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The Incorporation of Carbon Dioxide into Milk Citrate in the Isolated Perfused Goat Udder

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1. Isolated perfused goat udders supplied with glucose, acetate and amino acids were infused for several hours with $NaH^{14}CO_3$. 2. Lactose, milk-fat fatty acids and glycerol had very little radioactivity. The specific radioactivity (counts./min./mg. of C) of milk citrate was 9–16% that of the carbon dioxide in the perfusion fluid and 19% that estimated for tissue carbon dioxide. The specific radioactivity of tissue citrate resembled that of milk citrate. 3. The radioactivity in citrate was predominantly in C-6, suggesting some carboxylation of α-oxoglutarate in addition to carboxylation of C_3 compounds. 4. [1-^{14}C]Glutamate was infused in a similar experiment, and milk citrate radioactivity was predominantly in C-1 + C-5. 5. The results are discussed in relation to the contribution of glucose and acetate carbon to citrate. The implications of the carboxylation of α-oxoglutarate are considered.

Using the isolated perfused goat udder Hardwick, Linzell & Mepham (1963) confirmed that both glucose and acetate contribute significantly to milk citrate, though only acetate was a significant precursor of milk fatty acids. It has been suggested (Black & Kleiber, 1954) that carbon dioxide may enter the tricarboxylic acid cycle by carboxylation of pyruvate or propionate and may, by these routes, be incorporated into citrate in the lactating cow. Thus some of the labelled carbon from radioactive glucose or acetate may get into citrate via carbon dioxide. I have therefore estimated the contribution of carbon dioxide to milk constituents (Hardwick, 1963). Because the contribution of carbon dioxide to citrate was considerable, I have degraded the citrate to locate the activity and found it predominantly in C-6 (see below), a location not to be expected from the carboxylation of a C_3 to a C_4 compound. A further experiment with [1-^{14}C]-glutamate, used as a generator of α-oxo[1-^{14}C]glutarate, was made to test the possible carboxylation of α-oxoglutarate, and the result was in agreement with the possibility.

METHODS

Goat mammary glands were perfused as described by Hardwick, Linzell & Price (1961) by using a closed gas circuit (Hardwick *et al.* 1963). Perfusion fluid (whole blood with 1mg. of bromolysergic acid diethyl amide/l., 10000 units of heparin/l., 10mg. of neomycin/l. and 100000 units of penicillin/l.) was circulated through the artificial kidney in parallel to the circuit through the mammary gland.

Analyses and separation of milk constituents were made as described by Hardwick *et al.* (1963), except that in Expts. 3, 4 and 5 milk and blood CO_2 were transferred to NaOH under vacuum (Annison & Lindsay, 1961) and precipitated as $BaCO_3$, which was separated by centrifugation, washed with 5ml. of water and then 5ml. of 50% (v/v) ethanol, and finally dehydrated with 2ml. of ethanol.

Casein, obtained by precipitation from whole milk at pH4·7, was hydrolysed with 15ml. of a 1:1 (v/v) mixture of conc. HCl and 98% formic acid for 24hr., and the hydrolysate was extracted three times with equal volumes of light petroleum (b.p. 40–60°) to remove fat. Amino acids were isolated on an amino acid analyser (Spackman, Stein & Moore, 1958) and radioactivities measured after desalting by absorbing the amino acids on Zeo-Karb 225 and eluting with 5N-ammonia (J. L. Mangan, personal communication).

Note on citrate carbon numbering. Individual carbons of the citrate molecule are referred to according to the scheme of Freedman & Graff (1958):

$$\underset{1\ \ 2\ \ 3\ \ \ \ 6\ \ \ \ 4\ \ 5}{HO_2C\cdot CH_2\cdot C(OH)(CO_2H)\cdot CH_2\cdot CO_2H}$$

where C-4 and C-5 are derived from acetate, and C-1 and C-6 are lost as CO_2 in the tricarboxylic acid cycle. This numbering is consistent with the usual numbering of glutamate.

