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The Metabolism of Acetate and Glucose by the Isolated Perfused Udder

2. THE CONTRIBUTION OF ACETATE AND GLUCOSE TO CARBON DIOXIDE AND MILK CONSTITUENTS*

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Recent work has emphasized that both glucose and acetate are important metabolites in the ruminant. Annison & Lindsay (1961) estimate that 33% of expired CO_2 is derived directly from acetate in the fed sheep, and Annison & White (1961) find that, under these conditions, 23% comes from glucose; both these values are sensitive to the nutritional state of the animal. Considerable quantities of both glucose and acetate are taken up by the goat udder *in vivo* [Linzell (1960); some of the values for glucose uptake in this paper were wrong and an erratum has been published in *J. Physiol.* (1962), **163**, 1], but it is not easy to determine in the whole animal the relative amounts of CO_2 coming from each substance in the metabolism of the udder itself (Wood, Gillespie, Hansen, Wood & Hardenbrooke, 1959). It is technically easier in the isolated organ because (*a*) the addition of substrates can be controlled, (*b*) radioactive substances can readily be infused for long periods, so that steady-state levels are more likely to be reached, (*c*) the blood flow is known, and (*d*) the metabolism of only one organ is involved.

Although glucose is not a significant precursor of milk fatty acids in the ruminant (Balmain, Folley & Glascock, 1954; Kleiber *et al.* 1955), considerable amounts of milk citrate come from glucose in the cow (Kleiber *et al.* 1955; Tombropoulos & Kleiber, 1961), and half the CO_2 from glucose may be produced by routes other than the pentose phosphate pathway (Black, Kleiber, Butterworth, Brubacher & Kaneko, 1957*a*). These discordant results suggest that the relationship between glucose, acetyl-CoA and the tricarboxylic acid cycle in the ruminant udder is not clear.

Further work (D. C. Hardwick, J. L. Linzell & S. M. Price, unpublished work) to elucidate the metabolic routes of these substances by using inhibitors and substituents for glucose gave results that were difficult to interpret. In the presence of acetate and amino acids, the following substances were tested as glucose substitutes: lactose, galactose, mannose, fructose, ribose, glycerol, pyruvate, β-hydroxybutyrate, propionate, fumarate and malate. None supported milk secretion, and only mannose and propionate caused an oxygen uptake similar to that with glucose. Lactose, galactose, fructose, pyruvate and β-hydroxybutyrate were taken up by the gland, but at a rate lower than that for glucose. Transport into the cell or, in some cases, phosphorylation may have been limiting for many of these substances. With inhibitors, although milk secretion was decreased by potassium cyanide, 2,4-dinitrophenol and sodium fluoride, other inhibitors (e.g. fluoroacetate and fluorocitrate) could not be assessed because they had non-specific effects on other tissues of the gland (e.g. they caused vasoconstriction and oedema).

Hardwick, Linzell & Price (1961) have shown that in the isolated udder maximal milk production of normal composition depends on both glucose and acetate; it was not clearly shown, however, that lactose and fat were synthesized from the added glucose and acetate. We have therefore now studied the transfer of ^{14}C from [^{14}C]glucose and [^{14}C]-acetate, not only to CO_2, but also to lactose, triglyceride fatty acids, triglyceride glycerol and citrate in the milk formed *in vitro* by the perfused udder.

METHODS

Treatment of mammary glands. Mammary glands were taken from Welsh goats in early lactation (3–8 weeks) giving 22–34 ml./hr./gland. The perfusions and general analysis were carried out as described by Hardwick & Linzell (1960) and Hardwick *et al.* (1961), with modification to enable the total respired $^{14}CO_2$ to be collected. For this purpose the Hooker-type oxygenators were replaced by a single membrane-type 'lung' (Peirce, 1960) to provide an enclosed gas circuit and ensure that the venous blood reached the lung without any prior gas exchange. The membrane 'lung' was fed by a stromuhr-type pump that also measured the venous blood flow (Kisin & Tsaturov, 1961). The oxygen, supplied from a spirometer, was circulated through the 'lung' and two CO_2 traps in a closed circuit. (The first CO_2 trap, containing 200 ml. of 0·1 N-NaOH, was changed hourly, and the second, containing 200 ml. of N-NaOH, was checked at the end of the experiment.) The membrane 'lung' did not always supply sufficient oxygen and had to be supplemented from time to time by the Hooker-type oxygenator which was not completely gas-tight. To ensure that exchange of CO_2 between the blood and artificial kidney fluid did not vitiate the measure-

* Part 1: Hardwick, Linzell & Price (1961).

ments, the 'kidney' was excluded from the circuit during some periods of the experiment. Neomycin (10 μg./ml.) and penicillin (100 units/ml.) were used in the perfusion fluid and artificial kidney fluid because they gave a wider antibacterial cover than the streptomycin and penicillin previously used.

