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RAPID SEPARATION OF PARTICULATE COMPONENTS AND SOLUBLE CYTOPLASM OF ISOLATED RAT-LIVER CELLS

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Summary

A method is described for the rapid separation of mitochondria (plus other particulate components) from the soluble cytoplasm of isolated rat-liver cells. The cells were incubated briefly with a low concentration of digitonin. After rapid centrifugation, the pellet contained more than 90% of the total adenylate kinase and glutamate dehydrogenase activities and the supernatant at least 80% of the lactate dehydrogenase activity. About 60% of total adenine nucleotides in hepatocytes were found in the soluble cytoplasm. The ATP/ADP ratio in the particulate fraction 80 s after exposure to digitonin of hepatocytes metabolizing alanine was 2.0—2.4, and that in the soluble cytoplasm 6—19. In the presence of atractyloside, these values were 3.5—4.4 and 1.3—2.2, respectively.

The redox state and the phosphate potential in different cellular compartments are major factors influencing the direction and flow of metabolic processes in the cell [1]. The metabolite indicator method [2—4] has been developed to estimate both the redox state [1—4] and the phosphorylation state [1, 5] in the mitochondrial and cytosolic compartments (see also refs 6—8). In order to apply this method, it is essential to know the concentration of metabolites in the two compartments.

However, the time required to separate the compartments by conventional methods is too long to be sure that no reactions or redistribution of metabolites have taken place [9]. Chamalaun [10] attempted to isolate

Abbreviation: MOPS, morpholinopropane sulphonic acid.

TABLE I

LEAKAGE OF LACTATE DEHYDROGENASE FROM RAT-LIVER CELLS AFTER INCUBATION WITH DIGITONIN

A suspension (1.5 ml) of rat-liver cells (8.1 mg dry weight of cells; 0 °C) was added to 5 ml of ice-cold medium containing 0.25 M sucrose, 20 mM MOPS buffer (pH 7.0), 3 mM EDTA and digitonin as indicated. After 10, 20 or 40 s, the suspension was rapidly centrifuged as described in the text. The lactate dehydrogenase activity in the supernatant is the percentage of the activity in supernatant plus pellet. The recovery of activities in supernatant plus pellet was 90–115% as compared with the stock suspension of cells.

Digitonin (mg)	Lactate dehydrogenase in supernatant (% of total) after incubation for		
	10 s	20 s	40 s
0	3	5	5
2.5	11	48	75
5	76	70	85

mitochondria from freeze-clamped liver at a low temperature in dimethyl sulphoxide containing media, but was unable to obtain intact mitochondria consistently. A more promising approach is that of Elbers et al. [11], who have isolated mitochondria from freeze-clamped liver with the aid of organic solvents. However, the method is laborious.

In the past two years, increasing use has been made of preparations of isolated liver cells to study hepatic metabolism [12–16], the cells usually being isolated by the method of Berry and Friend [12].

In this paper we describe a method for the rapid separation of mitochondria and soluble cytoplasm of isolated rat-liver cells, based on the fact that the cholesterol content of the plasma membrane is greater than that of the mitochondrial membranes [17] and that digitonin reacts specifically with cholesterol. The cells are incubated with digitonin at a concentration such that the plasma membrane is damaged whereas the mitochondrial membranes remain intact. This principle has been successfully applied to separate the inner and outer mitochondrial membranes [18, 19] and to deplete rat-liver mitochondrial preparations of lysosomes [20].

