Concentration of free oxaloacetate in the mitochondrial compartment of isolated liver cells

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The concentration of metabolically active (i.e. 'free') oxaloacetate in the mitochondrial compartment of isolated liver cells was investigated by two independent approaches. On the basis of mitochondrial aspartate aminotransferase maintaining equilibrium and the direct measurements of mitochondrial aspartate, 2-oxoglutarate and glutamate, the concentration of free oxaloacetate was calculated to be $5\ \mu\text{M}$ after incubation of hepatocytes in the presence of $1.5\ \text{mM}$ -lactate and $0.05\ \text{mM}$ -oleate. Gradually increasing oleate up to $0.5\ \text{mM}$ decreased the free oxaloacetate to $2\ \mu\text{M}$. Very similar results were obtained when free oxaloacetate concentration was derived from the CO_2 production of hepatocytes as a measure of citrate flux through the tricarboxylic acid cycle, and the kinetic data on citrate synthase *in situ*. The decrease in free oxaloacetate on increasing oleate concentration was associated with lowered rates of cycle-dependent CO_2 output and O_2 uptake, indicating a decrease in the disposal of acetyl-CoA into the tricarboxylic acid cycle. This decrease could explain 25–30% of the increase in ketone-body production occurring at elevated fatty acid supply. This work documents on a quantitative basis the role of free oxaloacetate in the regulation of ketogenesis.

In a previous paper we demonstrated that the rate of ketone-body production from long-chain fatty acids in isolated hepatocytes is inversely correlated with the concentration of metabolically active (i.e. 'free') mitochondrial oxaloacetate, which had been derived from direct measurements of mitochondrial malate concentration and mitochondrial [3-hydroxybutyrate]/[acetoacetate] ratios (Siess *et al.*, 1982). The present study aims to corroborate these earlier findings by the use of two additional approaches for the measurement of free oxaloacetate. The first is based on the assumption that the reactants of mitochondrial aspartate aminotransferase (EC 2.6.1.1) are kept near equilibrium. The second uses the rate of CO_2 production in the tricarboxylic acid cycle as a measure of citrate synthase (EC 4.1.3.7) activity and, hence, of the degree of saturation of this enzyme with oxaloacetate. Both methods yielded very similar free oxaloacetate concentrations, which, however, were higher than those found previously under comparable conditions (Siess *et al.*, 1982). In agreement with our earlier findings (Siess *et al.*, 1982), the free oxaloacetate concentration proved to be

inversely correlated with the rate of ketone-body formation.

Experimental

Chemicals

Worthington collagenase type CLS came from Seromed, Munich, Germany. Bovine carbonic anhydrase (EC 4.2.1.1) was bought from Sigma, Taufkirchen, Germany. Other enzymes and co-enzymes were from Boehringer, Mannheim, Germany. L-Lactic acid was bought from Sigma. Dowex 1X8 (200–400 mesh) was obtained from Serva, Heidelberg, Germany. [*acetyl*- ^3H]Acetyl-CoA was purchased from New England Nuclear, Dreieich, Germany. Scintigel was bought from Roth, Karlsruhe, Germany. All other chemicals came from Merck, Darmstadt, Germany, and were of the highest purity commercially available.

Preparation of liver cells

Male Sprague–Dawley rats (A. Ivanovas, Kisslegg, Germany) weighing 200–250 g after starvation for 48 h were anaesthetized by intraperitoneal

injection of 0.8 ml of sodium pentobarbital (Abbott)/kg body wt., and liver cells were prepared essentially as described by Berry & Friend (1969). At least 95% of the cells excluded 0.2% Trypan Blue.

Incubation of hepatocytes for fractionation studies

The cells (about 25 mg dry wt.) were preincubated for 10 min in the presence of 2 mM-lactate as described by Siess *et al.* (1982) before albumin-bound oleate was added to yield the fatty acid concentrations indicated in Table 1, and incubation was continued for 7 min (some 30% of the lactate had been consumed during the preincubation period). Immediately after the addition of oleate, and 7 min later, 0.1 ml samples of the incubation mixture were deproteinized with 20 μ l of 70% (w/v) HClO_4 before neutralization with 10 M-KOH. Measurements of mitochondrial metabolites were performed after fractionation of hepatocytes into the cytosolic and mitochondrial compartments by the low-temperature modification (Brocks *et al.*, 1980) of the digitonin method (Zuurendonk & Tager, 1974).