The glands were starved for the first 2 hr. to remove as much of the endogenous substrates and intermediary metabolites as possible. After the second hour a substrate mixture was infused into the perfusion fluid continuously at a rate appropriate for the amount of milk being formed. The mixture consisted of glucose, acetate and amino acids approximately in the proportions taken up by the gland *in vivo* (Hardwick & Linzell, 1960); to this mixture was added either uniformly labelled [^{14}C]glucose ([U-^{14}C]-glucose) (20 μc/100 ml.; 6·63 μmc/mg. of glucose C) or [2-^{14}C]acetate (10 μc/100 ml.; 10 μmc/mg. of acetate C).

Carbon dioxide. The CO_2 output was determined every hour by titrating the NaOH in the first trap between pH 9·0 (phenolphthalein) and pH 3·3 (bromophenol blue). The contents of the second trap were titrated at the end of the experiment; about 10% of the CO_2 was trapped here.

A portion of the 0·1 N-NaOH from the first trap was acidified with HCl and the CO_2 evolved was trapped as $BaCO_3$ in 3 ml. of 0·1 N-$Ba(OH)_2$. The $BaCO_3$ was centrifuged, washed twice with water and suspended in ethanol. The CO_2 from samples of perfusion fluid was similarly evolved and collected with the use of cetyl alcohol to prevent frothing. Suspensions of $BaCO_3$ were plated out on 5 cm.^{2} aluminium planchets and dried, and the $BaCO_3$ was determined both by weighing and, after counting, by dissolving in an excess of 0·05 N-HCl and back-titrating with 0·01 N-NaOH with methyl red as indicator. The radioactivity of the respired gas was also monitored by passing the gas from the 'lung', before absorption in caustic soda, through a 1 l. flask in which were mounted a matched pair of Geiger–Müller tubes. The counting efficiency of this was 0·12%.

Acetate. This was isolated from plasma and kidney fluids by steam-distillation at acid pH (bromophenol blue), determined by titration and counted as described by Annison & Lindsay (1961).

Glucose. This was determined with glucose oxidase (Huggett & Nixon, 1957) and isolated as the phenylhydrazone (Baxter, Kleiber & Black, 1955), which was determined by its extinction at 390 mμ in ethanol, and this solution was dried on a planchet for counting.

Lactose. This was isolated, from 2 ml. of milk, as described by Lucas, Kaneko, Hirohara & Kleiber (1959). The first precipitation of whey proteins was omitted and the heating at pH 6·8–7 was at 100°. The cation- and anion-exchange resin columns were each 8 cm. × 0·6 cm. Lactose was further purified by chromatography on Whatman 3MM paper by using propan-2-ol–water (4:1, v/v) and eluting with water; it was determined by copper reduction with the reagents of Somogyi (1952) and Nelson (1944).

Citrate. This was eluted from the anion-exchange resin used, as described above, for de-ionizing the lactose. The elution was performed with a formic acid gradient made by running 6 N-formic acid into 100 ml. of water in a 100 ml. flask; the flow rate was about 0·5 ml./min. Citrate appeared between 60 and 90 ml. of effluent. The content of the fractions was determined by the method of McArdle (1955). The citrate was further purified by precipitation of the sodium salt from ethanol, and, in some cases, by rechromatographing the acid on Dowex 2 resin as above.