Degradation of citrate. Citrate was degraded by the method of Martin & Wilson (1951). A 4mg. portion of unlabelled citrate was added to 0·5–2mg. of citrate isolated from milk or tissue. Recoveries of carbon and counts were 70–100% (Table 2). Negligible quantities (0·05–0·15mg.) of $BaCO_3$ were obtained in the C-1 + C-5 and the C-6 fractions when a blank degradation was done, but the blanks for the C-2 + C-3 + C-4 fraction were high (6–12mg.) and variable. This made calculation of recovery of carbon in this fraction uncertain.

Specific radioactivity. All specific radioactivities were calculated as counts/min./mg. of C. The ratios of specific

radioactivities then measure the proportion of carbon in a product derived from a precursor, assuming that constant specific radioactivity has been reached.

RESULTS

Distribution of ^{14}C *in milk constituents after administration of* $NaH^{14}CO_3$. After four isolated mammary glands from Welsh or Welsh cross goats had been perfused for 2hr. without added substrates, 150μc of $NaH^{14}CO_3$ was added to the artificial kidney fluid (20l.), and 10μc to the perfusion fluid (1·3l). The $NaH^{14}CO_3$ was then infused with the usual substrate mixture at a rate calculated to maintain the specific radioactivity of carbon dioxide in the system at the level reached by the initial loading. The calculation involved an assumed rate of carbon dioxide production by the gland, derived from previous perfusion experiments.

In Expt. 1 the specific radioactivity of the perfusate carbon dioxide remained constant during the first 8hr. of the experiment, then rose; in the other three experiments the value during the first hour of infusion was high and fell during the second hour. The average values of specific radioactivity for the second to the fifth hour of $NaH^{14}CO_3$ infusion (the fourth to the seventh hour of perfusion) were taken for calculation. Individual values showed up to $\pm 35\%$ range about the mean. The individual specific radioactivities of milk carbon dioxide varied less than $\pm 10\%$ from their mean and became constant by the first hour after infusing $NaH^{14}CO_3$, showing that HCO_3^- diffuses rapidly through the tissue. Citrate specific radioactivity became constant 3–5hr. after infusion of $NaH^{14}CO_3$ had begun [cf. Lauryssens, Verbeke, Peeters & Donck (1959), who perfused for 2hr. with a presumably rising specific radioactivity of sodium hydrogen carbonate].

Table 1 gives the final specific radioactivities of carbon dioxide and citrate from milk, venous blood and tissue as percentages of the mean specific radioactivity of carbon dioxide in the arterial perfusion fluid. About 8–16% of the carbon in milk and tissue citrate came from carbon dioxide in the perfusion fluid. The values for lactose, glycerol and fatty acids were too low for accurate measurement, but seemed still to be rising at the end of the experiments. The values for fatty acids confirm the low incorporation in these compounds found by Cowie *et al.* (1951) in a perfused cow udder and by Kleiber, Smith & Black (1952) in the whole cow.

Table 1. *Final specific radioactivities of milk and tissue constituents as percentage of the specific radioactivity of carbon dioxide in arterial blood*

Experimental details are given in the text.

Constituent	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Venous CO_2	—	—	74	91
Milk CO_2	26*	25*	55†	85
Tissue CO_2	—	—	—	94
Milk citrate	10	9	11	16·5
Tissue citrate	9	7·5	—	15·5

* Low value (see the text).
† Oedematous tissue.

In Expts. 1 and 2 the measured specific radioactivity of milk carbon dioxide, 25% that of the carbon dioxide in the perfusion fluid, was underestimated, because of the small quantity (0·1–0·4mg.) of barium carbonate isolated (Hardwick, 1963). In Expts. 3 and 4 specific radioactivities of venous as well as arterial and milk carbon dioxide were measured. Oedema developed after only 2–3hr. in Expt. 3, both hindering the exchange of carbon dioxide and increasing the carbon dioxide pool in the gland. This is reflected in the low values of carbon dioxide specific radioactivity in venous blood and milk relative to those in arterial blood. In Expt. 4, tissue taken at the end of the experiment was quickly minced under paraffin with scissors and the carbon dioxide transferred to sodium hydroxide as above. The specific radioactivity of tissue carbon dioxide was 94% of the value for arterial blood. Thus values for milk carbon dioxide might be considered a valid estimate of those in tissue.