Incubation of hepatocytes for measurement of CO_2 production and O_2 consumption

The simultaneous measurement of CO_2 output and O_2 uptake was performed by the 'indirect method' of Warburg (1924) as described by Umbreit (1964). The incubation medium used in these experiments was as described by Siess *et al.* (1982), except that buffering by bicarbonate was replaced by 80 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], final pH 7.4, to minimize the influence of acetoacetic acid formation on CO_2 production, and that carbonic anhydrase (700 Wilbur-Anderson units/ml) was added. By gassing with 99.5% O_2 /0.5% CO_2 a bicarbonate concentration of 1.5 mM was achieved. In paired Warburg vessels (5–6 ml), 0.5 and 1.0 ml, respectively, of the incubation mixture containing about 8 mg dry wt. of hepatocytes/ml were preincubated in the presence of 3.5 mM-lactate for 20 min at 37°C before albumin (6%) bound oleate was tipped in from the side-arm to yield the final concentrations given in Table 2. (At the time of oleate addition, lactate concentration was around 1.5 mM.) After 5 min of further equilibration, six manometer readings were made at 3 min intervals. For metabolite measurements sufficient 70% (w/v) HClO_4 was added to the incubation mixture at 0 and 20 min of incubation with oleate to yield a final concentration of 12%.

Assays

Acetoacetate and 3-hydroxybutyrate were measured as described by Williamson *et al.* (1962). Lactate was determined as described by Wieland

& von Jagow-Westermann (1974), 2-oxoglutarate as described by Bergmeyer & Bernt (1974), and glutamate by the method of Bernt & Bergmeyer (1974). Aspartate was converted into citrate by using [*acetyl*- ^3H]acetyl-CoA under the conditions described by Parvin *et al.* (1980). Removal of excess acetyl-CoA by charcoal adsorption (Parvin *et al.*, 1980) yielded fairly high and variable backgrounds. Therefore acetyl-CoA was hydrolysed (0.5 M-KOH, 30 min) before the incubation mixture (0.2 ml) was added to 1 ml of a 10% suspension of Dowex 1X8 (acetate form) in water. After thorough mixing, the supernatant and the three following washings (10 ml each) with 0.5 M-acetic acid were discarded. The radioactive citrate was detached by two washings (1 ml each) with 2 M-HCl containing 0.5 M-citric acid, which were combined before a sample was measured by liquid-scintillation counting.

Calculations

The concentration of metabolites was calculated on the basis of 10^8 liver cells corresponding to 1 g of fresh liver (Zahlten *et al.*, 1973) or 255 mg dry wt. of cells. Osmotically active water in the mitochondrial matrix has been taken to be 0.216 ml/g dry wt. (Soboll *et al.*, 1976). The concentration of free oxaloacetate from the aspartate aminotransferase equilibrium was calculated by the equation:

$$[\text{Oxaloacetate}] = \frac{[\text{2-oxoglutarate}] \times [\text{aspartate}]}{[\text{glutamate}] \times 6.6}$$

by using the equilibrium constant given by Veech *et al.* (1969). The amounts of CO_2 and O_2 were computed as detailed by Umbreit (1964). Free oxaloacetate from CO_2 production was calculated on the basis of two CO_2 molecules corresponding to one of citrate. The V_{max} and K_m values for oxaloacetate of rat liver citrate synthase *in situ*, corresponding to 30 nmol/min per mg of mitochondrial protein at 25°C and 4.5 μM respectively, were taken from Matlib *et al.* (1978). It was assumed that the kinetic data were independent of the [oleate] used. On the basis of 60 mg of mitochondrial protein corresponding to 1 g of fresh liver (Scholz & Bücher, 1965) and the temperature coefficient given by Srere (1976), the V_{max} of 3.0 $\mu\text{mol/min}$ per g fresh wt. at 37°C used for calculation of free oxaloacetate concentration was obtained. The rate of O_2 consumption for β -oxidation has been derived from ketone-body production as described by Stadie *et al.* (1940). Subtraction of this value from the measured O_2 consumption essentially yields the tricarboxylic acid-cycle-dependent respiration [the respective values given (Table 3) have not been corrected for cyanide-insensitive O_2 utilization].