Fatty acids. The milk fat was extracted in a Soxhlet apparatus with ether–light petroleum (b.p. 40–60°) (1:1, v/v) either from 2 ml. of milk previously dried at 95° or from the dried casein precipitate obtained during the separation of lactose and citrate. This was hydrolysed with 5 ml. of methanolic 12% (w/v) KOH for 3 hr. under reflux on a boiling-water bath, and the residue was evaporated to dryness on a water bath. After dissolution in 5 ml. of water, the solution was acidified with about 1 ml. of 10 N-H_2SO_4, and the fatty acids and non-saponifiable material were extracted with ether which was washed well with water. The aqueous layer was preserved for glycerol extraction (see below). The fatty acids were extracted with N-NaOH, and the aqueous phase was washed with ether and acidified. The acid aqueous phase was extracted with ether which was washed well with water. This extraction method was used to ensure that no acetate was counted in the fatty acid fraction, so that any contamination of the milk by blood or tissue fluid would not interfere with the measurements. The recovery of [^{14}C]acetate added to milk fat was less than 0·2%. Some propionate and butyrate may also have been lost. The fatty acids were determined by weighing and by titrating 50–500 μg. in 2 ml. of methanol against aq. 0·01 N-NaOH in a Conway burette with phenol red as indicator. Normally these observations gave an equiv. wt. of about 230, which agrees with published values (Macy, Kelly & Sloan, 1953). In the acetate experiments the acids were divided into volatile and non-volatile fractions by steam-distilling 20–50 mg. of material in a 10 ml. flask for 20–30 min.; about 30 ml. of distillate was collected. The flask and residual fluid were extracted with ether to recover the non-volatile acids, and the condenser and distillate similarly treated to obtain the volatile acids. Determinations were made as above. The non-volatile acids gave an equiv. wt. of about 260, but the volatile acids often had an equiv. wt. higher than that calculated from published data on goat-milk fatty acids, suggesting the presence of a non-acidic impurity. Since the quantities were small it was decided to calculate the weight of material from the titration and an equiv. wt. of 135.

Glycerol. The specific radioactivity was determined after separation as the tribenzoate (Black *et al.* 1957*a*). This was filtered, washed with water and recrystallized from ethanol by adding 4 vol. of water. The ester was determined by its extinction at 275 mμ in ethanol. This method of benzoylating glycerol was not always successful.

Counting. All substances were counted on 5 cm.^{2} aluminium planchets in a Nuclear–Chicago gas-flow counter with a Micromil window. The counter efficiency was calculated to be 30%. Counts were corrected to infinite thinness by using curves for individual substances. Specific radioactivities are expressed as counts/min./mg. of C to ensure that the values are comparable for the various substances measured.

Calculations. The total counts in any one product (specific radioactivity × mg. of C in product) up to time t divided by the total counts added as precursor (specific radioactivity × mg. of C in precursor) up to time t and multiplied by 100 is referred to as the 'precursor quotient'. When the specific radioactivities of the precursor and product are constant this is a measure of the percentage of the precursor forming the product. In our experiments

such steady states were not always reached or maintained. We have therefore used the whole period of infusion for the calculation, and because of the slow appearance of radioactivity in the milk the estimates are minimal.

In contrast with this, the ratio of specific radioactivities of product to precursor (multiplied by 100) when precursor and product have constant specific radioactivities can be used to measure the percentage of product coming from the precursor. This is referred to as the 'product quotient', and is similar to the 'transfer quotient' of Kleiber (1954).

RESULTS AND DISCUSSION

Two experiments with [U-^{14}C]glucose and two with [2-^{14}C]acetate were done. Table 1 shows the precursor and product quotients in all four experiments and illustrates the essential similarity of the corresponding pairs of results. The detailed results of the two better experiments are given in Figs. 1 ([U-^{14}C]glucose) and 2 ([2-^{14}C]acetate), and results quoted in the text are calculated from these. The findings will be discussed under four headings: the production of CO_2, the synthesis of lactose, the synthesis of fatty acid and of glycerol, and the relation of glucose and acetate to the tricarboxylic acid cycle.

(1) *Carbon dioxide.* The specific radioactivity of CO_2 reached a constant level in experiments with both acetate and glucose, so that the ratios of specific radioactivities of CO_2 to those of precursors ('product quotients') are valid measures of the proportion of CO_2 coming from each substance. Thus, whereas about 40 % of expired CO_2 came from glucose, only 14 % came from acetate. The 'precursor quotient' suggests that 12 % of added glucose, but only 2·5 % of acetate, appeared as CO_2. However, these values are unreliable because total CO_2 output was not measured; during periods when the 'kidney' was excluded, accumulation of Na^+ ions from sodium acetate probably bound significant quantities of CO_2 in the plasma, a fact overlooked at the time. Table 1 also gives values calculated from the average CO_2 production in isolated perfused udders (36 observations) and may be more correct. In any case these are, of necessity, minimum figures, because of the time lag of 2–3 hr. between the entry of glucose or acetate into the udder and the reaching of steady-state levels of CO_2 specific radioactivity. The size of the initial pool of intermediates will affect the time required to reach the steady state, but not the value of the steady-state level, which is controlled by the relative entry of labelled and unlabelled precursors. We tried to reduce the initial pool size by starving the gland for 2 hr. Clearly at steady state 40–50 % of the CO_2 produced was from neither the glucose nor the acetate added. Possible sources of unlabelled carbon are the added amino acids (by