The specific radioactivity of tissue citrate was measured in Expts. 1, 2 and 4. In Expts. 1 and 2 the glands were allowed to cool slowly at $-10°$ and stored at this temperature; in Expt. 4 1–3g. slices of tissue were put immediately into liquid air, then stored at $-10°$. This tissue was subsequently broken with a percussion mortar under liquid air, and extracted at once with 0·35N-perchloric acid (Verbeke, Lauryssens, Peeters & James, 1959). Citrate was separated from the supernatant after removal of protein and potassium perchlorate by using a Dowex 2 column, as in the separation of milk citrate. In all cases the specific radioactivities of tissue citrate were slightly lower than that of the milk citrate.

Distribution of radioactivity within the citrate molecule after administration of $NaH^{14}CO_3$. Table 2 shows the distribution of radioactivity in the three fractions obtained by degradation. C-6 had the most radioactivity, being 1·3–3·4 times that of C-1+C-5. In Expt. 4 the distribution of radioactivity in the citrate isolated from quickly frozen tissue was similar to that in milk. In all cases the radioactivity in the C-2+C-3+C-4 fraction was low.

Carboxylation of α-oxoglutarate. In one experiment (5), after perfusion for 2hr. without addition of substrates, 70μc of DL-[1-^{14}C]glutamate was added to 20l. of kidney fluid (containing approx.

Table 2. *Distribution of radioactivity in milk citrate and tissue citrate isolated in experiments where* $NaH^{14}CO_3$ *was infused, beginning 2 hr. after the start of perfusion*

Experimental details are given in the text.

Expt. no.	Material	Time after start of perfusion (hr.)	Radioactivity (μc) recovered (% of total μc degraded)			Carbon recovered (% of total carbon degraded)		
			C-1+C-5	C-6	C-2+C-3+C-4	C-1+C-5	C-6	C-2+C-3+C-4
2	Milk	4–5	38	51	3	30	14·5	50
	Milk	6–7	30	47	5	17	15	50
3	Milk	6	22	74	1	32	18	19
	Milk	8	25	72	2	34	16	52
4	Milk	3	21·8	59	7·9	21	13·5	40
	Milk	4	11·5	29	6·5	17	12·5	57
	Tissue	10	19	46	12	32	16	55
Milk average		... ...	25	55	4	25	17·6	44·5

Table 3. *Distribution of radioactivity in milk citrate isolated in the experiment when* DL-[1-^{14}C]*glutamate was infused, beginning 2 hr. after the start of perfusion*

Experimental details are given in the text.

Time after start of perfusion (hr.)	Radioactivity (counts/min.) recovered (% of total counts/min. degraded)			Carbon recovered (% of total carbon degraded)		
	C-1+C-5	C-6	C-2+C-3+C-4	C-1+C-5	C-6	C-2+C-3+C-4*
3	70	4	9	23	13·5	37
4	77	7	7	28	22	44
5	52	9	6·5	18	14·5	42
6	75	10	4	26·5	16·7	56
7	70	7·5	3·5	29	10·5	85
8	81	9	5	29	12	45
Average ...	71	7·8	6	25·6	14·9	51·5

* Approximate values (see the text).

1·5g. of L-glutamic acid) and 30μc was added to 300ml. of substrate solution (containing 600mg. of L-glutamic acid), which was infused at 25ml./hr. (2·5μc/hr.). The specific radioactivity was 41000 counts/min./mg. of C. This experiment had to be done with an open gas circuit, allowing ingress of unlabelled carbon dioxide from outside. The specific radioactivity of the carbon dioxide produced by the gland, calculated by the method of Annison, Scott & Waites (1963) from the concentrations and specific radioactivities of arterial and venous carbon dioxide, was 1350 counts/min./mg. of C and that of the milk citrate 520 counts/min./mg. of C. Degradation of the milk citrate (Table 3) shows that radioactivity was predominantly in the C-1+C-5 fraction.

