Biochem. J. (1980) **188**, 207–212
Printed in Great Britain

Validity of the Digitonin Method for Metabolite Compartmentation in Isolated Hepatocytes

Dietrich G. BROCKS, Elmar A. SIESS and Otto H. WIELAND
Forschergruppe Diabetes and Klinisch-chemisches Institut des Städtischen Krankenhauses München-Schwabing, Kölner Platz 1, D-8000 München 40, Federal Republic of Germany

(*Received 25 September 1979*)

1. A modification of the digitonin method of Zuurendonk & Tager (1974) (*Biochim. Biophys. Acta* **333**, 393–399) (i.e. the 'conventional' method) was developed that allows the fractionation of isolated hepatocytes at −5°C (i.e. 'low-temperature' method). 2. With respect to compartmentation of adenine nucleotides, glutamate and citrate, the two methods yielded very similar results. 3. In contrast, the mitochondrial amounts of aspartate and malate, as revealed by the low-temperature method, were about twice as high as those found by the conventional procedure. No change in the total cellular content occurred. 4. With n-butylmalonate and glisoxepid present in the conventional digitonin medium, significantly higher amounts of malate and aspartate respectively were found in the mitochondrial pellets. The results obtained by the low-temperature method, however, were not influenced by these inhibitors. 5. It is concluded that under the conventional conditions of cell fractionation no appreciable redistribution of adenine nucleotides, glutamate and citrate occurs.

The present knowledge on the distribution of metabolites between the cytosolic and mitochondrial spaces of liver is based mainly on two different methods of fractionation applied to two different models. Elbers *et al.* (1974), working with the haemoglobin-free perfused liver, separated subcellular compartments by density-gradient centrifugation of freeze-dried tissue in organic solvents. Zuurendonk & Tager (1974) used the digitonin method, which takes advantage of the fact that the plasma membrane contains considerably more cholesterol than do the mitochondrial membranes (Colbeau *et al.*, 1971); thus isolated hepatocytes are incubated with digitonin under conditions that cause lysis of the cell membrane but not the mitochondrial inner membrane, thereby allowing the separation of the mitochondrial pellet fraction from the cytosolic supernatant by centrifugation.

The data on metabolite compartmentation accumulated so far by each approach agree reasonably well as far as malate, citrate, and 2-oxoglutarate are concerned. A marked discrepancy, however, exists with regard to the mitochondrial [ATP]/[ADP] ratio. Whereas the non-aqueous fractionation technique of Elbers *et al.* (1974) yielded values of between 0.2 and 0.7 (Soboll *et al.*, 1978), ratios of about 2 were obtained with the digitonin method (Zuurendonk & Tager, 1974; Siess & Wieland, 1975, 1976; Akerboom *et al.*, 1978). It has been argued (Soboll *et al.*, 1978) that phosphorylation of mitochondrial ADP might occur during digitonin fractionation, leading to artificially high mitochondrial [ATP]/[ADP] ratios. Although strong evidence against this view has been presented (Siess *et al.*, 1978), the present study was undertaken to obtain more information on metabolic stability during hepatocyte fractionation with digitonin. For this purpose we have developed a modification of the digitonin procedure that separates the cellular compartments at −5°C, i.e. at a temperature 10°C lower than that usually employed.

Materials and Methods

Animals

Male Sprague–Dawley rats (A. Ivanovas, Kisslegg, W. Germany), weighing about 200–220g after 48h of starvation, were used for hepatocyte isolation.

Chemicals

Enzymes and coenzymes were products of Boehringer (Mannheim, W. Germany). Dimethyl sulphoxide and toluene were from E. Merck (Darmstadt, W. Germany). Glisoxepid [1-(hexahydro-1*H*-azepin-1-yl)-3-({*p*-[2-(5-methyliso-oxazole-3-carboxamido)ethyl]phenyl}sulphonyl)urea] was kindly supplied by Farbenfabriken Bayer (Leverkusen, W.

0306-3283/80/040207-06$01.50/1

Germany). Other chemicals were the same as in an earlier study (Siess *et al.*, 1977). n-Butylmalonate was prepared from its ethyl ester by saponification.