Results and discussion

The influence of long-chain fatty acid supply on ketogenesis and mitochondrial metabolite concentrations with regard to aspartate aminotransferase has been studied in hepatocytes incubated with 1.5 mM-lactate as the basal substrate. From the experimental data and the assumption of the mitochondrial aspartate aminotransferase reaction being near equilibrium, it was attempted to gain a further insight into the role of oxaloacetate in the regulation of ketone-body production.

The data summarized in Table 1 indicate that isolated liver cells from 48 h-starved rats respond to oleate concentrations up to 0.3 mM with a linear increase in the rate of ketone-body production. Concomitantly, they display a decrease in the mitochondrial concentrations of 2-oxoglutarate and aspartate and an increase in glutamate (Table 1). From these reactants of aspartate aminotransferase, the concentration of free mitochondrial oxaloacetate could be calculated if the equilibrium condition for this enzyme is established. To gain information on this point, the mass-action ratios were computed by dividing the product of the concentrations of 2-oxoglutarate and aspartate by the product of the concentrations of glutamate and total mitochondrial oxaloacetate, by using the data in Table 1 and previous oxaloacetate measurements (Siess *et al.*, 1976b). Under the different substrate conditions used, the mass-action ratios were lower by a factor of 3–7 than the equilibrium constant. In this calculation, however, the fact has been neglected that the mitochondrial matrix contains a large number of oxaloacetate-binding sites (Sols & Marco, 1970). If this is taken into account, the value of the mass-action ratio becomes shifted towards the equilibrium constant. From this and the evidence discussed below, the assumption that in our experiments the mitochondrial aspartate aminotransferase operated near equilibrium seems warranted. It should be stressed that in the present study physiologically occurring concentrations of

both lactate (Williamson & Brosnan, 1974) and long-chain fatty acids (Hales & Kennedy, 1964; Brass & Hoppel, 1978) have been used, as under high-substrate conditions aspartate aminotransferase seems to be out of equilibrium. This is indicated by the fact that mass-action ratios considerably exceeding the equilibrium constant, owing to higher aspartate concentrations, have been observed under these circumstances (Zuurendonk *et al.*, 1976; Tischler *et al.*, 1977; Siess *et al.*, 1978).

Determination of free oxaloacetate from CO₂ production by hepatocytes

The rates of CO₂ production as determined by Warburg's 'indirect method' are listed in Table 2. Increasing [oleate] from 0.05 to 0.5 mM in the incubation medium caused a gradual decrease in CO₂ output. This result is qualitatively in agreement with the finding that CO₂ production by isolated mitochondria is decreased after addition of palmitoylcarnitine (Nicholls *et al.*, 1967). For the reasons detailed below, it is reasonable to conclude that under our experimental conditions the CO₂ measurements represent tricarboxylic acid-cycle activity. The CO₂ contributed by pyruvate dehydrogenase seems negligible because of the low activity of dephospho pyruvate dehydrogenase in the hepatocytes from starved rats (Siess & Wieland, 1976) and the inhibition of this enzyme by acetyl-CoA and NADH (for review see Wieland, 1983). In fact, nearly all of the lactate consumed was recovered as glucose, and there was no accumulation of malate (results not shown). With respect to the pentose phosphate cycle, its rate of CO₂ production (Kuehn & Scholz, 1982) corresponds to only 1% of that observed in our experiments. Moreover, the spontaneous decarboxylation of acetoacetate during 20 min incubation can be considered as being insignificantly low. The possibility that the decrease in CO₂ production by oleate was influenced by CO₂ fixation for urea formation has been excluded. Independent of the

Table 1. Effect of added oleate on the rate of ketone-body production and the mitochondrial concentrations of aspartate aminotransferase reactants in isolated hepatocytes

Mean values $\pm$ S.E.M. for nine different cell preparations are given. Ketone-body production was measured between 0 and 7 min of incubation with fatty acid. In pilot experiments the rates were found to be linear with time at least for 15 min. For further experimental details and calculation of [oxaloacetate]_{free} see the Experimental section.