Table 1. *Transfer of* ^{14}C *from uniformly labelled* [^{14}C]*glucose and* [2-^{14}C]*acetate to carbon dioxide and milk constituents*

Four perfusion experiments were carried out. Experimental details are given in the text. In Expt. 128 some unlabelled glucose (29 % of the total) was infused before the [U-^{14}C]glucose. In Expt. 130 the gland was anoxic between 1·5 and 5·5 hr. owing to partial 'lung' failure; the labelling of citrate was higher during this period. (The value in parenthesis is calculated from the specific radioactivity of the milk citrate obtained during the period of anoxia.)

Labelled substrate	[U-^{14}C]Glucose		[2-^{14}C]Acetate	
Expt. no.	128	134	130	136
Period of infusion (hr.)	4th–10th	2nd–8th	2nd–9th	2nd–11th
	Product quotients (percentage of product formed from precursor)			
CO_2	47	39	14	14
Lactose	49	77	0·1	0·27
Glycerol	9·7	> 23	0·57	> 2
Total fatty acids	0·15	0·47	> 34	> 48
Volatile fatty acids	—	—	> 42	90
Non-volatile fatty acids	—	—	> 20	> 34
Citrate	30	41	44 (72)	33
	Precursor quotients (percentage of precursor transferred to product)			
CO_2 (measured)	5	11·7	4·5	2·5
CO_2 (estimated from average CO_2 output)	12·5	12	10·5	5
Lactose	5·8	25·3	0·035	0·17
Glycerol	0·08	0·27	0·02	0·06
Total fatty acids	0·02	0·1	10·7	13·1
Citrate	0·1	0·38	0·54	0·5

transamination) and tissue stores of fat and carbohydrates. It was calculated that such a CO_2 production in a 10 hr. experiment represents less than 1% of the weight of the gland tissue. The utilization of unlabelled material can be variable, as illustrated in Fig. 2; between 5 and 9 hr. there was a fall in the specific radioactivities not only of products such as CO_2 and citrate but also of that of plasma acetate.

The greater amount of CO_2 coming from glucose than from acetate is consistent with the high uptake of glucose by lactating glands of conscious goats. From the finding of Black *et al.* (1957*a*) (and by assuming the validity of their calculations) and the present results, it may be calculated that 13% of the total CO_2 may come from added glucose via the tricarboxylic acid cycle; thus Black *et al.* (1957*a*) calculated that at least 50% of glucose-generated CO_2 comes from the pentose phosphate pathway in the cow, and, if the rest comes from the glycolysis route, one-third is produced before the tricarboxylic acid cycle and two-thirds within it; thus from our results two-thirds of one-half of 40%, i.e. 13%, of all the CO_2 produced comes from glucose via the tricarboxylic acid cycle. It seems likely therefore (on the assumption made by Black *et al.* 1957*a*) that glucose and the methyl carbon of acetate supply approximately equal amounts of the total CO_2 produced by the tricarboxylic acid cycle. It is possible that the carboxyl carbon of acetate may contribute more carbon to CO_2 than does the methyl carbon, because of possible exchange and synthetic reactions at the α-oxoglutarate level, which would dilute the methyl carbon after the carboxyl carbon had been released as CO_2 (Weinman, Strisower & Chaikoff, 1957). The results of Black, Kleiber, Smith & Stewart (1957*b*) suggest that such dilution is of minor importance.

(2) *Synthesis of lactose.* The importance of glucose as a precursor of lactose was first suggested by Foa's (1912) work with perfused sheep udders, and demonstrated by Folley, Popják & Malpress (1952) in the rabbit and by Barry (1952) in the goat. The present results showed that this is also true for the isolated perfused udder. The 'product quotient' shows that 77% of the lactose produced came from added glucose, whereas only 0·3% came from added acetate. The specific radioactivity of the plasma glucose fell during the experiment with [U-^{14}C]glucose, indicating a dilution by unlabelled glucose. Part at least of this dilution is accounted for by the influx of unlabelled glucose from the kidney, which was in circuit for 30 min. during the sixth