Preparation and incubation of hepatocytes

Hepatocytes were prepared as described earlier (Siess *et al.*, 1976). At least 95% of the cells excluded 0.2% Trypan Blue. The cells were incubated for 10min at 37°C as described (Siess *et al.*, 1976) in a medium composed of 1ml of Krebs–Henseleit (1932) bicarbonate buffer containing 1.5% gelatin, 0.01ml of 110mM-$CaCl_2$, 0.05ml of substrate solutions to give the final concentrations indicated in the Tables and 0.1ml of 10% defatted (Chen, 1967) bovine serum albumin in 0.9% NaCl, which was replaced by albumin-bound oleate where indicated. Incubations were commenced by the addition of 0.2ml of the hepatocyte suspension, corresponding to about 20mg dry wt.

Fractionation of hepatocytes

Fractionation by the conventional digitonin method (Zuurendonk & Tager, 1974) was performed as described earlier (Siess *et al.*, 1976). The low-temperature digitonin method was performed as follows. In the cold-room (2–4°C) 0.2ml of the incubation mixture was pipetted into a mixture of 1ml of digitonin medium (containing 15mg of digitonin, 3μmol of EDTA and 250μl of dimethyl sulphoxide, pH7) and 0.1ml of toluene, which had been precooled to −12°C. The temperature of the mixture was between −7°C and −5°C after the addition of the cell suspension. The mixture was shaken on an Eppendorf 'Rotationsmischer' type 3300 for 15s before 1.0ml was transferred to an Eppendorf cup containing 0.12ml of 12% (v/v) $HClO_4$ beneath a layer of 0.5ml of silicone oil (AR200/AR20, 2:1, w/w, which was kept at −12°C before use. Centrifugation for 10–15s in an Eppendorf centrifuge (type 3200) was started 30s after the addition of the cells to the fractionation medium.

For metabolite determinations in the supernatant fraction, 0.8ml of the supernatant was rapidly mixed with 0.2ml of 70% (v/v) $HClO_4$ and neutralized with 10M-KOH containing 0.1M-Tris. For metabolite measurements in the pellet fraction, the oil phase was carefully sucked off before a further 0.2ml of 12% (v/v) $HClO_4$ was added for pellet extraction. After centrifugation and re-extraction with 0.2ml of 12% (v/v) $HClO_4$ the combined supernatants were neutralized as described above.

For enzyme-activity measurements, 0.2ml of the incubation mixture was mixed in an Eppendorf cup with the fractionation medium, shaken as above and centrifuged (in the same cup) for 15s. The supernatant was poured off and both the supernatant and the pellet were quickly frozen in liquid N_2.

Determination of metabolites

The determination of metabolites was performed as described earlier (Siess *et al.*, 1976, 1977). Enzyme extraction and determination was done as described by Siess & Wieland (1975). Results are expressed on a dry-weight basis as mean values ±S.E.M. for the numbers of cell preparations given in parentheses. Statistical significance was calculated by Student's *t* test for paired data.

Results

The experimental procedure for the low-temperature digitonin method differs from that for the conventional method (Zuurendonk & Tager, 1974) in the increase in the digitonin concentration from 4 to 15mg/ml and in the addition of an anti-freeze that allows the digitonin medium to be precooled to −12°C. Whereas at +5°C lysis of hepatocytes was complete at 4mg of digitonin/ml (Zuurendonk & Tager, 1974; Siess & Wieland, 1975, 1976), a concentration of 15mg of digitonin/ml was necessary to achieve fractionation at −5°C. Further, the inclusion of 9% toluene (Hilderman *et al.*, 1975) proved to be useful, as the release of α-glycerophosphate, a commonly used cytosol marker (Zuurendonk *et al.*, 1976), was increased from 69.4±3.6 to 84.7±1.5% (means±S.E.M., $n=7$) when toluene was present in the digitonin medium. Table 1 summarizes the results obtained for leakage of α-glycerophosphate and marker enzymes of the cytosol, intermembrane and mitochondrial matrix spaces as measured by both the conventional and low-temperature digitonin methods. It appears that low-molecular-weight constituents of the cytosol, such as α-glycerophosphate, are released to nearly the same extent at the two temperatures, whereas

