has been applied to these results, and certain discrepancies have been observed. It is suggested that these might be due to competition for diphosphopyridine nucleotide and this possibility has been tested by the use of added diphosphopyridine nucleotide and nicotinamide.

4. From a comparison of the relative rates of oxidation of the C-1 atom of glucose and the methyl atom of acetate it is concluded that most of the $C_{(1)}$ atoms must be catabolized by a route other than glycolysis, this route probably being the direct oxidative pathway.

5. If the direct oxidative pathway is therefore quantitatively important in this tissue the depressing effect of pyruvate on the rate of oxidation of the $C_{(1)}$ atom of glucose is anomalous. Possible reasons for the effect are discussed.

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Studies on the Metabolism of Mammary Tissue *in vitro*

2. OXIDATION OF ACETATE AND GLUCOSE BY SLICES OF LACTATING SHEEP MAMMARY GLAND

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Earlier work in which the manometric technique was used has shown that ruminant mammary tissue metabolizing acetate as the only substrate has a respiratory quotient (R.Q.) above unity (about 1.2), which is increased (to about 1.4) by the addition of glucose (Folley & French, 1950). In a medium containing only glucose as substrate, however, the R.Q. was found to be less than unity. These results were interpreted as meaning that the tissue is virtually inert to carbohydrate, whereas acetate can supply both the carbon and the energy for fatty acid synthesis. The possibility was recognized, however, that in the presence of both substrates glucose carbon might also be used. Later work in which isotopes were used showed that lactating-sheep mammary tissue actively incorporates the carboxyl carbon of acetate into fatty acids when it is the only substrate, and even more actively when glucose is also present (Balmain, Folley & Glascock, 1952). Further (Balmain, Folley & Glascock, 1954), it was found that sheep mammary slices use about 30 times as much

acetate carbon as glucose carbon for fatty acid synthesis when both substrates are present in the medium.

As noted in Part 1 of this series (Duncombe & Glascock, 1956), the manometric technique does not indicate the relative amounts of each substrate oxidized when more than one is present, and in the later work only isotope incorporation into fatty acids was measured. Similar experiments to those carried out on lactating-rat mammary tissue have therefore been carried out on sheep tissue. Some of this work has already been communicated in preliminary form (Duncombe & Glascock, 1953), and the delay in accumulating sufficient results for this paper has been due chiefly to the fact that lactating-sheep tissue can be obtained only in the spring.

EXPERIMENTAL

Animals. All the ewes were in their first lactation and each with a single lamb. They were killed by shooting through the brain on the 21st to 28th day of lactation.

Substrates. As before, the labelled substrates used were doubly labelled sodium acetate ($^{14}\text{CH}_3$, $^{13}\text{CO}_2\text{Na}$) of specific activity 40 $\mu\text{mc}/\text{mg.}$ of methyl C and ^{13}C abundance 68 atom % excess in carboxyl carbon; uniformly ^{14}C -labelled and 1- ^{14}C -labelled glucose of specific activity 8.5 $\mu\text{mc}/\text{mg.}$ Unlabelled sodium acetate, sodium pyruvate and glucose were also used. The concentrations used in the media were: glucose 300 mg./100 ml. (0.0187 M); sodium acetate and sodium pyruvate, 0.02 M. The specific activity of the CO_2 obtained by combustion of the substrates was determined with the same gas counter as was used to measure the specific activity of the CO_2 produced in the experiments. This was of the order of 200 counts/min./ml. (about 0.15 $\mu\text{mc}/\text{ml.}$) of CO_2 in the experiments with labelled glucose and 1000 counts/min./ml. (about 0.7 $\mu\text{mc}/\text{ml.}$) of CO_2 in the experiments with labelled acetate. In the latter the ^{13}C abundance in the CO_2 was from 1 to 4 atoms % excess.

Incubation. The udder tissue was sliced with a hand microtome and batches of slices (0.5 g.) were incubated for 3 hr. at 37° with shaking in bicarbonate-saline (Krebs & Henseleit, 1932), in which the necessary substrates had been dissolved; the gas phase was $\text{O}_2 + \text{CO}_2$ (95:5). The procedure was exactly as described for rat mammary tissue (Duncombe & Glascock, 1956). Briefly, it consists in the use of an incubation unit fitted with stopcocks and standard joints. At the end of the incubation the unit is attached to a high-vacuum apparatus, acid is introduced through a side arm and the liberated CO_2 separated, measured, and the abundance of isotopes in it determined. ^{14}C was determined with a gas counter and ^{13}C determined in the mass spectrometer. The whole procedure has been described in detail by Glascock (1954).

