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The Fate of Acetyl Groups Derived from Glucose in the Isolated Perfused Goat Udder

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1. In the isolated perfused goat mammary gland, both glucose and acetate contribute 20–30% of carbon to citrate via acetyl-CoA. 2. Carbon dioxide does not contribute significantly to the formation of acetyl-CoA. 3. ATP citrate lyase, which, in rats, yields acetyl-CoA for fatty acid synthesis, is absent from or very low in high-speed supernatant fraction of ruminant tissue. 4. It is reasonable to infer that intramitochondrial acetyl-CoA, to which both glucose and acetate contribute, is separated from extramitochondrial acetyl-CoA to which glucose does not contribute and which is used for fatty acid synthesis.

It is well known that glucose contributes negligible quantities of carbon to fatty acid synthesis in the ruminant udder and the simplest explanation (Folley & McNaught, 1961; Barry, 1959) seemed to be that glucose does not form acetyl-CoA in this tissue, possibly because of the large quantity of acetate available. However, glucose and acetate contribute equal quantities of carbon to milk citrate (Hardwick, Linzell & Mepham, 1963) and there is a considerable incorporation of carbon dioxide into tricarboxylic acid-cycle intermediates (Hardwick, 1965); thus glucose contributes to the formation of citrate via oxaloacetate, but this does not exclude the possibility that glucose could also contribute to the formation of intramitochondrial acetyl-CoA which would not be available for fatty acid synthesis (Spencer & Lowenstein, 1962). This has been tested by degrading the citrate and glutamate from milk during perfusions in which [U-^{14}C]-glucose was given (Expts. 128 and 134 by Hardwick *et al.* 1963); the results show that glucose does indeed contribute to the formation of citrate via acetyl-CoA. Such acetyl-CoA is not used for fatty acid synthesis and I have correlated this fact with the absence of ATP citrate lyase (formerly citrate-cleavage enzyme, EC 4.1.3.8), which could make intramitochondrial acetyl-CoA available for extramitochondrial fatty acid synthesis (Srere & Bhaduri, 1962).

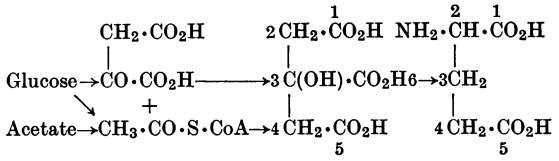
METHODS

Perfusion of goat udders was done as described by Hardwick *et al.* (1963). Glucose, acetate and amino acids were infused in all perfusions at appropriate rates. Milk citrate was isolated as described by Hardwick *et al.* (1963), and degraded as described below. In every experiment the citrate degraded was secreted after the specific radioactivity had reached a constant value.

A preparation containing ATP citrate lyase was obtained from the high-speed supernatant of liver homogenates from Wistar WAG rats given a low-fat diet for 3 days (Kornacker & Lowenstein, 1965). The liver was extracted and the enzyme estimated by the technique of Spencer & Lowenstein (1962). Radioactive citrate from milk was added to the reaction mixture these authors describe (Table VI of their paper) and the acetylhydroxamate thus formed separated by paper chromatography on Whatman no. 531 paper (Stadtman & Barker, 1950). The chromatogram was sprayed with $FeCl_3$ reagent, the acetylhydroxamate spot cut out, the radioactivity measured in a Nuclear–Chicago gas-flow counter with a Micromil window, and the colour extracted and measured. The counting efficiency on this paper was estimated to be 6·7% compared with 15·5% for infinitely thin layers on lens tissue.

The difference in counting efficiency seemed unsatisfactory, and so the method was checked by degrading the citrate in the presence of phosphate acetyltransferase (EC 2.3.1.8) (Novelli, 1955), which converted the acetyl-CoA into acetyl phosphate. Acetate from the hydrolysis of this was isolated as the free acid by steam-distillation (Annison, 1954) and the distillate titrated on an automatic recording titrator (Radiometer, Copenhagen, Denmark). The incubation mixture was: tris buffer, 100mM; cysteine hydrochloride, 10mM; K_2HPO_4, 5mM; $MgCl_2$, 5mM; citrate, 1mM; ATP, 6mM; CoA, 1mM; phosphate acetyltransferase, 1mg./ml.; ATP citrate lyase, 1mg. of protein N/ml.; the final pH was 7·5–7·7. The ready hydrolysis of the acetyl phosphate suggested the presence of an active phosphatase.