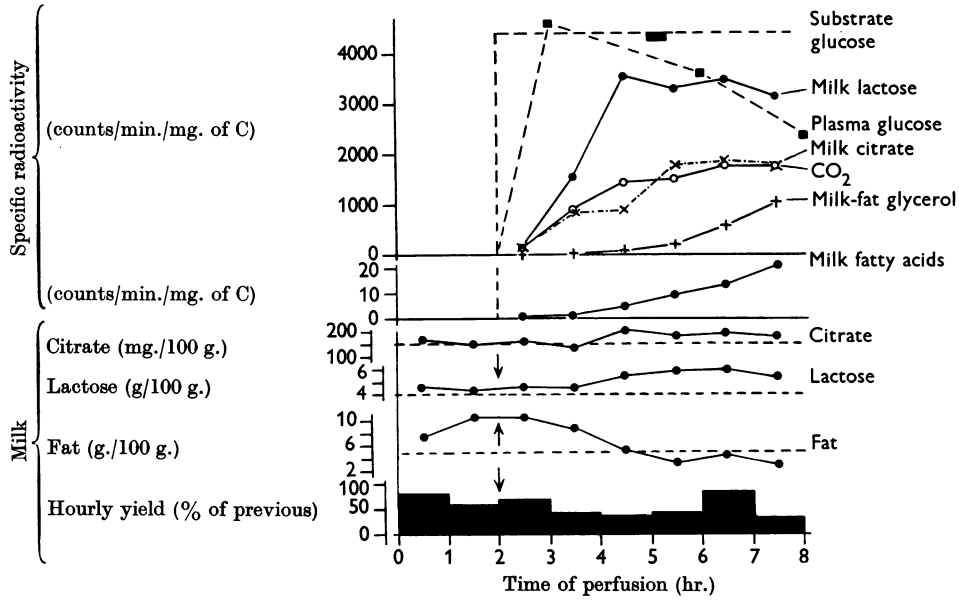


Fig. 1. Perfusion of one goat mammary gland with [U-^{14}C]glucose (Expt. 134). The gland, weighing 360 g., yielded 22 ml. of milk/hr. before the experiment (average of 7 days, shown as 100% on the Figure). The infusion of substrates with [U-^{14}C]glucose was started at 2 hr. In the next 6 hr. 9·75 g. of glucose and 3·4 g. of acetate were taken up. Some non-labelled glucose entered the circuit in the sixth hour (black bar). The mean blood flow during perfusion was 39 ml./100 g./min.; the O_2 consumption was about 2·2 ml./100 g./min., at maximum. The horizontal broken lines for milk composition represent the average contents for 3 days before the experiment, and those for specific radioactivity the level of radioactivity of the infused glucose.

hour of perfusion. If the 'product quotient' is calculated from the plasma specific radioactivity, rather than from that of the infused substrate, 100% of lactose came from plasma glucose (Fig. 1); 25% of the added glucose was converted into lactose, the highest 'precursor quotient' observed in these experiments. The specific radioactivity was constant after the third hour, suggesting small pools of intermediates relative to the rate of lactose secretion.

(3) *Synthesis of fat.* The importance of acetate as a precursor of fatty acids in the ruminant udder was first demonstrated in the whole animal by Popják, Folley & French (1951), and the same group showed that glucose was not an important precursor of fatty acids (Balmain *et al.* 1954).

(*a*) Fatty acids. The present results also show that acetate was an important precursor of fatty acids, whereas negligible quantities of glucose were incorporated into these compounds, the reverse of the findings with lactose. The 'product quotients' indicate that at least 50% of the total fatty acids were derived from acetate, whereas 0·5% came from glucose. Constant specific radioactivity was not reached so that the values are minimal. It is unlikely that the results with [1-^{14}C]acetate would be different, because the C_2 moiety is not believed to be broken during fatty acid synthesis. The volatile fatty acids, unlike the non-volatile fraction, did reach a constant specific radioactivity which was equal to that of the acetate being given; thus 90–100% of the volatile fatty acids came from acetate in our perfusion (Fig. 2).

It should be emphasized that these experiments were done in the absence of added triglycerides and higher fatty acids. When whole blood is used, relatively small quantities of these substances are available from this source, and variable amounts will be present in the udder, though it is not certain how much of this is available for milk fat. The 'product quotient' indicates that at least 30% of the non-volatile fatty acids can be synthesized from added acetate *in vitro*, but we do not yet know whether all the fatty acids present in this fraction are labelled.