RESULTS

The rate of substrate oxidation is reported here as $\mu\text{g.}$ of carbon/mg. dry wt. of tissue/hr., and is designated in the tables by the symbol q with the appropriate subscripts. Thus $q_{\text{CO}_2\text{H}}$ and q_{Me} refer to the rates of oxidation of the carboxyl and methyl-carbon atoms of acetate; q_{Ac} refers to the rate of oxidation of acetate total carbon (so that $q_{\text{Ac}} = q_{\text{CO}_2\text{H}} + q_{\text{Me}}$); q_{Gt} refers to the rate of oxidation of glucose total carbon and $q_{\text{G-1}}$ to that of the $\text{C}_{(1)}$ atom of glucose. In the tables, L and R refer to left and right udder halves of the same animal.

Oxidation of glucose

Although the absolute level of oxidation of glucose total carbon is quite variable between animals, there is, in nearly all experiments, a well-marked depression when pyruvate is added (Table 1). By contrast the oxidation of the $\text{C}_{(1)}$ atom of glucose is unaffected (Table 2).

The oxidation of both glucose total carbon and the C-1 atom is increased several times on the addition of acetate (Tables 3 and 4). The apparently low metabolic activity of animal 80 is attributed to the abnormally high fat content of the tissue.

Table 1. *Effect of pyruvate on the oxidation of glucose total carbon by lactating-sheep mammary slices*

Expt. no.	Pyruvate absent q_{Gt}	Pyruvate present q_{Gt}
54	1.64	0.89
	2.19	0.91
	2.06	0.90
Mean	1.96	0.90
80 L	0.103	0.048
	0.103	*
80 R	0.118	0.042
	0.065	0.093
Mean	0.109	0.061

* Sample lost.

Table 2. *Effect of pyruvate on the oxidation of the glucose $\text{C}_{(1)}$ atom by lactating-sheep mammary slices*

Expt. no.	Pyruvate absent $q_{\text{G-1}}$	Pyruvate present $q_{\text{G-1}}$
54	0.19	0.21
	0.21	0.21
	0.20	0.18
Mean	0.20	0.20
80 L	0.015	0.017
	0.014	0.017
80 R	0.034	0.043
	0.017	0.028
Mean	0.020	0.025

Table 3. *Effect of acetate on the oxidation of glucose total carbon by lactating-sheep mammary slices*

Expt. no.	Acetate absent q_{Gt}	Acetate present q_{Gt}
30 L	1.89	3.33
30 R	1.39	2.94
31	1.51	3.24
32 L	1.08	3.44
32 R	1.11	2.60
33 L	2.31	11.20
33 R	1.24	4.83
Mean	1.50	4.51

Table 4. *Effect of acetate on the oxidation of the glucose $\text{C}_{(1)}$ atom by lactating-sheep mammary slices*

Expt. no.	Acetate absent $q_{\text{G-1}}$	Acetate present $q_{\text{G-1}}$
53	0.22	0.93
	0.20	1.08
	0.23	0.84
	0.24	0.93
Mean	0.223	0.945
80 L	0.015	0.029
	0.014	0.046
80 R	—	0.028
	0.017	0.023
Mean	0.0153	0.0315

Oxidation of acetate

As will be seen from Table 5, the addition of glucose to the incubation medium considerably increased the rates of oxidation of both carbon atoms of acetate. These were affected about equally, so that although q_{Ac} rose from 0.783 to 1.145 the ratio q_{CO_2H}/q_{Me} was unaltered.

In the work on rat tissue (Duncombe & Glascock, 1956) the formula of Strisower, Kohler & Chaikoff (1952) was used to calculate the degree of dilution of isotopic tricarboxylic acid cycle intermediates which occurs by an influx of unlabelled molecules during the metabolism of labelled acetate. This is given by

$$x = \frac{(q_{CO_2H}/q_{Me} - 1)}{(q_{CO_2H}/q_{Me} + 1)} \times 100,$$

where x , the degree of dilution, is defined by the statement that after dilution $x\%$ of the molecules of cycle intermediates have been derived from unlabelled molecules. On applying this equation to the results in Table 5, it is found that x has values of 19.0 and 17.0 in the absence and presence of added glucose. Thus, whereas in rat tissue the effect of glucose was to depress the rate of oxidation of acetate and to increase the degree of dilution (x) in sheep tissue there is an increase in the rate of acetate oxidation and virtually no change in the degree of dilution.