The procedure used was as follows. Rat-liver cells were isolated according to the collagenase–hyaluronidase method of Berry and Friend [12] with the following modification*: the perfusion medium was Krebs–Ringer bicarbonate buffer in which the phosphate concentration was 2.38 mM and Ca^{2+} was omitted. The cells were washed twice and taken up finally in Krebs–Ringer bicarbonate buffer containing 2.5% dialysed bovine serum albumin (Fraction V, Sigma Chemical Co.). The cells were either stored at 0 °C or incubated at 37 °C as indicated. A 1.5-ml sample of the cell suspension was rapidly mixed with 5 ml of an ice-cold medium (pH 7.0) containing 0.25 M sucrose, 20 mM potassium morpholinopropane sulphonate (MOPS), 3 mM EDTA and 0.5 or 1.0 mg/ml digitonin (Calbiochem.). After 10, 20 or 40 s, the suspension was centrifuged for a total time of approximately 20 s at 3000 x *g* maximum in an M.S.E. Mistral 6 L centrifuge using a swing-out rotor

*This modification was suggested by Prof. Sir H.A. Krebs and Dr P. Lund.

TABLE II

INTRACELLULAR DISTRIBUTION OF ADENINE NUCLEOTIDES IN RAT-LIVER CELLS

Rat hepatocytes (11 mg dry weight of cells) were incubated at 37 °C in a medium (1.5 ml) containing Krebs—Ringer bicarbonate buffer, 2.5% bovine serum albumin, 10 mM alanine and (where indicated) 150 μg atractyloside. After 5 min, the reaction mixture was diluted with 5 ml ice-cold medium containing 0.25 M sucrose, 20 mM MOPS buffer (pH 7.0), 3 mM EDTA and digitonin as indicated. After 20 or 40 s, the suspension was rapidly centrifuged as described in the text. For determination of adenine nucleotides the pellet and supernatant were acidified with $HClO_4$. In parallel incubations with digitonin, lactate dehydrogenase and adenylate kinase activities were assayed in pellet and supernatant. The weight of cells was determined after drying a sample at 110 °C for 18 h. It was assumed that 1 g wet weight of cells was equal to 220 mg dry weight.

Digitonin (mg)	Incubation time (s)	Particulate fraction				Soluble fraction				Particulate + soluble	% leakage of	
		ATP	ADP	AMP	Σ	ATP	ADP	AMP	Σ		lactate dehydrogenase	adenylate kinase
Atractyloside absent		(μmoles/g wet weight of cells)										
0	40	1.93	0.25	0.02	2.20	0.04	0.03	0.00	0.07	2.27	5	5
2.5	20	0.83	0.36	0.04	1.23	1.29	0.07	0.07	1.43	2.66	77	4
5	20	0.75	0.31	0.03	1.04	1.43	0.15	0.06	1.64	2.72	81	5
2.5	40	0.73	0.37	0.03	1.13	1.29	0.20	0.00	1.49	2.52	88	5
5	40	0.65	0.31	0.02	0.93	1.38	0.22	0.05	1.65	2.63	90	8
Atractyloside present												
0	40	1.38	0.33	0.22	1.93	0.00	0.00	0.00	0.00	1.93	6	4
2.5	20	0.83	0.19	0.10	1.12	0.53	0.42	0.25	1.20	2.32	50	5
5	20	0.86	0.25	0.07	1.18	0.49	0.35	0.22	1.06	2.24	87	5
2.5	40	0.99	0.27	0.07	1.33	0.49	0.32	0.22	1.03	2.36	81	7
5	40	0.99	0.25	0.08	1.32	0.80	0.37	0.20	1.37	2.69	88	5

TABLE III

ATP/ADP RATIO IN PARTICULATE AND SOLUBLE FRACTIONS OF RAT-LIVER HEPATOCYTES

Data taken from Table II. Abbreviation: Atract., atractyloside.

Digitonin (mg)	Incubation time (s)	ATP/ADP in particulate fraction		ATP/ADP in soluble fraction	
		— Atract.	+ Atract.	— Atract.	+ Atract.
2.5	20	2.3	4.4	19	1.3
5	20	2.4	3.5	10	1.4
2.5	40	2.0	3.7	6	1.5
5	40	2.1	4.0	6	2.2

(No. 59550). The temperature in the centrifuge was 0 °C.

