A SENSITIVE AND SIMPLE METHOD FOR THE STUDY OF OXALOACETATE COMPARTMENTATION IN ISOLATED HEPATOCYTES

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1. Introduction

Data on the subcellular distribution of oxaloacetate in liver as calculated from mass action equilibria [1–3] have not been reliably proven so far by direct measurements. In order to determine oxaloacetate in the cytosolic and the mitochondrial fractions of isolated hepatocytes [4,5], we have worked out an assay superior to that reported earlier [6] in being sensitive enough to detect 5 pmol of oxaloacetate per ml. This assay is also easier to perform and a large number of determinations can be accomplished by one person. We present also data on subcellular distribution of oxaloacetate in isolated liver cells from fed rats.

2. Materials and methods

L-[4-^{14}C]Aspartic acid was purchased from the Radiochemical Centre Amersham, England. Acetyl-CoA, 2-oxoglutaric acid, oxaloacetic acid, enzymes and coenzymes were bought from Boehringer, Mannheim, Germany. Citric acid, ethylene glycol, ethanol, naphthalene, KOH, $HClO_4$ and Tris were products of the highest quality from Merck, Darmstadt, Germany. Dioxane, Rotitainer quality, came from Roth, Karlsruhe, Germany. Permablend III was bought from Packard Instruments, Frankfurt, Germany. Bio-Rad AG 50 W × 8 (200–400 mesh, H^+ form) was purchased from Bio-Rad Laboratories, Munich, Germany. The scintillator used contained in 1000 ml, 5.5 g of Permablend III, 50 g of naphtalene, 20 g of ethylene glycol, 100 g of ethanol and dioxane.

For oxaloacetate determination in whole liver, the tissue was removed with a Wollenberger clamp [7] from normal fed or 36–48 h starved Sprague-Dawley rats (W. Gassner, Sulzfeld, Germany) anaesthetized with ether and ground in a mortar under liquid nitrogen cooling. The frozen tissue powder was extracted by homogenization for 30–40 s with a 6-fold volume of ice-cold 12% (w/v) $HClO_4$ using a motor-driven glass-Teflon homogenizer. After centrifugation for 5 min at 15 000 × g at 2°C the precipitate was extracted as before. The two extracts were combined and assayed as soon as possible. Perchloric acid extracts from isolated hepatocytes were prepared as described earlier [5]. For complete hydrolysis of the variable amounts of acetyl-CoA contained in the extracts, they were brought to pH 12 with 10 N KOH and kept at room temperature for 30 min. The pH was then lowered to 6.2–6.4 with 70% (w/v) $HClO_4$ and 0.1 ml of 1 M Tris–Cl buffer, pH 7.2, was added per ml. As a blank the perchloric acid used for extraction was carried through the same procedure.

[^{14}C]oxaloacetate was prepared from [^{14}C]aspartate essentially by the method of [8]: A mixture consisting of 20 μl of 1 M Tris–Cl buffer, pH 7.2, 200 μl of 0.2 M 2-oxoglutarate (Na^+), 20 μl of L-[^{14}C]aspartate (Na^+) (17.8 mCi/mmol) and 10 μl of glutamic oxaloacetic transaminase (EC 2.6.1.1) corresponding to 4 U is incubated at 25°C for 20 min. For separation of oxaloacetate from remaining aspartate, the incubation mixture was put on a small column (5 × 50 mm) containing 700 mg of Bio-Rad AG 50 W × 8. Oxaloacetate was eluted with 0.6 ml of distilled water and neutralized with 1 N KOH (about 50 μl). The concen-

trations of stock solutions of oxaloacetate, freshly prepared prior to use and of acetyl-CoA were determined according to [9] and [10], respectively.

Assay of oxaloacetate: To plastic scintillation vials 100 μl of 1 M Tris–Cl buffer, pH 7.2, 20 μl of a mixture of [^{14}C]oxaloacetate and acetyl-CoA containing 85 and 63 pmol, respectively, 40 μl of citrate synthase (EC 4.1.3.7) corresponding to 13 U, and 1 ml of the sample appropriately diluted with the blank were added and incubated in a shaking water bath at 30°C for 90 min. Oxaloacetate not converted to citrate was removed by adding 50 μl of a 1:1 mixture of 12% (w/v) $HClO_4$ and 1 M citric acid and heating the vials at 120°C for about 75 min (until almost dry) before the residue was suspended in 10 ml of scintillator by vigorous shaking or ultrasonication ('Schoellerschall Reinigungswanne', Schoeller, Frankfurt, Germany). Under these conditions 99% of the [^{14}C]oxaloacetate was removed from incubation mixtures lacking acetyl-CoA in the absence, and 97% in the presence, of liver extract. To correct for the remaining background radioactivity incubation mixtures containing water instead of acetyl-CoA were run in parallel and the radioactivity due to acetyl-CoA was taken as [^{14}C]citrate. This correction was omitted in the case of extracts from isolated liver cells, as the error was considered to be negligible. Radioactivity was determined in a Packard TriCarb liquid scintillation spectrometer model 574. All assays were run in duplicate. Calibration curves were established for each assay usually with 5–50 pmol of unlabelled oxaloacetate in 1 ml of the blank.