Effect of pyruvate. Pyruvate has an effect quite different from that of glucose (Table 6). The rate of oxidation of both carbon atoms of acetate is depressed so that q_{Ac} is reduced from 0.783 to

Table 5. *Effect of glucose on acetate oxidation by lactating-sheep mammary slices*

Expt. no.	Glucose absent			Glucose present		
	q_{CO_2H}	q_{Me}	q_{CO_2H}/q_{Me}	q_{CO_2H}	q_{Me}	q_{CO_2H}/q_{Me}
30 L	2.17	—	—	2.45	—	—
30 R	0.61	—	—	1.56	—	—
31	0.89	—	—	1.34	—	—
32 L	1.34	—	—	2.22	—	—
32 R	1.17	—	—	1.56	—	—
33 R	1.44	—	—	2.78	—	—
57	0.540	0.390	1.38	0.590	0.450	1.31
	0.560	0.400	1.40	0.950	0.690	1.38
	0.420	0.290	1.45	0.520	*	—
	0.500	0.340	1.47	0.730	0.550	1.33
75 L	0.278	0.180	1.54	0.611	0.426	1.44
	0.284	0.175	1.62	0.364	0.233	1.56
75 R	0.629	0.414	1.52	0.883	0.599	1.47
	0.514	0.344	1.49	0.628	0.452	1.39
Mean†	0.466	0.317	1.48	0.659	0.486	1.41
Mean† q_{Ac}		0.783			1.145	
x		19.0			17.0	

* Sample lost.

† Mean Expts. 57, 75 L and 75 R.

Table 6. *Effect of pyruvate on acetate oxidation by lactating-sheep mammary slices*

Expt. no.	Pyruvate absent			Pyruvate present		
	q_{CO_2H}	q_{Me}	q_{CO_2H}/q_{Me}	q_{CO_2H}	q_{Me}	q_{CO_2H}/q_{Me}
57	0.540	0.390	1.38	0.490	0.270	1.81
	0.560	0.400	1.40	0.410	0.230	1.78
	0.420	0.290	1.45	0.480	0.280	1.71
	0.500	0.340	1.47	*	—	—
75 L	0.278	0.180	1.54	0.362	0.190	1.91
	0.284	0.175	1.62	0.269	0.146	1.84
75 R	0.629	0.414	1.52	0.258	0.142	1.82
	0.514	0.344	1.49	0.368	0.168	2.19
Mean	0.466	0.317	1.48	0.377	0.204	1.86
Mean q_{Ac}		0.783			0.581	
x		19.0			30.1	

* Sample lost.

0.581. At the same time the ratio q_{CO_2H}/q_{Me} is increased from 1.48 to 1.86, the corresponding values of x being 19.0 and 30.1.

DISCUSSION

The appreciable rate of oxidation of glucose by sheep mammary tissue found here is in contrast to the findings of Folley & French (1950), whose respiratory experiments indicated that this tissue is almost inert to glucose in the absence of other substrates. It is found further that the addition of pyruvate considerably depresses the rate of oxidation of glucose total carbon while having no effect on the rate of oxidation of the $C_{(1)}$ atom. On this evidence alone it might be concluded that glucose is catabolized by the direct oxidative pathway (Dickens, 1953) in which the conversion of the $C_{(1)}$ atom into carbon dioxide does not involve pyruvate. However, consideration of the relative rates of oxidation of glucose total carbon and the $C_{(1)}$ atom place serious difficulties in the way of accepting this mechanism. For each molecule of carbon dioxide derived from the $C_{(1)}$ atom of glucose by the direct oxidative pathway there is left a 5-carbon residue. It is highly improbable that this would be completely oxidized, but, if it were, the ratio of the rate of oxidation of glucose total carbon (q_{Gt}) to that of the $C_{(1)}$ atom (q_{G-1}) could not be more than 6:1. If any of the 5-carbon residue escaped oxidation, this ratio would be smaller. The results show, however (Tables 1 and 2), that the ratio is as much as 10:1 (Expt. 54). Thus a major part of the glucose oxidized cannot be catabolized by way of the direct oxidative pathway. In support of this is the fact (Glock & McLean, 1954 and private communication) that the levels

of enzymes of this pathway are much lower in sheep than in rat mammary tissue.

It thus seems likely that glycolysis may be the major pathway of glucose catabolism in sheep mammary tissue, and indeed Dr C. Terner (private communication) has found evidence for it from manometric experiments. However, several inconsistencies remain to be resolved: the effect of added pyruvate reported here and the fact that glucose is a poor source of C_2 units in this tissue (Balmain *et al.* 1954) would not be expected if glycolysis were the major catabolic pathway. Furthermore, the great increase in the rate of oxidation of glucose on the addition of acetate cannot be accounted for on the present evidence.

The effects of glucose and of pyruvate on acetate oxidation are also inconsistent. If the theory of Strisower *et al.* (1952) is tenable, then the fact that the degree of dilution x (see Results) is not increased on the addition of glucose means that glucose does not give rise to tricarboxylic acid cycle intermediates. Pyruvate, on the other hand, does increase the value of x , implying that it can give rise to such intermediates. This suggests a block between glucose and pyruvate which, again, would not be expected if glycolysis were the major catabolic pathway.