Goat tissues were also homogenized and both ATP citrate lyase and acetyl-CoA synthetase (EC 6.2.1.1) measured in the high-speed supernatant ($3 \times 10^6 g$-min.) by the method of Spencer & Lowenstein (1962) after dialysis against the sucrose medium used for homogenizing (0·25M-sucrose for liver, 0·44M-sucrose for mammary gland, both made 10mM with respect to EDTA) for 5–20hr. at 2°. Assays for ATP citrate lyase were also done by the method of Srere (1959). For both assays the enzymes, when present, were shown to depend on ATP (2·4–6μmoles/ml.), CoA (0·4–1μmole/ml.), Mg^{2+} (5μmoles/ml.) and potassium citrate



Scheme 1. Derivation and numbering of carbon atoms in citric acid and glutamic acid.

(6 μmoles/ml.) or sodium acetate (6 μmoles/ml.). Where ATP-citrate-lyase activity was low or negligible, the effect of the extract on the ATP-citrate-lyase activity of rat-liver extract was measured.

Protein was determined by the micro-Kjeldahl technique. Enzyme activity was measured as mμmoles of product formed/min./mg. of protein N.

Glutamate was separated from milk casein hydrolysates and degraded by the Schmidt reaction to give C-5 as CO_2. The resulting 2,4-diaminobutyric acid was then treated with ninhydrin, evolving C-1 as CO_2 (Pigretti & Stoppani, 1961). Separation of glutamate and measurement of CO_2 radioactivity were as described by Hardwick, (1965).

Specification of citrate carbons. Scheme 1 indicates the way citrate carbon atoms are referred to.

RESULTS

Degradation of citrate. Table 1 gives the specific radioactivities of the labelled substrate infused, of the milk citrate and of the acetyl group obtained by the action of ATP citrate lyase on this citrate. It is important to be sure that this enzyme removes C-4 and C-5 (Scheme 1) and not C-1 and C-2. The present results (Table 1) indicate that less than 1% of radioactivity present in citrate and derived from $NaH^{14}CO_3$ is present in the acetyl group removed by ATP citrate lyase. This group contains either C-1 or C-5. Isolation of glutamate from the experiment with $NaH^{14}CO_3$ (Table 2) shows that the radioactivity is substantially in C-1 and not in C-5. Thus the carbon atom of low radioactivity isolated by the action of ATP citrate lyase on citrate in the experiment with $NaH^{14}CO_3$ must be C-5. This confirms the observation by Spencer & Lowenstein (1962) that ATP citrate lyase has the same stereospecificity as citrate synthase.

The experiments with [U-^{14}C]glucose show that the specific radioactivity of the citrate carbon atoms derived from acetyl-CoA is 20–30% of that of the glucose used (last column of Table 1). This is similar to the equivalent ratio in the experiments in which [1-^{14}C]- or [2-^{14}C]-acetate was used and indicates that both glucose and acetate contribute 20–30% of the acetyl groups used in the tricarboxylic acid cycle. The source of the other 40–60% is unknown. The perfused goat udder may resemble the perfused rat heart, in which (Shipp, Matos, Knizely & Crevasse, 1964) endogenous material supplies half the respiratory carbon dioxide when glucose and palmitate are infused.

Degradation of glutamate. I tried to confirm that glucose contributes to citrate via acetyl-CoA by examining the radioactivity of the two carboxyl groups of milk glutamate. Here, because of dilution arising from milk glutamine and added glutamate, the ratio of the specific radioactivities of the two carboxyl carbon atoms should be considered rather than the absolute values. Table 2 gives the specific radioactivities of the whole glutamate molecule and of the two carboxyl carbon atoms. The test degradation of [1-^{14}C]glutamate shows that the Schmidt reaction evolves a negligible proportion (1·5%) of C-1 of glutamate as carbon dioxide, assuming that C-5 has no intrinsic radioactivity. The experiment with $^{14}CO_2$ confirms that carbon dioxide does not

Table 1. *Specific radioactivity of milk citrate, and of the acetyl group obtained enzymically from it, after experiments in which radioactive substances were infused into the isolated perfused goat udder, together with the normal substrate mixture*

Further details are given in the text.