[Oleate] (mM)	Ketone-body production (μ mol/min per g dry wt.)	[3-Hydroxybutyrate]/ [acetoacetate]	Concns. of mitochondrial metabolites (mM)			
			2-Oxoglutarate	Aspartate	Glutamate	Oxaloacetate _{free}
0.05	3.57 $\pm$ 0.30	0.41	1.60 $\pm$ 0.16	0.297 $\pm$ 0.072	13.4 $\pm$ 1.5	5.4 $\times 10^{-3}$
0.1	4.73 $\pm$ 0.25	0.67	1.46 $\pm$ 0.20	0.245 $\pm$ 0.038	14.2 $\pm$ 1.4	4.5 $\times 10^{-3}$
0.2	6.38 $\pm$ 0.34	1.58	1.27 $\pm$ 0.20	0.211 $\pm$ 0.041	14.2 $\pm$ 1.3	2.9 $\times 10^{-3}$
0.3	8.31 $\pm$ 0.47	2.76	1.15 $\pm$ 0.09	0.192 $\pm$ 0.026	15.6 $\pm$ 1.3	2.1 $\times 10^{-3}$
0.5	9.46 $\pm$ 0.45	6.40	1.13 $\pm$ 0.07	0.214 $\pm$ 0.038	17.0 $\pm$ 1.6	2.2 $\times 10^{-3}$

Table 2. *Effect of oleate on CO₂ production by hepatocytes and mitochondrial free oxaloacetate concentration calculated therefrom*

Mean values $\pm$ S.E.M. for the numbers of different liver cell preparations in parentheses are given. For further details see the Experimental section.

[Oleate] (mM)	Ketone-body production (μ mol/min per g dry wt.)	CO ₂ production (μ mol/min per g dry wt.)	[Oxaloacetate] _{free} (μ M)
0.05	3.05 $\pm$ 0.41 (4)	13.5 $\pm$ 0.4 (4)	5.70
0.1	3.99 $\pm$ 0.38 (6)	12.4 $\pm$ 1.1 (6)	4.73
0.2	4.78 $\pm$ 0.23 (5)	11.1 $\pm$ 0.8 (5)	3.83
0.3	5.93 $\pm$ 0.19 (7)	11.7 $\pm$ 1.1 (7)	4.26
0.5	6.64 $\pm$ 0.31 (8)	10.4 $\pm$ 1.1 (8)	3.26

oleate concentration used, the rate of urea production was constantly $15.2 \pm 2.2 \mu\text{mol}/20\text{min}$ per g dry wt. ($n = 4$), being equivalent to some 8% of the observed CO₂ production. Furthermore, it was ascertained that the underestimation of CO₂ output owing to oleate-stimulated glutamate and citrate formation was only about 1% (results not shown). Thus the measured rate of CO₂ production (Table 2) can be regarded as a reliable index for the estimation of citrate flux through the tricarboxylic acid cycle. This provides a basis for the calculation of oxaloacetate metabolically active at citrate synthase, since the mitochondrial acetyl-CoA concentration (Siess *et al.*, 1976a, 1978) by far exceeds the K_m value of rat liver citrate synthase *in situ* (Matlib *et al.*, 1978) for this substrate. The data on free oxaloacetate obtained in this way are recorded in Table 2. They clearly show that this metabolite becomes considerably decreased by increased oleate supply.

Comparison of data on free oxaloacetate obtained by different methods

The values for free oxaloacetate arrived at in the present study at the different fatty acid concentrations were in the range of 2–6 μM (Tables 1 and 2). Thus they are at least by one order of magnitude higher than the results obtained by the metabolite indicator methods (Krebs & Veech, 1969; Williamson, 1969; Greenbaum *et al.*, 1971). They were by a factor of 3–6 also higher (depending on the oleate added) than those derived from measurements of mitochondrial malate concentration and mitochondrial [3-hydroxybutyrate]/[acetoacetate] ratios (Siess *et al.*, 1982), when [lactate] in the medium was about 1.5 mM. However, the values previously found (Siess *et al.*, 1982) at lactate concentrations of 3–6 mM (plus 0.3–0.6 mM-pyruvate) are in good agreement with the present results (Tables 1 and 2). This possibly indicates that the assumed equilibrium condition for the malate (and 3-hydroxybutyrate) dehydrogenase(s) may not be

