CALCULATION OF THE INTRACELLULAR DISTRIBUTION OF ACETYL CoA AND CoA, BASED ON THE USE OF CITRATE SYNTHASE AS AN EQUILIBRIUM ENZYME

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1. Introduction

Knowledge of the acetyl CoA/CoA ratio of the cytosolic and mitochondrial compartments of the cell is important in the understanding of control mechanisms in carbohydrate and lipid metabolism. The relative amounts of acetyl CoA and free CoA have been implicated in the control of pyruvate dehydrogenase [1, 2], pyruvate carboxylase [3], pyruvate kinase [4] and citrate cleavage enzyme [5, 6] while recent studies by Pontremoli [7] have shown effect of free CoA on fructose diphosphatase activity.

There have been two major approaches to examining the problem of the compartmentation of CoA derivatives. Firstly, the relative impermeability of the mitochondrial inner membrane to CoA derivatives has permitted the direct fractionation of tissues such as liver and the determination of the distribution of acetyl CoA, CoA and long chain acyl CoA compounds [8]. Secondly, attempts have been made to derive the acetyl CoA/CoA ratio of the mitochondrial compartment by use of the calculated intracellular distribution of citrate and oxaloacetate and by the assumption that citrate synthetase was functioning as an equilibrium enzyme [9]. Clearly, both methods have certain disadvantages, the former because of the possibility that the ratio of acetyl CoA/CoA alters during the fractionation procedure and the latter because of the basic assumption of citrate synthase equilibrium, a point of some controversy [11 and see also 1, 10] as well as the assumptions used in arriving at the mitochondrial citrate and oxaloacetate [12, 13].

A direct experimental approach to the mitochondrial acetyl CoA/CoA ratio can be made using ruminant mammary tissue. This tissue has an extremely low citrate cleavage enzyme. Thus, glucose can readily give rise to mitochondrial acetyl CoA and mitochondrial and cytosolic citrate (as shown by the incorporation of glucose carbons into milk citrate [14]) but does not form cytosolic acetyl CoA (as shown by the lack of incorporation of glucose carbon into fatty acids [15]). It therefore follows that, if ruminant mammary tissue slices are incubated with glucose as sole substrate and the metabolite profile determined, it is probable that virtually the whole of the acetyl CoA determined will be located within the mitochondria. Fractionation studies can then be used to obtain the distribution of the (acetyl CoA + CoA) in the cytosol and mitochondria, while the distribution of citrate and oxaloacetate may be calculated by the methods previously established for liver [12, 13]. From these data, the acetyl CoA/CoA ratio in each of the cell compartments may be calculated.

Using sheep mammary gland, evidence has been obtained for the close approximation of the $K_{eq.}$ and mass action ratio of citrate synthase which may be valuable in the further interpretation of pathways of metabolism in this tissue.

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2. Methods

Lactating sheep mammary tissue was obtained with the kind collaboration of Dr. J. Linzell; glands from the 7th–21st days of lactation were used. Tissue slices were prepared with a Stadie–Riggs cutter and were incubated with 10 vol of Krebs–Ringer bicarbonate medium and a glucose concentration of 20 mM; the gas phase was O_2/CO_2 (95:5) and gassing was continued throughout the 1 hr incubation period. At the end of this time, the slices were rapidly filtered from the medium, frozen and extracted with 0.5 N $HClO_4$ containing 25% ethanol at a temperature of $-10°$. After removal of the protein, neutralization and separation of the $KClO_4$, the metabolites and CoA derivatives were estimated as previously described [13].

Fractionation studies for the distribution of (acetyl CoA + CoA) between the cytosolic and mitochondrial compartments were run in parallel. The mammary gland slices, after incubation in 20 mM glucose for 1 hr, were homogenized in ice-cold 0.25 M sucrose. The debris and nuclei were removed by centrifugation at 600 *g* for 10 min and the large particle fraction collected by centrifugation at 12 000 *g* for 10 min; the supernatant fraction was designated cytosol. The large particle fraction and cytosol were deproteinised and the metabolites extracted and estimated as described above.

Citrate synthase activity of sheep mammary gland was estimated in the washed large particle fraction treated either by freezing and thawing or by Triton-X-100 (1% final conc. for 1 hr). The flux through this enzyme in intact tissue slices was estimated from the incorporation of [6-^{14}C]glucose, [2-^{14}C]pyruvate and [1-^{14}C]acetate into $^{14}CO_2$ during a 1 hr incubation procedure.

The calculation of the distribution of malate and oxaloacetate between the two cell compartments followed the method of Williamson [12] since that proposed by Greenbaum et al. [13] was not applicable in a tissue such as sheep mammary gland which has a very low activity of malic enzyme. The distribution of 2-oxoglutarate, isocitrate and citrate was calculated using both the method of Williamson [12] and that of Greenbaum et al. [13], the two procedures giving values in good agreement.

The $K_{eq.}$ of citrate synthase, taken from Stern et al. [16] is

$$\frac{[Cit]\,[CoA]}{[AcCoA]\,[OAA]\,[H_2O]} = 0.83 \times 10^4 \text{ M}$$

3. Results

The mass action ratio of citrate synthase for both whole cell content and the calculated mitochondrial content of metabolites is as follows. (Data derived from sheep mammary gland slices incubated for 1 hr in 20 mM glucose.)