DISCUSSION

The ready diffusion of carbon dioxide through the tissue suggested by the attainment of constant specific radioactivity of carbon dioxide in the milk in less than 1hr. agrees with the findings of Annison & Linzell (1964) in the goat udder *in vivo* and of Soesja (1963) in the rat brain. It is therefore reasonable to assume that the specific radioactivity of milk carbon dioxide is a measure of that of tissue carbon dioxide, although intracellularly this may vary from site to site. The values for tissue citrate (Tables 1 and 2) suggest that no large pool of citrate in the tissue had a very different specific radioactivity from that of milk citrate. Assuming that the values for milk carbon dioxide and citrate represent the values at the site for citrate synthesis, in both Expts. 3 and 4 the specific radioactivity of the citrate was 19·5% of that of the milk carbon dioxide. Thus about 20% of citrate carbon comes from intracellular carbon dioxide. The relation between the specific radioactivities of blood and intracellular carbon dioxide will depend on blood flow, metabolic activity and extent of oedema.

Hardwick *et al.* (1963) showed that 43% of

carbon dioxide carbon and 35% of citrate carbon came from glucose. Therefore 20% of 43%, i.e. 8·6%, of all citrate carbon could have come from glucose carbon via carbon dioxide; (8·6/35) × 100, i.e. 25%, of the carbon in citrate originating in glucose comes via carbon dioxide. Similarly, 20% of 14%, i.e. 2·8%, of all citrate carbon could have come from the methyl carbon of acetate via carbon dioxide; and (2·8/38) × 100, i.e. 7·5%, of the citrate carbon originating in acetate comes via carbon dioxide. Thus the contribution of carbon dioxide is considerable, indicating that a substantial part of the glucose carbon enters the tricarboxylic cycle by carboxylation reactions.

The most likely routes of carbon dioxide incorporation are:

$$\text{Pyruvate} + CO_2 \rightarrow \text{oxaloacetate} \quad (1a)$$

$$\text{Oxaloacetate} \rightleftharpoons \text{fumarate} \quad (1b)$$

$$\text{Propionate} + CO_2 \rightarrow \text{succinate} \quad (2)$$

In reaction (1*a*) the radioactivity would be only in C-1 of citrate; if both reactions (1*a*) and (1*b*) occur radioactivity will be in both C-1 and C-6, and if equilibrium is complete the radioactivity in these two carbon atoms will be equal. Reaction (2) would again give equal activities in C-1 and C-6 of citric acid. Therefore the predominant radioactivity in C-6 (Table 2) cannot be explained by these routes, and the most likely explanation is the reversal of the isocitrate-dehydrogenase reaction. This postulate is strengthened by the result of the experiment with DL-[1-^{14}C]glutamate, where (ignoring any strange metabolism of the D-isomer) a conversion into α-oxoglutarate and decarboxylation to succinate (or direct decarboxylation) would immediately lose the radioactive carbon as carbon dioxide. If decarboxylation were the sole fate of either D- or L-glutamate, radioactivity in citrate, synthesized from oxaloacetate and acetyl-CoA, could come only from carbon dioxide and show the same distribution within the molecule as for the $NaH^{14}CO_3$ experiments. The high radioactivity (Table 3) in the C-1 + C-5 fraction in the experiment with labelled glutamate is most easily explained by carboxylation of α-oxoglutarate to isocitrate.

Decarboxylation of isocitrate is catalysed by two enzymes, one NADP-dependent and reversible, the other NAD-dependent and believed to be irreversible (Plaut & Sung, 1954). Hathaway & Atkinson (1963) have shown that conditions exist in which the NAD-dependent isocitrate dehydrogenases from *Acetobacter* and yeast do catalyse this reverse reaction slowly, and Sanwal, Zink & Stachow (1964) have extended this observation to *Neurospora crassa*. Clearly the NADP-dependent enzyme could account for the labelling in the citrate molecule by an exchange process. Such an exchange was noted by Grisolia & Vennesland (1947) in an acetone-dried powder extract of pigeon liver, and more recently by D'Adamo & Haft (1962) in perfused rat livers. Madsen, Abraham & Chaikoff (1964) have shown the carboxylation of α-oxoglutarate in rat mammary tissue and calculated that 20–30% of the glutamate metabolized in the tricarboxylic acid cycle is carboxylated, provided that glucose is present. In the goat udder the extent of interchange of carbon between glutamate and α-oxoglutarate is suggested by the considerable dilution of casein glutamate ^{14}C compared with that of infused glutamate.