The cow udder takes up neutral fat from the blood *in vivo* (Lintzel, 1934), and the findings of Riis, Luick & Kleiber (1960) suggest that 50% of the fatty acids of the milk come from colloidal fatty material in the plasma. On the other hand, in

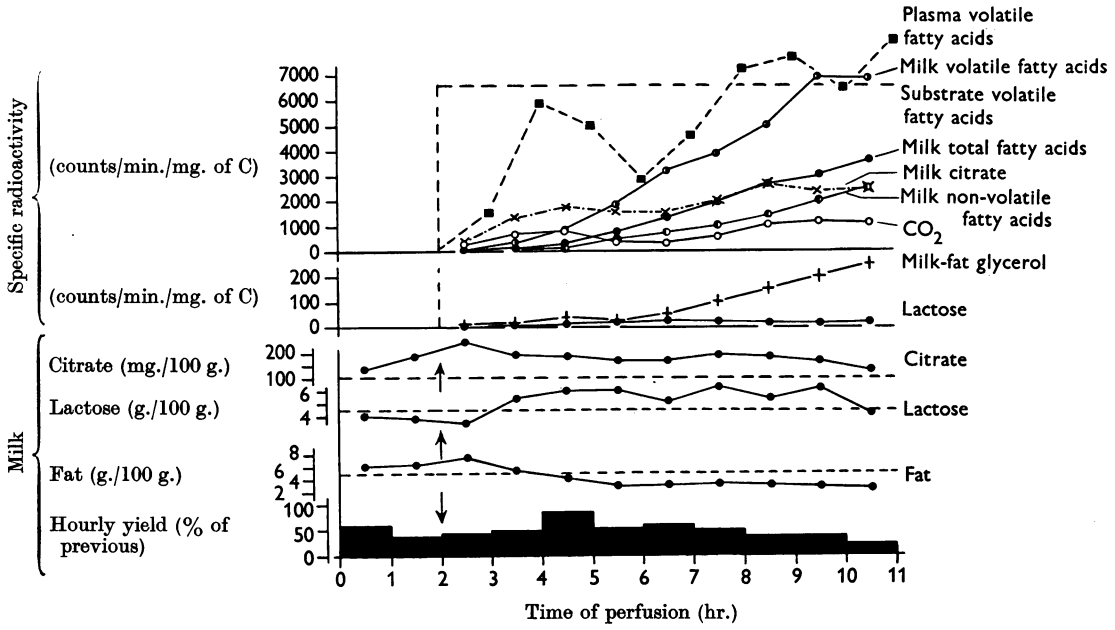


Fig. 2. Perfusion of one goat mammary gland with [2-^{14}C]acetate (Expt. 136). The gland, weighing 413 g., yielded 34·4 ml. of milk/hr. before the experiment (average of 7 days, shown as 100% on the Figure). The infusion of substrates with [2-^{14}C]acetate was started at 2 hr. In the next 9 hr. 28·2 g. of glucose and 9·6 g. of acetate were taken up. The mean blood flow during perfusion was 65 ml./100 g./min.; the O_2 consumption was about 4 ml./100 g./min. at maximum. The horizontal broken lines for milk composition represent the average contents for 3 days before the experiment, and those for the specific radioactivity the level of radioactivity of the infused acetate.

the isolated perfused udder, without added triglyceride, D. C. Hardwick, J. L. Linzell & P. V. F. Ward (unpublished work) have shown that milk triglyceride secreted in the presence, or even the small quantity secreted in the absence, of acetate does not differ markedly in fatty acid composition from that of normal milk. Ganguly (1960) suggests that the cow udder is capable of synthesizing all the milk fatty acids from acetate, but he did not distinguish between C_{16} and C_{18} acids. Such ability would be surprising, in view of the findings by Popják, French, Hunter & Martin (1951) that [^{14}C]acetate contributed largely to acids up to C_{16} but little to C_{18} acid synthesis for milk fat in the whole goat.

(*b*) Glycerol synthesis. Glucose is generally accepted as the likely precursor of milk-fat glycerol, but Glascock (1958) concluded that this does not take place in the mammary gland whereas Luick (1960) considered that it does, although Luick & Kleiber (1961) indicated that this is difficult to prove in the whole animal. We found that the perfused udder can indeed synthesize glyceride glycerol from glucose; at least 23 % came from glucose but only 2 % from acetate. The rate of rise in glycerol specific radioactivity resembled that of fatty acids: in both cases very little radioactivity appeared in the first 3–4 hr., after which the specific radioactivity rose throughout the experiment and did not reach steady state in 8 hr., in contrast with the curves for lactose, CO_2 and citrate. The much slower rise compared with that of lactose indicates that the gland has a larger store of milk triglyceride or precursors than of lactose or precursors either in the cells or in the alveoli. The similarity between the glycerol and fatty acid curves suggests either that the pools are of triglyceride or that the glycerol and fatty acid-precursor pools are of similar size. In addition the initial period of 3–4 hr. when little or no radioactivity appeared in the fat may also mean that there is a pool of preformed triglyceride, possibly in the alveoli, with which newly synthesized material does not readily mix.