Table 1. *Release of α-glycerophosphate and of marker enzymes into the supernatant fraction during fractionation of isolated hepatocytes by the conventional and low-temperature digitonin methods*

Hepatocytes were incubated at 37°C in the presence of 10mM-glycerol for 10min before their simultaneous fractionation at +5°C and −5°C as described in the Materials and Methods section. The total cellular amount is taken as 100%. Results are means±S.E.M. for 36 different cell preparations.

	Release into the supernatant fraction (%)	
	+5°C	−5°C
α-Glycerophosphate	87.4±0.6	82.6±0.8
Lactate dehydrogenase	91.9±1.1	28.0±3.0
Adenylate kinase	37.2±3.4	4.5±0.5
Glutamate dehydrogenase	3.5±0.6	1.9±0.2

high-molecular-weight components, e.g. lactate dehydrogenase (EC 1.1.1.27), are retained much more at −5°C. Further, the amount of adenylate kinase (EC 2.7.4.3) found in the supernatant after fractionation at −5°C is markedly reduced, indicating that the mitochondrial outer membrane is much less affected by digitonin at the lower temperature. Moreover, the small release of glutamate dehydrogenase (EC 1.4.1.3) (Table 1) suggests that the preservation of the mitochondrial inner membrane at the lower temperature is at least as good as under conventional conditions. It should be noted that the possibility of enzyme inactivation by the toluene/digitonin medium had been excluded (results not shown).

Subcellular distribution of adenine nucleotides

The results obtained for the simultaneous performance of the conventional and low-temperature digitonin methods with the same hepatocyte suspension incubated with different substrates to perturb the subcellular adenosine nucleotide concentrations are recorded in Table 2. Apparently, the temperature change had no effect on the amounts of the adenine nucleotides in the supernatant fraction. In the mitochondrial pellet fraction the amount of ADP was also not altered by lowering the fractionation temperature to −5°C, whereas the concentration of ATP in hepatocytes incubated with endogenous substrate or with adenosine was slightly higher. With fructose as the substrate no difference in mitochondrial ATP was discernible. As to the mitochondrial [ATP]/[ADP] ratio, it is noteworthy that it was not lowered by changing the fractionation temperature, and hepatocytes incubated with adenosine even displayed a slightly elevated mitochondrial [ATP]/[ADP] ratio after fractionation at −5°C (Table 2).

Subcellular distribution of malate

Fractionation at −5°C led to malate concentrations in the pellet (P) fraction that were markedly higher ($P < 0.0005$) than those found after fractionation at +5°C (Table 3). No difference in total

Table 2. *Subcellular distribution of adenine nucleotides in isolated hepatocytes fractionated simultaneously at +5°C and −5°C*

Isolated hepatocytes were incubated for 10 min with the substrates indicated as described in the Materials and Methods section. Then the conventional (+5°C) and low-temperature (−5°C) digitonin fractionation procedures were carried out simultaneously. The difference between the results obtained by the two methods were statistically significant at $*P < 0.025$; $**P < 0.005$; $***P < 0.0005$.