For measuring the enzyme activities the pellets in one set of samples were suspended in sucrose—MOPS—EDTA medium to which Lubrol WX (I.C.I.; 0.3 mg per mg dry weight of cells) had been added. The supernatants were untreated. For determining adenine nucleotides, both pellets and supernatants in another set of samples were acidified with $HClO_4$ (6% final concentration). After removal of protein by centrifugation the extracts were neutralized with 2 M KOH—0.2 M phosphate. The total time taken for mixing the cell suspension with the digitonin medium, incubating for 40 s, centrifuging, and acidifying the media was approximately 80 s. Lactate dehydrogenase, adenylate kinase (more than 90% of the total adenylate kinase of the cell is found in the particulate fraction; see below) and glutamate dehydrogenase, which were used as markers for the cytosol [21], intermembrane space [22, 23] and mitochondrial matrix [24], respectively, were assayed by methods described in ref. 25. Adenine nucleotides were determined enzymically [25] at 350—375 nm using an Aminco—Chance Dual Wavelength spectrophotometer.

In Table I, the release of lactate dehydrogenase from rat-liver cells after incubation with digitonin is shown. Without digitonin little lactate dehydrogenase was released. At the lower level of digitonin the release of lactate dehydrogenase increased as the time of incubation was increased. As the level of digitonin was increased, more lactate dehydrogenase was found in the super natant. At still higher levels of digitonin the percentage of lactate dehydrogenase that leaked into the supernatant was maximally 90% (not shown). In the experiment of Table I, less than 2% of the total adenylate kinase and less than 1.5% of the total glutamate dehydrogenase appeared in the supernatant under the conditions tested.

These results show that brief incubation of the cells with low levels of digitonin, followed by centrifugation, leads to separation of cytosolic and mitochondrial components. The addition of EDTA was essential in order to obtain a satisfactory fractionation. In the absence of EDTA, longer incubation times were necessary in order to cause release of lactate dehydrogenase (not shown). Prolonged incubation led to damage of the outer mitochondrial membrane, as indicated by the fact the adenylate kinase appeared in the supernatant (not shown).

The digitonin-treated cells could be spun down using the same centrifugal force as for intact cells. Furthermore, electron microscopic examination of the precipitate revealed that it consisted of cells with a heavily damaged plasma membrane and with the other cell constituents embedded in endoplasmic reticulum (P.J. Weijers, unpublished observations). It is concluded that only the plasma membrane was damaged by the digitonin treatment and that the particulate constituents were spun down as a "leaky" cell.

Table II shows the distribution of adenine nucleotides between the particulate and soluble fractions of cells metabolizing alanine. With increasing amounts of digitonin and increasing incubation times, the total content of adenine nucleotides in the pellet reached a minimum (40% of total) and that in the supernatant a maximum (60% of total). The total adenine nucleotide content was similar to that found by Quistorff et al. [26] but somewhat lower than the value reported by Hommes et al. [27] for cells isolated by a different method.

In freeze-clamped whole livers, total adenine nucleotide contents of 2.7—3.7 μmoles/g wet weight have been reported [5, 28—31]. Since the adenine nucleotide content of isolated rat-liver mitochondria is 17 nmoles/mg protein [32] and since 1 g wet weight of liver contains 60 mg mitochondrial protein [33], it may be calculated that 28—38% of the total adenine nucleotides in whole liver is present in the mitochondrial fraction. The difference between this calculated value and that of 40% found by direct measurement (Table II) may be due in part to the fact that the isolated cell preparation consists of hepatocytes whereas whole liver contains a mixture of cell types, and in part to uncertainties in the calculation factors used. In addition, approximately 7% of the total adenine nucleotides in the cell is present in the nuclei [34].