The extent of carboxylation of α-oxoglutarate is considerable; in the $NaH^{14}CO_3$ experiments the ratio of counts in C-6 to counts in C-1 + C-5 is 1·3–3·4. Assuming all the ^{14}C in C-1 + C-5 is in C-1 and that there is complete equilibration between oxaloacetate and fumarate, the carboxylation of α-oxoglutarate accounts for 13–55% of the entry of carbon dioxide into citrate. The ratio of citrate to carbon dioxide radioactivities is rather greater than 1:6, indicating that at least 1 mol. of carbon dioxide is incorporated/mol. of citrate formed. Therefore 1 mol. of carbon dioxide is supplied by the reversal of the isocitrate-dehydrogenase reaction for every 2–8 mol. of citrate formed. At maximum, the reverse reaction is proceeding at half the total forward reaction by both isocitrate dehydrogenases.

The functions of the two isocitrate dehydrogenases has been the subject of much speculation. Lowenstein (1961*a*,*b*) has suggested that the NADP-dependent enzyme present outside mitochondria may provide NADPH for fatty acid synthesis in rat liver, but not rat mammary gland. Estimates based on the work of Annison & Linzell (1964) suggest that, in the goat mammary gland, NADPH output of the pentose phosphate pathway is at least adequate for fatty acid synthesis.

Any excess of NADPH, produced outside mitochondria, could very well transfer its hydrogen to α-oxoglutarate, forming isocitrate or equivalent metabolite, which could carry the hydrogen into mitochondria and there transfer it to NAD^+. For this, α-oxoglutarate or an equivalent substance would have to leave the mitochondrion. Such a reaction might be kinetically possible because McLean (1958) has observed that the NADPH/$NADP^+$ ratio may be 5·5 in the soluble fraction of the lactating rat mammary gland, whereas the NAD^+/NADH ratio is 1·5 in the mitochondria (and 5·2 in the soluble fraction). Unfortunately, the concentrations of citrate, isocitrate and α-oxoglutarate inside and outside mitochondria are not known, although data (Ochoa, 1945) suggest that the isocitrate/α-oxoglutarate ratio would have to be very low outside mitochondria to permit the net back reaction.

The possibility that the reverse reaction is driven by NADPH is strengthened by the finding of Madsen *et al.* (1964) that the carboxylation of α-oxoglutarate is dependent on glucose. This may be because only glucose can produce the NADPH required to drive the NADP-dependent isocitrate dehydrogenase backwards, or possibly because NADPH-dependent fatty acid synthesis is required to remove acetyl-CoA in their system.

Devlin & Bedell (1960) have discussed the possibility that β-hydroxybutyrate is a hydrogen carrier across the mitochondrial membrane of rat liver, and other authors (see Zebe, Delbrüch & Bücher, 1959) have suggested α-glycerophosphate for such a role in rat liver, brain and skeletal muscle. Both these oxidize NADH extramitochondrially. Hurlock & Talalay (1958) have suggested an extramitochondrial transhydrogenase system with 3-hydroxy steroids as hydrogen carriers. The present results do not demonstrate a transhydrogenase system across the mitochondrial membrane, although the extent of carboxylation, the high extramitochondrial NADPH/NADP ratio and the possible excess of production of NADPH combined to make it conceivable. A clear demonstration of its existence in a relatively 'physiological' situation such as the isolated perfused udder is difficult and will ultimately rest on evidence that mitochondria are sufficiently permeable to citrate (or isocitrate) and α-oxoglutarate or suitable derivatives, and that the extra- and intra-mitochondrial concentrations of these and the nicotinamide nucleotides would permit the cycle.

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