Species differences. The present results, together with those of Part 1 (summarized for convenience in Table 7), give further evidence of the considerable metabolic differences between mammary tissue of rats and sheep. In agreement with earlier work on acetate utilization (e.g. Folley & French, 1949, 1950), it is now found that the rate of acetate oxidation is several times as fast in sheep as in rat mammary tissue; at the same time the effects on acetate oxidation of added pyruvate or glucose are

Table 7. Summary of observed species differences between lactating rat and sheep mammary slices in the oxidation of acetate and glucose, and in the interaction of substrates

Additions	Effect on acetate metabolism	
	Rat tissue	Sheep tissue
Acetate	$q_{Ac} = 0.273$	$q_{Ac} = 0.783$
Acetate + glucose	q_{Ac} reduced by 50%; ratio* increased	q_{Ac} increased by 50%; ratio unaffected
Acetate + pyruvate	q_{Ac} unaffected; ratio increased from 1.54 to 1.93	q_{Ac} reduced by 25%; ratio increased from 1.48 to 1.86
Additions	Effect on glucose metabolism	
	Rat tissue	Sheep tissue
Glucose	$q_{Gt} = 6.5$ $q_{G-1} = 1.03$	$\dagger q_{Gt} = 1.96$ $\dagger q_{G-1} = 0.2$
Glucose + acetate	No effect	q_{Gt} and q_{G-1} increased threefold
Glucose + pyruvate	q_{Gt} and q_{G-1} reduced by a factor of 2-3	q_{Gt} reduced by 50%; q_{G-1} not affected

* Ratio = ratio of rates of oxidation of acetate carbon atoms q_{CO_2H}/q_{Me} .

† Taken from Tables 1 and 2 respectively (results for Expt. 54).

qualitatively different in the two species. By contrast, sheep tissue oxidizes considerably less glucose than does rat tissue, though in the presence of acetate the levels are comparable; as already mentioned, this level of glucose oxidation was not suspected from the results of earlier manometric experiments. The effects on glucose oxidation of added acetate or pyruvate are also very different in the two species. It is now clear, however, that glucose must serve as an important source of energy in the lactating mammary gland of the sheep especially when, as in the living animal, acetate is present.

These marked species differences are of particular interest in view of the conclusions drawn by Hirsch, Baruch & Chaikoff (1954) from experiments on lactating-rat mammary gland which were confirmed in this laboratory (see Duncombe & Glascock, 1956). They say: 'The increase in glucose oxidation that results from the addition of glucose to the medium shifts the metabolism of the C_3 intermediate, derived from acetate and pyruvate, from an oxidative fate to one involving synthesis.'

It is now clear that this statement does not apply even approximately to what is observed in sheep tissue under identical experimental conditions. As already noted, the addition of glucose causes an increase in acetate oxidation (Table 5), and also in the rate of synthesis of fatty acids from acetate (Balmain *et al.* 1952). Thus there is no question of a shift in the metabolic fate of acetate; glucose increases its utilization for both synthesis and oxidation.

It may therefore be that the statement of Hirsch *et al.* (1954) is only a brief summary of their observations and is not a precise description of what is believed to occur in the tissue. This seems all the more likely since, as shown by their own work, the decrease in the rate of acetate oxidation consequent on the addition of glucose is, at the most, only about one-tenth of that needed to compensate for the increased rate of fatty acid synthesis.

SUMMARY

1. The rates of oxidation of the two carbon atoms of sodium acetate, glucose total carbon and the $C_{(1)}$ atom of glucose have been measured in lactating-sheep mammary slices in the presence and absence of other substrates.

2. The rates of oxidation of the two acetate carbon atoms are increased by about 50% on addition of glucose to the medium, the ratio of their rates of oxidation being unaffected. Pyruvate, on the other hand, causes a reduction in the rate of acetate oxidation and an increase in the ratio of the rates of oxidation of the two carbon atoms (q_{CO_2H}/q_{Me}).

3. The formula of Strisower *et al.* (1952) applied to these results indicates that whereas pyruvate can give rise to tricarboxylic acid cycle intermediates, glucose cannot do so and suggests that there is a metabolic block between glucose and pyruvate in this tissue.

4. Studies on the oxidation of glucose in the presence and absence of pyruvate, however, do not confirm this conclusion, neither do they indicate that the direct oxidative pathway is a major route of glucose catabolism.

5. The effect of acetate on glucose oxidation has also been investigated, and is found to increase it threefold, glucose and acetate thus increasing the rate of oxidation of each other.

6. The metabolic behaviour of sheep mammary tissue now observed is compared with that of rat mammary tissue previously reported.

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