		Amount (μmol/g dry wt.)			
		Pellet		Supernatant	
Substrate	Metabolite	+5°C	−5°C	+5°C	−5°C
Endogenous	ATP	2.25 ± 0.18 (8)	3.01 ± 0.28* (8)	7.12 ± 0.39 (9)	7.86 ± 0.53 (9)
	ADP	1.48 ± 0.21 (7)	1.55 ± 0.20 (7)	1.20 ± 0.13 (9)	1.16 ± 0.15 (9)
	ATP/ADP	1.70 ± 0.23 (7)	2.12 ± 0.42 (7)	6.58 ± 0.9 (9)	8.10 ± 1.28 (9)
	ATP + ADP	3.85 ± 0.31 (7)	4.54 ± 0.37* (7)	8.28 ± 0.36 (9)	9.01 ± 0.56 (9)
Adenosine (5 mM)	ATP	2.02 ± 0.16 (7)	2.73 ± 0.17*** (7)	11.68 ± 0.44 (9)	11.66 ± 0.69 (9)
	ADP	1.92 ± 0.26 (8)	2.02 ± 0.20 (8)	1.59 ± 0.11 (9)	1.75 ± 0.16 (9)
	ATP/ADP	1.09 ± 0.15 (7)	1.40 ± 0.20* (7)	7.60 ± 0.52 (9)	6.82 ± 0.41 (9)
	ATP + ADP	3.95 ± 0.44 (7)	4.76 ± 0.31** (7)	13.16 ± 0.54 (9)	13.41 ± 0.79 (9)
Fructose (10 mM)	ATP	1.11 ± 0.10 (9)	1.27 ± 0.16 (9)	4.33 ± 0.39 (9)	4.22 ± 0.42 (9)
	ADP	1.41 ± 0.09 (9)	1.75 ± 0.14 (9)	1.06 ± 0.12 (9)	0.92 ± 0.16 (9)
	ATP/ADP	0.76 ± 0.05 (9)	0.76 ± 0.08 (9)	4.45 ± 0.57 (9)	5.83 ± 0.86 (9)
	ATP + ADP	2.52 ± 0.18 (9)	3.03 ± 0.24* (9)	5.39 ± 0.37 (9)	5.13 ± 0.45 (9)

Table 3. *Influence of n-butylmalonate present during fractionation on the subcellular distribution of malate in isolated hepatocytes fractionated simultaneously at +5°C and −5°C*

Isolated hepatocytes were incubated for 10 min with the substrate(s) indicated. The fractionation was carried out as described in the Materials and Methods section. Where indicated n-butylmalonate (1 mM) was present in the digitonin medium. Five hepatocyte preparations were used in each case.

		Malate (nmol/g dry wt.)					
	n-Butyl-malonate in digitonin	Pellet (P)		Supernatant (S)		Total (P + S)	
Substrate(s) added	medium	+5°C	−5°C	+5°C	−5°C	+5°C	−5°C
Lactate (8 mM)	−	155 ± 30	239 ± 27	1657 ± 170	1567 ± 180	1812	1806
	+	206 ± 34	239 ± 6	1404 ± 170	1752 ± 260	1610	1991
Lactate (8 mM)	−	683 ± 119	1408 ± 156	5764 ± 480	5774 ± 240	6477	7182
+ oleate (0.7 mM)	+	1114 ± 137	1376 ± 94	5640 ± 430	5463 ± 290	6754	6839

Table 4. *Subcellular distribution of aspartate in isolated hepatocytes fractionated simultaneously at +5°C and −5°C*

Isolated hepatocytes were incubated for 10 min with the substrate(s) indicated. The fractionation was carried out as described in the Materials and Methods section. Where indicated glisoxepid (1 mM) was present in the digitonin medium.

		Aspartate (μmol/g dry wt.)					
	Glisoxepid in digitonin	Pellet (P)		Supernatant (S)		Total (P + S)	
Substrate(s) added	medium	+5°C	−5°C	+5°C	−5°C	+5°C	−5°C
Lactate (8 mM)	−	103 ± 12 (8)	192 ± 39 (8)	1241 ± 147 (8)	1210 ± 174 (8)	1344	1402
Alanine (7 mM)	−	179 ± 23 (9)	388 ± 53 (9)	3142 ± 396 (9)	2991 ± 342 (9)	3321	3379
Lactate (8 mM) + alanine (7 mM)	−	188 ± 43 (7)	561 ± 74 (7)	5350 ± 619 (7)	5600 ± 801 (7)	5538	6161
	+	273 ± 55 (7)	471 ± 79 (7)	5466 ± 668 (7)	5675 ± 773 (7)	5739	6146

cellular amount of malate was discernible, either at normal or markedly elevated concentrations, as obtained after incubation with lactate or lactate plus oleate respectively (Table 3). From these results it appears that efflux of mitochondrial malate occurs during the conventional digitonin fractionation procedure. It was therefore decided to see whether, under conventional conditions, n-butylmalonate, an inhibitor of the dicarboxylate carrier (Robinson & Chapell, 1967; Meyer & Tager, 1969), yields the same values as those obtained at −5°C. Table 3 indicates that n-butylmalonate increased the concentration of malate in the pellet fraction by 33% ($P < 0.0005$) under conventional conditions, but was ineffectual at −5°C.

