bovine udders*

<table>
<thead>
<tr>
<th>Perfusion No.</th>
<th>Duration of perfusion</th>
<th>Volume of blood used</th>
<th>Times blood passed through perfused half*</th>
<th>Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min.</td>
<td>ml.</td>
<td>mg./100 ml.</td>
<td>In blood</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>120</td>
<td>6</td>
<td>3</td>
<td>33.8</td>
</tr>
<tr>
<td>7</td>
<td>95</td>
<td>6</td>
<td>6.8</td>
<td>54.9</td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>3</td>
<td>5.4</td>
<td>62.8</td>
</tr>
<tr>
<td>10</td>
<td>130</td>
<td>7</td>
<td>5.9</td>
<td>37.4</td>
</tr>
</tbody>
</table>

* Calculated from blood flow rates.


### Table IIA

**Summary of three udder perfusions indicating competition between acetate and β-hydroxybutyrate**

<table>
<thead>
<tr>
<th>Perfusion No.</th>
<th>Mean rate of blood flow as percentage of estimated rate</th>
<th>Mean uptake of β-hydroxybutyrate</th>
<th>Mean acetate uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg./100 ml.)</td>
<td>(mg./100 ml. blood)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Before adding acetate</td>
<td>After adding acetate</td>
<td>Before adding acetate</td>
</tr>
<tr>
<td>76</td>
<td>75 %</td>
<td>5.2 (3)*</td>
<td>1.4 (3)</td>
</tr>
<tr>
<td>80</td>
<td>80 %</td>
<td>5.3 (3)</td>
<td>0.5 (2)</td>
</tr>
<tr>
<td>82</td>
<td>82 %</td>
<td>3 % (3)</td>
<td>0.8 (1)</td>
</tr>
</tbody>
</table>

Mean: ........................ 4.8 ........................ 1.0 ........................ 5.6

Standard deviation of the mean: ±0.25 ........................ ±0.15 ........................ ±0.12

* Numbers in parentheses refer to number of arteriovenous differences.

### Table IIIB

**Effect of addition of acetate on utilization of β-hydroxybutyric acid by perfused udder (perfusion 82)**

<table>
<thead>
<tr>
<th>Perfusion time</th>
<th>Rate of blood flow</th>
<th>Arterial</th>
<th>Venous</th>
<th>Difference</th>
<th>Acetic acid in blood</th>
<th>Arterial</th>
<th>Venous</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>min. 0</td>
<td>ml./min.</td>
<td>mg./100 ml.</td>
<td>mg./100 ml.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>800</td>
<td>56.7</td>
<td>54.3</td>
<td>2.4</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>1000</td>
<td>53.4</td>
<td>49.8</td>
<td>3.6</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1100</td>
<td>47.4</td>
<td>43.9</td>
<td>3.5</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>1300</td>
<td>42.2</td>
<td>37.2</td>
<td>5.0</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Arterial acetate was too low to be measured (less than 0.05 mg./100 ml.).
† 2 gm. of sodium acetate were added at 52 minutes perfusion time.

### Table III

**Specific activities of milk components isolated from milk recovered after perfusion of isolated bovine udder with 1 mc. of CH₃CHOH.CH₂COOH in the blood**

<table>
<thead>
<tr>
<th>Component</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor in blood..........</td>
<td>315 × 10⁶ c.p.m./µmole</td>
</tr>
<tr>
<td>Milk fat</td>
<td>501 c.p.m./mg.</td>
</tr>
<tr>
<td>Casein</td>
<td>399 c.p.m./mg.</td>
</tr>
<tr>
<td>Lactose</td>
<td>52 c.p.m./µmole</td>
</tr>
<tr>
<td>Total VSFA* from milk fat</td>
<td>770 c.p.m./µmole</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>590 c.p.m./µmole</td>
</tr>
<tr>
<td>Caproic acid</td>
<td>1118 c.p.m./µmole</td>
</tr>
<tr>
<td>Caprylic acid</td>
<td>1396 c.p.m./µmole</td>
</tr>
<tr>
<td>Capric acid</td>
<td>1226 c.p.m./µmole</td>
</tr>
<tr>
<td>Lauric acid†</td>
<td>19 c.p.m./µmole</td>
</tr>
<tr>
<td>Longer chain acids..........</td>
<td>Negligible</td>
</tr>
</tbody>
</table>

* Volatile soluble fatty acids.
† Volatile insoluble fatty acids.