Table II also shows that only 8% of the adenylate kinase is recovered in the supernatant after the digitonin treatment. The finding that adenylate kinase is predominantly a mitochondrial enzyme in rat-liver hepatocytes is supported by the observation of Pette [22] that adenylate kinase is extracted with difficulty from rat liver.

The ATP/ADP ratio in the particulate fraction was 2.0—2.4 and that in the soluble fraction 6—19 (Table III). Similar results have been obtained by Elbers et al. [11] in freeze-clamped liver fractionated with organic solvents. This difference in ATP/ADP ratio in the two compartments is much less than that predicted by results obtained with isolated mitochondria (see ref. 32 and 35).

When atractyloside was present, the ATP/ADP ratio in the particulate fraction increased to 3.5—4.4, whereas that in the soluble fraction decreased to 1.3—2.2 (Table III). This result is to be expected, since the adenine nucleotide translocator is inhibited, and cytosolic ATP would be rapidly used up in synthetic processes.

In the presence of atractyloside, the AMP content of the soluble fraction was greatly increased (Table II). The loss of total adenine nucleotides observed

in the soluble fraction in the presence of atractyloside (Table II) may be due to AMP deaminase (cf. ref. 14).

Since EDTA is present in the medium when the cells are exposed to digitonin, adenine nucleotide-dependent reactions in the cytosol will be inhibited as soon as the plasma membrane is damaged. Thus the ATP/ADP ratios found in the cytoplasm are probably very similar to those existing in the intact cell. On the other hand, the values observed in the particulate fraction are a less reliable measure of the situation in vivo, since changes may occur after addition of digitonin even at the low temperature used. It should be stressed that the measurements of adenine nucleotides give information on the mass—action ratio, which may not necessarily relate to the thermodynamic phosphate potential (see ref. 5).

We hope to obtain information about the distribution of other metabolites by carrying out the incubation with digitonin in the presence of inhibitors of mitochondrial substrate ion translocators. In addition, experiments are in progress aimed at reducing the time needed for separation still further.

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References

1 Krebs, H.A. and Veech, R.L. (1969) in The Energy Level and Metabolic Control in Mitochondria (Papa, S., Tager, J.M., Quagliariello, E. and Slater, E.C., eds), pp. 329—382, Adriatica Editrice, Bari
2 Holzer, H., Schultz, G. and Lynen, F. (1956) Biochem. Z. 328, 252—263
3 Bücher, Th. and Klingenberg, M. (1958) Angew. Chem. 70, 552—570
4 Hohorst, H.J., Kreutz, F.H. and Bücher, Th. (1959) Biochem. Z. 332, 18—46
5 Krebs, H.A. and Veech, R.L. (1970) in Pyridine Nucleotide-Dependent Dehydrogenases (Sund, H., ed), pp. 413—434, Springer Verlag, Berlin
6 Hoek, J.B. (1972) Glutamate dehydrogenase and the oxidoreduction state of nicotinamide nucleotides in rat-liver mitochondria. A problem of extrapolation, Ph.D. thesis, University of Amsterdam, Mondeel, Amsterdam
7 Williamson, J.R. (1969) in The Energy Level and Metabolic Control in Mitochondria (Papa, S., Tager, J.M., Quagliariello, E. and Slater, E.C., eds), pp. 385—400, Adriatica Editrice, Bari
8 Greenbaum, A.L., Gumaa, K.A. and McLean, P. (1971) Arch. Biochem. Biophys. 143, 617—663
9 Delaney, V.B. and Slater, T.F. (1970) Biochem. J. 116, 299—302
10 Chamalaun, R.A.F.M. (1971) Interrelationship between intra- and extramitochondrial processes during nitrogen metabolism in the perfused rat liver, M.D. thesis, University of Amsterdam, Academic Service, Amsterdam
11 Elbers, R., Heldt, H.W., Schmucker, P. and Wiese, H. (1972) Hoppe-Seylers Z. Phys. Chem. 353, 702—703
12 Berry, M.N. and Friend, D.S. (1969) J. Cell Biol. 43, 506—520
13 Berry, M.N. (1971) Biochem. Biophys. Res. Commun. 44, 1449—1456
14 Tager, J.M., Zuurendonk, P.F. and Akerboom, T.P.M. (1973) in Developmental Biochemistry — Inborn Errors of Metabolism (Hommes, F.A. and van den Berg, C.J., eds), pp. 177—200, Academic Press, London
15 Meijer, A.J. and Williamson, J.R. (1974) Biochim. Biophys. Acta, in the press
16 Krebs, H.A., Hems, R. and Lund, P. (1973) in Advances in Enzyme Regulation (Weber, G., ed.), Vol. 11, pp. 361—377, Pergamon Press, Oxford