Subcellular distribution of aspartate

Table 4 summarizes our results on the compartmentation of aspartate obtained by simultaneous fractionation of hepatocytes at +5°C and at −5°C. With lactate or alanine as the substrate, the lowering of the fractionation temperature caused a 2-fold increase ($P < 0.05$) in the mitochondrial aspartate concentration [the total cellular (P + S) aspartate concentration remained constant]. This effect was even more pronounced when the hepatocytes had been incubated in the presence of lactate plus alanine ($P < 0.005$). As for malate, it is tempting to assume that under conventional conditions of fractionation the transport of aspartate also is still going on, since the pellet fraction contained a significantly higher amount of aspartate ($P < 0.05$) (Table 4), when it was prepared at +5°C with glisoxepid, a known inhibitor of the aspartate carrier (Söling & Seck, 1975), present in the digitonin medium. At −5°C glisoxepid was virtually ineffective (Table 4).

Subcellular distribution of citrate

Experiments wtih hepatocytes incubated for 10 min with 8 mM-lactate revealed that lowering the fractionation temperature had no effect on the

amount of citrate, either in the pellet (1209 ± 53 and 1054 ± 32 nmol/g dry wt. at +5°C and −5°C respectively; $n = 7$) or in the supernatant fraction (1336 ± 294 and 1425 ± 222 nmol/g dry wt. at +5°C and −5°C respectively; $n = 7$). Further, benzene-1,2,3-tricarboxylate, an inhibitor of the tricarboxylate transporter (Robinson *et al.*, 1970), when present at 5 mM in the digitonin medium at both temperatures, did not influence the citrate concentration in the pellet fraction (results not shown).

Subcellular distribution of glutamate

With hepatocytes incubated for 10 min with 8 mM-lactate ($n = 13$) or 7 mM-alanine ($n = 13$) as the substrate no effect of the fractionation temperature on the amounts of glutamate in the subcellular fractions was demonstrable (results not shown). Consistent with this result is the finding that *N*-ethylmaleimide, an inhibitor of the glutamate carrier (Meijer *et al.*, 1972), did not change the glutamate concentration in the pellet fraction. This confirms and extends our previous results on the compartmentation of glutamate in hepatocytes from fed rats (Siess *et al.*, 1978).

Discussion

The present study shows that two of the most active carriers, namely those for malate and aspartate (Palmieri *et al.*, 1971; Tischler *et al.*, 1976), apparently display considerable activity at +5°C, yielding erroneously low amounts of these metabolites in the pellet fraction after conventional digitonin fractionation. The possibility that the higher amounts of aspartate and malate found in the pellets after fractionation at −5°C are caused by a mere increase in the carry-over of these metabolites from the cytosol seems unlikely, since only 0.27, 0.32 and 0.68% of labelled aspartate, sucrose and ADP respectively added to the digitonin medium were recovered in the pellet fraction after fractionation at −5°C. Further, if this were so, the effect of the transport inhibitors would have been superimposed. If these interpretations are valid, it appears that the mitochondrial concentrations of aspartate, malate and free oxaloacetate as reported earlier (Siess *et al.*, 1978) are underestimated by a factor of about 2. In this case also the values for the pH gradient across the mitochondrial membrane previously reported (Siess *et al.*, 1978) to be 0.14 and 0.06 for hepatocytes incubated in the presence of lactate and lactate plus oleate respectively have to be corrected. On the basis of the malate distribution, as revealed by the low-temperature digitonin method, ΔpH values of 0.25 and 0.20 for hepatocytes incubated with lactate and lactate plus oleate respectively result, which are close to those derived from the subcellular distribution of citrate (Siess *et al.*, 1978).