The presence of acetate depresses the uptake of β-hydroxybutyrate by the perfused bovine udder (Tables IIA and IIIB). The limitations and advantages of the perfusion technique are too well recognized to merit detailed discussion here. A study of the present type is not readily conducted without the aid of the perfusion but the application of these findings to the behavior of the udder in situ in the live cow must be made with the proper reservations.

β-Hydroxybutyrate is present in the normal bovine arterial blood to the extent of 3 to 6 mg./100 ml. Normally there is no marked diurnal variation in the blood levels of β-hydroxybutyrate (1), whereas arterial acetate levels exhibit diurnal tides of considerable magnitude (4). The competition between acetate and β-hydroxybutyrate may be a mechanism whereby acetate may be utilized efficiently in spite of the large diurnal tides in the blood. Butyrate appears to participate in the net synthesis of carbohydrate in the ruminant by pathways not involving acetate (6, 7); hence an additional advantage of such competition might be in the sparing of the glucose precursors in an animal that has no direct dietary source of glucose. The low specific activity of lactose (Table III) indicates that there is no appreciable carbohydrate synthesis from four carbon intermediates in the isolated udder or that the carboxyl group is lost during the synthesis.

Since glucose is the precursor of lactose, the differences between the data recorded herein and those reported by Kleiber et al. (6) and McCarthy et al. (7) can best be explained on the basis that C₄ intermediates are converted to carbohydrate elsewhere.

The data are consistent with the beliefs that the substrate was reduced directly to butyrate and that the C₂ pool in the perfused udder is negligible in size (5). The C₂ moieties of β-hydroxybutyrate could not have been utilized identically; a preferential utilization of the carboxyl end of the molecule for lipogenesis (17) is the possible explanation for the higher specific activities of the C₆, C₈, and C₁₀ compared to the C₂ volatile fatty acids of the milk fat. Laurie and higher acids were negligibly labeled;
this is in contrast with the work of Cowie et al. (5) on acetate, where appreciable labeling was found up to and including palmitic acid. Popjak et al. (18) suggested, on the basis of earlier studies by Shaw and Knodt (1), that \( \beta \)-hydroxybutyrate was perhaps the \( \text{C}_4 \) precursor for the volatile fatty acids of milk which was even more important than acetate. The work of Kleiber et al. (6) and McCarthy et al. (7) would preclude butyrate as such from being this precursor; it is now certain that this precursor is \( \beta \)-hydroxybutyrate.

The diminution in the molar percentage of short chain fatty acids of low ruminant milk fat during fasting (15) or during ketosis (16) is not attributable simply to the absence of dietary acetate and the change takes place in the presence of high levels of \( \beta \)-hydroxybutyrate in blood. It seems logical to assume that mammary lipogenesis from \( \text{C}_2 \) fragments is inhibited by fasting, by a mechanism similar to that operating in the inhibition of hepatic lipogenesis (19) by fasting. This assumption is strengthened by the fact, now well recognized, that the molar percentage of butyrate in bovine milk fat is not reduced as markedly or as quickly by fasting as the molar percentage of caproic, caprylic, and capric acids (15), indicating that, while mammary lipogenesis from \( \text{C}_2 \) units is inhibited, direct reduction of \( \beta \)-hydroxybutyrate can maintain butyrate levels relatively high in the milk fat. This is further strengthened by the observation by Keeney (13) that the molar percentage of butyrate in commercial butter is relatively uniform in spite of the wide fluctuations in the Reichert-Meissl value (which is an arbitrary measure of the \( \text{C}_4 \) and \( \text{C}_6 \) fatty acids) and the Polenske value (which is an arbitrary measure of the volatile insoluble \( \text{C}_8 \), \( \text{C}_{10} \), and \( \text{C}_{12} \) volatile fatty acids).

**SUMMARY**

The carboxyl carbon of \( \text{C}^{14} \)-labeled \( \beta \)-hydroxybutyrate was incorporated appreciably into milk fat and casein by the perfused lactating bovine udder. Negligible incorporation was found in lactose. The volatile fatty acids of milk fat were labeled in such a manner as to indicate an asymmetrical utilization of the substrate, the carboxyl end being preferentially utilized.

The uptake of \( \beta \)-hydroxybutyrate from the blood by the perfused lactating bovine udder was shown to be depressed by the presence of acetate; acetate was taken up preferentially.

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Soma Kumar, S. Lakshmanan and J. C. Shaw


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