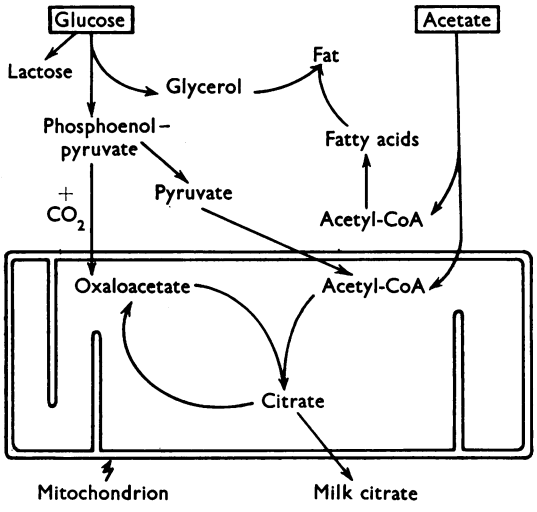
(4) *Formation of citrate.* Kleiber *et al.* (1955) were the first to demonstrate that glucose contributes to milk citrate in the whole cow. In our experiments both glucose and acetate contributed to the synthesis of milk citrate, and the 'product quotient' shows that approx. 30 % of this substance came from each precursor (Figs. 1, 2). This is consistent with the relative quantity of carbon from glucose and acetate estimated to be entering the tricarboxylic acid cycle on the basis of CO_2 labelling. This similarity in citrate labelling was in marked contrast with the different extents of fatty acid synthesis from acetate and glucose. Our results in the ruminant may be compared with those of Spencer & Lowenstein (1962) in a non-ruminant. They observed incorporation of [^{14}C]citrate into fatty acids in the supernatant fraction of rat-mammary-gland homogenates.

Both fatty acids and citrate are generally believed to be synthesized from acetyl-CoA and, if both glucose and acetate contribute to these compounds solely via acetyl-CoA in the mammary gland, then the ratios of the specific radioactivities of milk fatty acid and citrate should be the same, whether the glucose or the acetate of the substrate mixture is labelled. That this is not so suggests that the assumptions made, in particular that acetyl-CoA is the sole precursor of these substances and that the cell contains an homogeneous pool of acetyl-CoA, should be examined.

Since citrate is synthesized from oxaloacetate and acetyl-CoA, glucose may contribute to citrate via either or both of these substances. The rate at which new oxaloacetate (or other C_4 acid) is required is determined by losses of cycle acids from the system either by synthetic reactions or into the milk. According to Deuel (1957) and Lindsay (1959) glucose is the most likely source of such C_4 acids. Utter & Keech (1960) have shown that mammalian liver will carboxylate pyruvate to oxaloacetate (cf. Utter, 1959), and Freedman & Nemeth (1961) have obtained evidence for a similar reaction in young guinea-pig liver. The reaction has not been demonstrated in mammary tissue but is included in Scheme 1 to indicate a possible route from glucose to citrate.

The rate of loss of citrate to the milk can be calculated for the normal lactating udder, by assuming that the calculations of Black *et al.* (1957*a*) are approximately correct and that glucose and acetate are the major CO_2 precursors. The result shows that, for every 15 carbon atoms converted into CO_2 by the tricarboxylic acid cycle, only one is lost as milk citrate. However, the CO_2 labelling suggests that glucose and acetate contribute approximately equal amounts of carbon to the cycle, so that the loss of citrate to the milk represents only about two-fifteenths of the carbon contributed by glucose to the tricarboxylic acid cycle. The loss of citrate is not enough therefore to account for the entry of all the glucose, and, if all this glucose enters the cycle via oxaloacetate, there should be other, much greater, losses of cycle acids. Such losses are rendered less likely by the conclusion of Black & Kleiber (1957) that dilution of cycle acids by the influx of C_4 components is slight compared with the turnover in the cycle.

The results presented indicate that glucose and acetate do not both contribute to the same pool of acetyl-CoA. It is possible that pyruvate derived from glucose is decarboxylated to acetyl-CoA at a site where it is not available for the synthesis of milk fatty acids. This possibility is indicated in



Scheme 1. Possible relationships of glucose and acetate metabolism in the lactating goat mammary gland.