The results reported in the present study further pertain to the problem of the correct measurement of adenine nucleotide compartmentation by the digitonin method. From studies with isolated liver-mitochondria it was inferred (Soboll *et al.*, 1978) that digitonin fractionation at 2–5°C might yield erroneously high mitochondrial [ATP]/[ADP] ratios as a result of the continued phosphorylation of ADP. This view, however, is not compatible with our finding that lowering the temperature by 10°C during fractionation apparently does not decrease the [ATP]/[ADP] ratio in the mitochondrial pellet fraction (Table 2). Earlier studies had already shown that the subcellular distribution of adenine nucleotides was not influenced by the temperature of the digitonin medium, as long as it was kept below +6°C (Siess & Wieland, 1976). Moreover, the identity of the cellular [ATP]/[ADP] ratio of unfractionated liver cells with that arrived at by summing the adenine nucleotide contents in the subcellular compartments measured after fractionation (Siess *et al.*, 1978) provides strong evidence for the view that the high mitochondrial [ATP]/[ADP] ratio compared with that in the perfused liver (Elbers *et al.*, 1974; Soboll *et al.*, 1978) is a characteristic of the isolated hepatocytes rather than a failure of the digitonin method.

The excellent technical assistance of Mrs. C. Tews and Miss B. Kasparek is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, W. Germany.

References

Akerboom, T. P. M., Bookelman, H., Zuurendonk, P. F., van der Meer, R. & Tager, J. M. (1978) *Eur. J. Biochem.* **84**, 413–420

Chen, R. F. (1967) *J. Biol. Chem.* **242**, 173–181

Colbeau, A., Nachbaur, J. & Vignais, P. M. (1971) *Biochim. Biophys. Acta* **249**, 462–492

Elbers, R., Heldt, H. W., Schmucker, P., Soboll, S. & Wiese, H. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* **355**, 378–393

Hilderman, R. H., Goldblatt, P. J. & Deutscher, M. P. (1975) *J. Biol. Chem.* **250**, 4796–4801

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Meijer, A. J., Brouwer, A., Reijngoud, D. J., Hoek, J. B. & Tager, J. M. (1972) *Biochim. Biophys. Acta* **283**, 421–429

Meyer, A. J. & Tager, J. M. (1969) *Biochim. Biophys. Acta* **189**, 136–139

Palmieri, F., Prezioso, G., Quagliariello, E. & Klingenberg, M. (1971) *Eur. J. Biochem.* **22**, 66–74

Robinson, B. H. & Chapell, J. B. (1967) *Biochem. Biophys. Res. Commun.* **28**, 249–255

Robinson, B. H., Williams, G. R., Halperin, M. L. & Lezneff, C. C. (1970) *Eur. J. Biochem.* **15**, 263–272

Siess, E. A. & Wieland, O. H. (1975) *FEBS Lett.* **52**, 226–230

Siess, E. A. & Wieland, O. H. (1976) *Biochem. J.* **156**, 91–102

Siess, E. A., Brocks, D. G. & Wieland, O. H. (1976) *FEBS Lett.* **69**, 265–271

Siess, E. A., Brocks, D. G., Lattke, H. K. & Wieland, O. H. (1977) *Biochem. J.* **166**, 225–235

Siess, E. A., Brocks, D. G. & Wieland, O. H. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 785–798

Soboll, S., Scholz, R. & Heldt, H. W. (1978) *Eur. J. Biochem.* **87**, 377–390

Söling, H. D. & Seck, A. (1975) *FEBS Lett.* **51**, 52–59

Tischler, M. E., Pachence, J., Williamson, J. R. & La Noue, K. F. (1976) *Arch. Biochem. Biophys.* **173**, 448–462

Zuurendonk, P. F. & Tager, J. M. (1974) *Biochim. Biophys. Acta* **333**, 393–399

Zuurendonk, P. F., Akerboom, T. P. M. & Tager, J. M. (1976) in *Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies* (Tager, J. M., Söling, H. D. & Williamson, J. R., eds.), pp. 17–27, North-Holland, Amsterdam.