17 Colbeau, A., Nachbaur, J. and Vignais, P.M. (1971) Biochim. Biophys. Acta 249, 462—492
18 Lévy, M., Toury, R. and André, J. (1967) Biochim. Biophys. Acta 135, 599—613
19 Schnaitman, C., Erwin, V.G. and Greenawalt, J.W. (1967) J. Cell Biol. 32, 719—735
20 Loewenstein, J., Scholte, H.R. and Wit-Peeters, E.M. (1970) Biochim. Biophys. Acta 223, 432—436
21 Delbrück, A., Schimassek, H., Bartsch, K. and Bücher, Th. (1959) Biochem. Z. 331, 297—311
22 Pette, D. (1966) in Regulation of Metabolic Processes in Mitochondria (Tager, J.M., Papa, S., Quagliariello, E. and Slater, E.C., eds), BBA Library, Vol. 7, pp. 28—49, Elsevier, Amsterdam
23 Klingenberg, M. and Pfaff, E. (1966) in Regulation of Metabolic Processes in Mitochondria (Tager, J.M., Papa, S., Quagliariello. E. and Slater, E.C., eds), BBA Library, Vol. 7, pp. 180—201, Elsevier, Amsterdam
24 Brdiczka, D., Pette, D., Brunner, G. and Miller, F. (1968) Eur. J. Biochem. 5, 294—304
25 Bergmeyer, H.A. (1970) in Methoden der Enzymatischen Analyse, Band I and Band II, Verlag-Chemie, Weinheim/Bergstr.
26 Quistorff, B., Bondesen, S. and Grunnet, N. (1973) Biochim. Biophys. Acta 320, 503—516
27 Hommes, F.A., Draisma, M.I. and Molenaar, I. (1970) Biochim. Biophys. Acta 222, 361—371
28 Heldt, H.W. (1963) Biochem. Z. 337, 397—413
29 Bücher. Th., Krejci, K., Rüssmann, W., Schnitger, H. and Wesemann, W. (1964) in Rapid Mixing and Sampling Techniques in Biochemistry (Chance, B., Eisenhardt, R.H., Gibson, Q.H. and Lonberg-Holm, K.K., eds), pp. 255—264, Academic Press, New York
30 Lagunas, R., McLean, P. and Greenbaum, A.L. (1970) Eur. J. Biochem. 15, 179—190
31 Veech, R.L., Raijman, L. and Krebs, H.A. (1970) Biochem. J. 117, 499—503
32 Slater, E.C., Rosing, J. and Mol, A. (1973) Biochim. Biophys. Acta 292, 534—553
33 Scholz, R. and Bücher, Th. (1965) in Control of Energy Metabolism (Chance, B., Estabrook, R.W. and Williamson, J.R., eds), pp. 393—414, Academic Press, New York
34 Siebert, G. (1961) Biochem. Z. 334, 369—387
35 Klingenberg, M., Heldt, H.W. and Pfaff, E. (1969) in The Energy Level and Metabolic Control in Mitochondria (Papa, S., Tager, J.M., Quagliariello, E. and Slater, E.C., eds), pp. 237—253, Adriatica Editrice, Bari