3. Results and discussion

The principle of the method is the enzymatic conversion of [^{14}C]oxaloacetate to [^{14}C]citrate in the presence of a limiting amount of acetyl-CoA. Oxaloacetate of an unknown sample is determined by its competition with added [^{14}C]oxaloacetate for acetyl-CoA, thereby lowering the specific radioactivity of the [^{14}C]citrate formed. It is important, therefore, that the amount of acetyl-CoA employed in the assay does not exceed that of [^{14}C]oxaloacetate. Routinely, about 75% of the stoichiometric amount of acetyl-CoA, determined as shown in fig.1, was used in our assays. The incorporation of radioactivity from

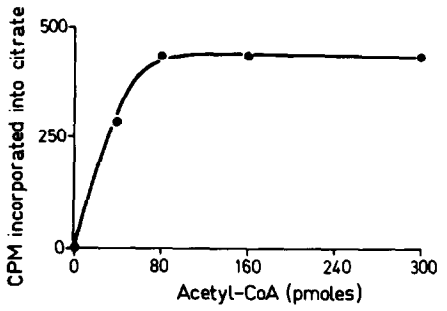

Fig.1. Determination of the limiting amount of acetyl-CoA. Freshly prepared dilutions of a 10 mM stock solution of acetyl-CoA containing the amounts indicated by the abscissa were incubated with a constant amount (83 pmol) of [4-^{14}C]oxaloacetate (sp. act. = 7.6 mCi/mmol) for 30 min at 30°C and further processed as described in the Materials and methods section. The minimum amount of acetyl-CoA giving maximal citrate yield is regarded as to be stoichiometric to the amount of [4-^{14}C]oxaloacetate.

a constant amount of [^{14}C]oxaloacetate is illustrated in fig.2, which shows the means of 10 calibration curves, ranging from 0–50 pmol. Figure 3 documents that there was a linear relationship between the amount of (a) liver tissue or (b) cell extract and the oxaloacetate contents of the samples, as read off from

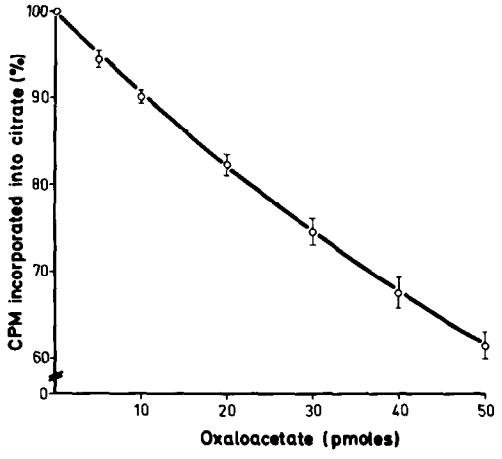

Fig.2. Effect of unlabelled oxaloacetate on [^{14}C]oxaloacetate incorporation into [^{14}C]citrate. The mean values ± S.E. of 10 calibration curves established as described in the Materials and methods section are given. To get a common ordinate the citrate radioactivity of each blank was set 100%, corresponding to 730–1015 cpm.

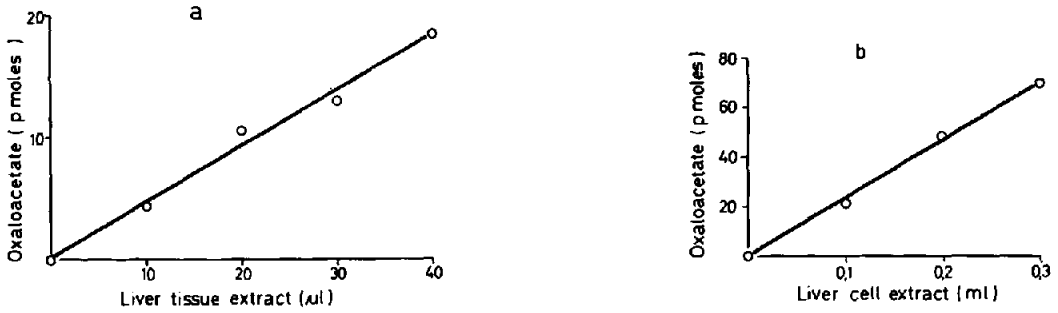

Fig.3. Linear relationship between oxaloacetate found and the amount of (a) liver tissue or (b) liver cell extract employed.

the calibration curves. The results of five determinations of one liver cell extract are given in table 1, indicating a variation coefficient of 6% (relative variation coefficient 3%). In order to test the specificity of the assay, aliquots of liver cell extracts were treated as indicated in table 2 prior to analysis. The preincubation with malate dehydrogenase (EC 1.1.1.37) or citrate synthase together with the appropriate cofactors resulted in an almost quantitative removal of oxaloacetate from the sample as expected. The recovery of oxaloacetate added to the perchloric acid extracts from liver cells or liver tissue is shown in table 3.