Radioactive compound infused	Specific radioactivity (counts/min./mg. of C)			Specific radioactivity of acetyl group (% of that of substrate)
	Substrate	Citrate	Acetyl group	
[U-^{14}C]Glucose	930	280	193	21
	930	280	190	20
[U-^{14}C]Glucose	4100	1870	1280	31
	4100	1870	1100	27
$NaH^{14}CO_3$	9500	803	45	0·5
[1-^{14}C]Acetate	51000	12400	11600	23
	51000	6000	10500	20
[2-^{14}C]Acetate	23000	8400	6200	26
	14600	3120	3700	25

Table 2. *Specific radioactivities of* C-1 *and* C-5 *of milk casein glutamate formed during perfusions of the isolated goat udder infused with radioactive substances together with the normal substrate mixture*

Further details are given in the text.

Radioactive compound infused	Specific radioactivity of milk glutamate (counts/min./mg. of C)			
	Whole molecule	C-1	C-5	Counts in C-5 ratio / Counts in C-1 ratio
[1-^{14}C]Glutamate*	42000	167000	2400	0·015
[U-^{14}C]Glucose	326	260	300	1·2
[1-^{14}C]Acetate	7400	6700	16300	2·4
$NaH^{14}CO_3$	189	650	70	0·1

* Test substance.

contribute to acetyl-CoA available to the tricarboxylic acid cycle in the goat mammary gland.

The values from the experiment with glucose show that approximately equal quantities of carbon are contributed by glucose to the two carboxyl groups of glutamate and therefore of α-oxoglutarate. Since C-5 is derived from acetyl-CoA via citrate in the normal functioning of the tricarboxylic acid cycle (Scheme 1), glucose must contribute to acetyl-CoA. C-1 can derive label from acetyl-CoA via oxaloacetate by the normal functioning of the tricarboxylic acid cycle. The C-5/C-1 ratio should be at least 2·0 if there is no influx of labelled carbon other than acetyl-CoA to the tricarboxylic acid cycle (Weinman, Streisower & Chaikoff, 1957). The observed value is lower, showing that glucose must contribute to an influx of carbon at points other than acetyl-CoA: this is in agreement with the carboxylation of pyruvate previously reported (Hardwick, 1965).

ATP citrate lyase in goat tissues. In rat tissue, citrate contributes acetyl groups to fatty acids synthesized outside mitochondria (Spencer & Lowenstein, 1962; Spencer, Corman & Lowenstein, 1964) by using ATP citrate lyase. In the ruminant udder citrate does not contribute significantly to fatty acids, though it must leave the mitochondrion because it appears in milk. It follows that ATP-citrate-lyase activity should be low or absent. This point was checked in goat mammary tissue and liver and the activities of the enzyme observed were very low (0·5–2·5 mμmoles/min./mg. of protein N) and only just above blank readings. A value of about 0·5 mμmole/min./mg. of protein N would account for the slight labelling in fatty acids observed during a perfusion with [U-^{14}C]glucose (Hardwick *et al.* 1963). The extracts did not inhibit rat-liver ATP-citrate-lyase activity, which was about 200 mμmoles/min./mg. of N.

ATP citrate lyase was observed in goat retinal tissue (20 mμmoles/min./mg. of protein N), but not in caudate nucleus or superior colliculus. Acetyl-CoA synthetase was present in rat liver (25–40 mμmoles/min./mg. of N), goat liver (5–20 mμmoles/min./mg. of N) and goat mammary gland (65–100 mμmoles/min./mg. of N).