Using whole cell content (Values as nmoles/g tissue $Cit_t = 805$; $OAA_t = 0.432$; $AcCoA_t = 6.54$; $CoA_t = 78.2$.

$$\Gamma = \frac{805 \times 78.2}{0.432 \times 6.54 \times 55} = 0.40 \times 10^3 \text{ M}$$

Using compartmented metabolites

i) $Mal_t = (OAA_t - OAA_m) \times Lac/Pyr \times K_{LDH}/K_{MDH} + OAA_m \times BOHB/AcAc \times K_{BOHD}/K_{MDH}$

$Mal_t = 272$; $OAA_t = 0.432$; $Lac = 1576$; $Pyr = 13.5$; $BOHB = 54.4$; $AcAc = 3.45$.

$$OAA_m = 0.0026$$

ii) a. $Asp_m = OAA_m \times BOHB/AcAc \times NH_4^+ \times K_{BOHD} \times K_{GOT}/K_{GDH}$

b. $$2\text{-}OG_t = \frac{Glut_c \times OAA_c \times 6.61}{Asp_c} + \frac{(Glut_t - Glut_c) \times OAA_m \times 6.61}{Asp_m}$$

c. $$Cit_c = \frac{Glut_c \times OAA_c \times K_{GOT} \times K_{6PG\text{-}DH} \times 6PG}{Asp_c \times K_{AH} \times K_{1CDH} \times Ru\ 5P}$$

$Asp_t = 2012$; $NH_4^+ = 174$; $2\text{-}OG_t = 8.25$; $Glut_t = 5815$; $Cit_t = 805$; $6PG_t = 67.0$; $Ru\ 5P_t = 12.4$.

$$Cit_m = 427$$

iii) Calculation of mitochondrial free CoA

	Acetyl CoA	Free CoA
Freeze-clamped tissue slices	6.54	78.2
Whole homogenate	6.48	71.1
Cytosol	< 1	53.2
Large particle fraction	5.70	20.1

$Acetyl CoA_m = 6.54$

$CoA_m = 20.1$

$$\Gamma = \frac{427 \times 20.1}{0.0026 \times 6.54 \times 55} = 0.91 \times 10^4 \text{ M}$$

A requirement for the possibility that citrate synthase acts as an equilibrium enzyme is that the V_{max} should considerably exceed the flux through the enzyme. A comparison of the V_{max} and flux is shown in table 1. From this table it is apparent that the activity exceeds the flux by a factor of approx. 20.

Table 1
Relative values for citrate synthase activity and flux through the enzyme in lactating sheep mammary gland.

	(μmoles/g/hr at 37°)
1. Citrate synthase activity	108
2. Flux through citrate synthase	
(a) Citrate oxidised	4.4
(b) Citrate accumulated	1.4
(c) Citrate cleaved for lipogenesis	0
	5.8

(a) Was estimated from the oxidation of [2-^{14}C]pyruvate by lactating sheep mammary gland slices.
(b) Was estimated from citrate accumulated in slices and medium during a 1 hr incubation of sheep mammary gland slices.
(c) Was assumed to be negligible because of virtual absence of citrate cleavage enzyme.

4. Discussion

The absence of citrate cleavage enzyme in the ruminant mammary gland makes this tissue eminently suited to an experimental approach to the determination of the acetyl CoA/CoA ratio in mitochondria as it is this enzyme which provides the cytosolic acetyl CoA. Since it may be taken that, in this tissue incubated with acetate alone, the mitochondrial acetyl CoA is equivalent to the total cell acetyl CoA and the total mitochondrial (acetyl CoA + free CoA) can be determined in fractionated extracts, the mitochondrial acetyl CoA/CoA ratio can be readily obtained. When this value is inserted into the calculation of the mass action ratio of citrate synthase, together with the calculated values for the mitochondrial citrate and oxaloacetate, as shown in Results, excellent agreement between the mass action ratio and the published $K_{eq.}$ is found. A more recent determination of the $K_{eq.}$ gives a value of 4.05×10^4 M in the presence of 1 mM Mg^{2+} (R.W. Guynn, H. Gelberg and R.L. Veech, personal communication) and the calculated mass action ratio is reasonably consistent with this also. The agreement between the mass action ratio and the $K_{eq.}$ values available suggests that, at least in ruminant mammary gland, citrate synthase acts as an equilibrium enzyme.

Some support for the idea that this approach might have a more general application and not be a peculiarity of ruminant tissue, may be deduced from the data of Williamson [12] on rat liver. These data, derived from experiments on whole perfused liver and on isolated mitochondria, provide values for the mitochondrial contents of the reactants of the citrate synthase reaction and these, when calculated, yield a mass action ratio of 0.8×10^4 M compared with the published value for $K_{eq.}$ of 0.83×10^4 M. When, however, the same treatment is applied to the values obtained from freeze-clamped whole liver in a number of hormonal and dietary states, although a close relationship is found between the whole cell acetyl CoA/CoA ratio and the mitochondrial citrate and oxaloacetate [9], which would be mandatory if citrate synthase were acting as an equilibrium enzyme, a discrepancy of about 50 is found between the calculated mass action ratio and the $K_{eq.}$ of citrate synthase. But when the same procedures are applied to the data from rat mammary gland, a much closer agreement between mass action ratio and $K_{eq.}$ is obtained.

The marked disparity between the $K_{eq.}$ and mass action ratio in freeze-clamped livers stands in sharp contrast to the striking agreement of these parameters in the perfused liver preparation. One explanation that may be advanced is a difference in the intramitochondrial pH in the two systems. It may be noted that if the overall reaction including malate dehydrogenase and citrate synthase is considered, then, as shown by Stern et al. [16] a difference of 0.4 pH units would lead to a 10-fold change in the $K_{eq.}$ (see also Srere [17]). Another factor which may operate to produce the discrepancy is the over-estimation of the mitochondrial oxaloacetate. As pointed out by Sols and Marco [18], it is apparent that only a small fraction of the total content of oxaloacetate can be in the free form so that further information on the true free oxaloacetate and the intramitochondrial pH are necessary before considering hepatic citrate synthase to be an equilibrium enzyme.

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