Table 1
Variability of the oxaloacetate assay

Assay (Number)	Oxaloacetate (pmol/assay)
1	42
2	46.5
3	45.2
4	47.2
5	48.7

Mean = 45.9
SD = 2.5

Results of five analyses of one liver cell preparation are given.

The concentration of oxaloacetate in the livers of normal fed rats was determined to be 4 nmol/g fresh wt (table 4). This value is in agreement with earlier measurements, where different methods were employed [2,6,11]. Table 4 further indicates that starvation for 36–48 h caused a significant ($p < 0.01$) reduction of hepatic oxaloacetate.

The subcellular distribution of oxaloacetate between the mitochondrial and cytosolic compart-

Table 2
Specificity of the oxaloacetate assay

Number of experiment	Blank (cpm)	Pretreatment of the liver cell extract: None (cpm)	Malate dehydrogenase + NADH (cpm)	Citrate synthase + Acetyl-CoA (cpm)	$HClO_4$ extract boiled for 60 min (cpm)
1	883	607	852	884	–
2	975	699	934	947	–
3	975	834	911	1020	–
4	1069	899	–	1010	1016
5	833	614	870	864	–

To 1 ml of neutralized liver cell extract 10 µl of malate dehydrogenase (24 U) and 5 µl of 10 mM NADH, or 20 µl of citrate synthase (4 U) and 0.15 nmol of acetyl-CoA, respectively, were added prior to incubation for 30 min at 25°C. The reaction was terminated by the addition of 50 µl of 70% (w/v) $HClO_4$. Further processing as described in the Materials and methods section.

Table 3
Recovery of oxaloacetate from the perchloric acid extracts from isolated hepatocytes or liver tissue[a]

Number of experiment		Amount of oxaloacetate (pmol/assay)				
		Endogenous	Added	Calculated	Found	Yield (%)
1	(a)	21	6.4	27.4	27.5	100
	(b)	16.2	6.4	22.6	23.9	106
2		26.2	3.7	29.9	31.9	107
3[a]		45.6	18.2	63.8	62.2	97

Table 4
Concentration of oxaloacetate in freeze-clamped liver tissue from normal fed and 36–48 h starved rats

Nutritional state	Oxaloacetate (nmol/g fresh wt)
Fed	4.4 ± 0.3 (10)
Starved	2.6 ± 0.5 (5)

Mean values ± S.E.M. are given for the numbers of rats in parentheses.

ments of liver cells isolated from fed rats is shown in table 5. When incubated without exogenous substrate some 90% of cellular oxaloacetate was found to be located in the cytosol, yielding a concentration ratio between the two spaces of about 1 on the basis of the parameters given earlier [5] except that one gram wet liver cells from a fed rat corresponds to 0.42 ± 0.02 g (n = 15) dry wt. Much lower ratios were reported by others [1–3], using an indirect approach for the estimation of matricial oxaloacetate. On incubation with different substrates large varia-

Table 5
Subcellular distribution of oxaloacetate in hepatocytes isolated from normal fed rats

Substrate added	mM	Amount (nmol/g dry wt)			Concentration (μM)		Concentration gradient mit./cyt.
		Total	Pellet	(T–P)	mit.	cyt.	
None		17.6 ± 2.1 (5)	1.7 ± 0.6 (3)	15.9	14.4	16.2	0.9
Lactate	10	23.4 ± 2.1 (4)	1.4 ± 0.3 (4)	22.0	11.9	22.4	0.5
Oleate	0.9	44.2 ± 6.8 (4)	1.9 ± 0.3 (5)	42.3	16.1	43.0	0.4
Oleate + lactate	0.9 10	51.9 ± 4.1 (5)	2.6 ± 0.3 (4)	49.3	22.0	50.1	0.4
Pyruvate	7	51.0 ± 6.2 (5)	2.5 ± 0.3 (5)	48.5	21.2	49.3	0.4
Ethanol	12	2.9 ± 0.9 (5)	0.9 ± 0.4 (4)	2.0	7.6	2.0	3.8

Mean values ± S.E.M. are given for the number of different cell preparations in parentheses. The cells were prepared and incubated as described [5].

tions of the oxaloacetate concentration occurred in the cytosol, while it was kept fairly constant within the mitochondria (table 5). The same observation was made earlier with hepatocytes isolated from fasted animals [5].

The sensitive assay described here allows the determination of oxaloacetate in the mitochondrial and extramitochondrial compartments of isolated hepatocytes. This opens the possibility to study the role of oxaloacetate availability in the two spaces for the regulation of important liver cell functions such as ketogenesis and gluconeogenesis.

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