Scheme 1 by an intramitochondrial pool of acetyl-CoA synthesized from both pyruvate and acetate and an extramitochondrial pool synthesized only from acetate. The Scheme assumes that milk citrate is made in the mitochondria. This has not been investigated but seems likely, although Srere & Lipmann (1953) have shown the possibility of citrate synthesis in cell-supernatant fractions of pigeon liver.

The two possibilities, synthesis of oxaloacetate from pyruvate and the existence of two intracellular pools of acetyl-CoA, are not mutually exclusive, and Scheme 1 suggests possible metabolic pathways and sites for these. Whether either or both of these suggestions is true, it seems that there is a block between pyruvate and the acetyl-CoA that can be used for fatty acid synthesis. This is supported by two experiments where pyruvate was substituted for acetate in the full substrate mixture in one of two mammary glands. The fat concentration of the milk and the total milk secreted were lower on the side receiving pyruvate than on the side receiving acetate. The result suggests that pyruvate cannot be used for fat synthesis, but this will have to be confirmed by, for example, experiments with [^{14}C]pyruvate. It would also seem to be worth while to investigate whether an enzyme is absent or an inhibitor present.

SUMMARY

1. A substrate mixture of glucose, acetate and amino acids containing either uniformly labelled [^{14}C]glucose ([U-^{14}C]glucose) or [2-^{14}C]acetate has been infused for 6–9 hr. into the perfusion fluid of four isolated perfused mammary glands of lactating goats.

2. In an experiment with [U-^{14}C]glucose, 77% of the milk lactose, 23% of milk triglyceride glycerol, 41% of milk citrate, 0·5% of milk triglyceride fatty acids and 39% of the expired CO_2 came from added glucose.

3. In another experiment with [2-^{14}C]acetate, only 0·3% of the milk lactose, 2% of milk triglyceride glycerol and 14% of expired CO_2 came from added acetate. However, 48% of the milk triglyceride fatty acids, 90% of the volatile fatty acids and about 33% of the milk citrate came from this precursor.

4. The labelling of milk fatty acids by [2-^{14}C]-acetate but not by [U-^{14}C]glucose is contrasted with the equal labelling of milk citrate by both. The possibilities of two acetyl-CoA pools and of the carboxylation of pyruvate are discussed.

5. The slow initial rise in the specific radioactivities of both milk glycerol and fatty acids, as compared with that of lactose, suggests the existence of larger stores of triglyceride and precursors than of lactose and precursors.

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The Arrangement of the Peptide Chains in γ-Globulin

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Although *N*-terminal amino acid analysis of γ-globulin suggested that the number of peptide chains/molecule depended on the species of origin (Porter, 1960), other workers (Edelman, 1959; Edelman & Poulik, 1961; Franěk, 1961; Ramel, Stellwagen & Schachman, 1961) showed that, with all the γ-globulins examined, reduction in 6 M-urea led to a uniform fall in molecular weight from about 150 000 to about 50 000. This evidence indicated that all γ-globulins have the same number of chains/molecule and that therefore some of the *N*-terminal amino acids must be unreactive. The products obtained after reduction in urea are insoluble except in urea solutions, and hence are difficult to fractionate and have lost all biological activity. It was observed, however (Porter, 1962; Fleischman, Pain & Porter, 1962), that, when γ-globulin was reduced with mercaptoethanol in the absence of urea, five out of twenty disulphide bonds were broken, and that, though the molecular weight at neutral pH was unchanged, acidification caused the dissociation into two products which could be separated on Sephadex G-75 columns with 100% recovery and which were soluble and retained biological activity. The molecular weights of the two components (Pain, 1963) indicated that there were two larger chains A and two smaller chains B in a molecule of γ-globulin. As chains A and B retained their antigenic activity their relation to the three pieces formed by papain digestion of γ-globulin (Porter, 1959) could be established and led to the postulation of a diagrammatic structure for γ-globulin (Porter, 1962), shown in Scheme 1. The papain-digestion pieces I and II both contain an antibody-combining site and are considered to be identical in their general structure (Palmer, Mandy & Nisonoff, 1962; Stelos, Radzimski & Pressman, 1962).

We now describe a more detailed characterization of the products of reduction of whole γ-globulin and of the papain-digestion pieces. The results substantiate most of the features of the four-chain structure, the location of papain hydrolysis and the position of the antibody-combining site.

EXPERIMENTAL

Materials

γ-Globulin from horse, human and rabbit serum was prepared by chromatography on diethylaminoethylcellulose (Sober & Peterson, 1958). Some preparations of rabbit γ-globulin were also made by precipitation with Na_2SO_4