ideally fulfilled at the lower lactate supply. The other possibility, that aspartate aminotransferase is out of equilibrium under our low-lactate conditions, is less likely because of the close similarity between the free oxaloacetate values obtained on the basis of the presumed equilibrium and from the rate of CO₂ production. The validity of the free-oxaloacetate data derived from the aspartate aminotransferase equilibrium is further documented by the fact that they lead to theoretical rates of cycle-dependent respiration that are in good agreement with those experimentally found. Thus from a value of 5.4 μM free oxaloacetate (e.g. at 0.05 mM-oleate; Table 1) and the kinetic data given in the Experimental section, the resulting rate of citrate formation at 37°C is $1.6 \mu\text{mol}/\text{min}$ per g fresh wt. As citrate essentially does not accumulate under our conditions, and also fatty acid synthesis from citrate in the starved cells can be neglected, the rate of citrate utilization in the tricarboxylic acid cycle corresponds to that of its formation. Therefore the expected rate of cycle-dependent respiration corresponds to $2 \times 1.6 \mu\text{mol}$ of O₂/min per g fresh wt., which is the experimental value given in Table 3.

Relationship between free oxaloacetate and the rate of ketone-body formation

The free mitochondrial oxaloacetate concentrations plotted against the corresponding rates of ketone-body production yielded the curve shown in Fig. 1. Evidently, a reciprocal interrelationship between the two variables exists, analogous to that obtained under similar conditions when the free oxaloacetate concentration was derived on the premise of malate dehydrogenase and 3-hydroxybutyrate dehydrogenase catalysing equilibrium reactions (Siess *et al.*, 1982). According to the present data, even at a low oleate concentration free mitochondrial oxaloacetate can achieve at best half-saturation of citrate synthase operating in intact hepatocytes, as the respective K_m value has

Table 3. Decrease by oleate in tricarboxylic acid-cycle-dependent respiration of hepatocytes
The same experiments were evaluated as in Table 2. For details see the Experimental section.

[Oleate] (mM)	Total O ₂ consumption (μmol/min per g dry wt.)	β-Oxidation-dependent O ₂ uptake (μmol/min per g dry wt.) calculated from:		Cycle-dependent O ₂ uptake (μmol/min per g dry wt.) (% of total)	
		Acetoacetate	3-Hydroxybutyrate		
0.05	16.6	2.99	1.42	12.2	73
0.1	16.3	3.47	1.77	11.1	68
0.2	16.9	3.43	3.07	10.4	62
0.3	18.1	3.94	4.10	10.1	56
0.5	18.4	4.26	4.81	9.3	51

Fig. 1. Relationship between mitochondrial concentration of free oxaloacetate and the rate of ketone-body production
The data on the mitochondrial concentration of free oxaloacetate from Table 1 (○) and Table 2 (●) are plotted against the values for ketone-body production shown in Tables 1 and 2 respectively.

been determined by different means to be 2–7 μM (Wieland *et al.*, 1964; Shepherd & Garland, 1969; Moriyama & Srere, 1971; Srere, 1976; Matlib *et al.*, 1978; Siess *et al.*, 1978). Therefore the gradual decrease in free oxaloacetate observed after increasing oleate (Table 2) can be expected to contribute to ketogenesis by decreasing the conversion of acetyl-CoA into citrate. This view is supported by the decrease in CO₂ production after addition of oleate (Table 2), and is further substantiated by the fact that the tricarboxylic acid-cycle-dependent respiration appeared as an inverse function of the fatty acid concentration (Table 3). Thus, on the basis of the data shown in Tables 1 and 2, 25–30% of the increase in ketone-body production seen at elevated fatty acid supply could be explained by the decrease in acetyl-CoA utilization

by citrate synthase. These results provide a quantitative basis for the estimation of the role of oxaloacetate availability in the regulation of ketone-body production, qualitatively documented by several earlier studies (Lehninger, 1946; Breusch, 1948; Wieland, 1968; Lopes-Cardozo & Van den Bergh, 1972; Lopes-Cardozo, 1978; Blackshear *et al.*, 1975; Nosadini *et al.*, 1980; Demaugre *et al.*, 1982). In connection with our previous measurements of mitochondrial [3-hydroxybutyrate]/[acetoacetate] ratios (Siess *et al.*, 1982) the present work furthermore proves the previously expressed view (Wieland, 1968) that the decrease in oxaloacetate in ketosis is caused by an increase in the mitochondrial redox state.

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