DISCUSSION

Spencer & Lowenstein (1962) suggested a 'compartmentation' of acetyl-CoA, based on the low diffusion of acetyl-CoA through the mitochondrial membrane. It is generally accepted that pyruvate is oxidized to acetyl-CoA only inside the mitochondrion, so that any acetyl-CoA formed from pyruvate must be changed to a more diffusible compound if it is to contribute extensively to extramitochondrial fatty acid synthesis. Spencer & Lowenstein (1962) suggest citrate as the most likely compound and have shown that this substance is broken down extramitochondrially in rat liver and mammary gland to give acetyl-CoA at a rate comparable with that of fatty acid synthesis. Bartley, Abraham & Chaikoff (1965) have confirmed this hypothesis.

The present paper shows that glucose contributes acetyl groups to citrate in the goat udder. The specific radioactivity of the citrate carbon atoms derived from acetyl groups is similar to that of the whole citrate molecule. The contribution of glucose to acetyl groups appears to be of the same order as that of acetate. Glucose also contributes considerably to oxaloacetate; A. L. Black (personal communication) finds that glucose contributes equally to acetyl-CoA and to oxaloacetate in the whole lactating cow, in which most glucose is probably metabolized in the udder. Annison & Linzell (1964) have shown, however, that, in the udder of the goat, 21–33% of the carbon dioxide comes from acetate: in the perfused gland only 14% comes from

infused acetate (Hardwick *et al.* 1963). The reason for the difference is unknown, but the estimated percentage of acetyl-CoA derived from infused acetate in perfused glands may be lower than it is *in vivo*. The proportions of carbon dioxide derived from glucose are, however, similar in the two experimental conditions.

The failure of glucose to contribute carbon to fatty acids in the ruminant udder is not therefore because glucose does not form acetyl-CoA, but because the acetyl carbon so formed is not transferred from the site of formation to the site of fatty acid synthesis. Citrate is formed from acetyl-CoA, and since citrate is found in considerable quantity (1–2g./l.) in the milk it can presumably leave the mitochondrion. It is assumed that milk citrate is synthesized in the mitochondria; milk and tissue citrates have similar specific radioactivities (Hardwick, 1965).

ATP-citrate-lyase activity seems to be low in or absent from most ruminant tissues, and this may be an enzymic difference between the adult ruminant relying largely on acetate and higher fatty acids with only minimal quantities of glucose available, and the non-ruminant with a larger glucose intake. In the mammary gland and probably the liver of the goat acetyl-CoA formed in the mitochondrion from either acetate or pyruvate cannot be used outside the mitochondrion for fatty acid synthesis. Acetyl-CoA synthetase is present in considerable quantity in the high-speed supernatant of these tissues, permitting acetate to be used for fatty acid synthesis in the cell sap. Such 'compartmentation' has been illustrated both in cows (Tombropoulos & Kleiber, 1961) and in a goat (J. L. Linzell, T. B. Mepham & D. C. Hardwick, unpublished work), where the amount of carbon contributed by glucose to milk fatty acids in the whole animal is only about 1% of that contributed to lactose. Some neural tissue in the ruminant may, however, contain ATP citrate lyase: this may be correlated with the specific requirement of the ruminant brain for glucose (McClymont & Setchell, 1956).

This 'compartmentation' of acetyl-CoA, which decreases the metabolic flexibility of the ruminant compared with the non-ruminant, may have a bearing on the problem of ketosis. In the non-ruminant, ATP citrate lyase provides oxaloacetate which could be returned (in some suitable form) to the mitochondrion to help sustain the tricarboxylic acid cycle: in the ruminant any citrate leaving the mitochondrion would not be compensated by a return of oxaloacetate and acetyl-CoA is therefore more likely to accumulate in the mitochondrion. It may be significant that the citrate content of some non-ruminant milks (horse, human, pig) is about half that of ruminant milks. Krebs (1965) has postulated that oxaloacetate concentrations fall in ketosis, and this is confirmed in cows by the findings of Bach & Hibbitt (1959) that blood citrate is lowered in ketosis. Thus, in saving scarce glucose from making fatty acids, ruminants are more likely to have lowered oxaloacetate concentrations and therefore a greater probability of ketone-body formation than non-ruminants.

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