Dependence of mitochondrial and cytosolic adenine nucleotides on oxygen partial pressure in isolated hepatocytes

Application of a new rapid high pressure filtration technique for fractionation

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By using a new rapid high pressure filtration technique, mitochondrial and cytosolic ATP and ADP contents were determined in isolated hepatocytes at different oxygen partial pressures. At 670 mmHg, subcellular adenine nucleotide contents and ATP/ADP ratios were comparable with values obtained with the digitonin fractionation technique. However at lower oxygen partial pressure ADP appears to be rephosphorylated during digitonin fractionation whereas with high pressure filtration fractionation rephosphorylation of ADP is avoided due to shorter fractionation times. Cytosolic and mitochondrial ATP/ADP ratios decrease if oxygen partial pressure is lowered. However the absolute values of ATP/ADP ratios depend critically on the incubation conditions. Thus incubation of hepatocytes in an oxystat system, where oxygen partial pressure is maintained constant by infusing oxygen-saturated medium and the hepatocyte suspension is continuously stirred, yields much higher subcellular and overall ATP/ADP ratios than incubation in Erlenmeyer flasks gassed with different gas mixtures and shaken in a water bath. This is ascribed to limited diffusion of oxygen from the medium into the cell if the suspension is not mixed thoroughly by stirring. The strong dependence of subcellular ATP/ADP ratios on incubation conditions indicates that oxygen may be one rate-controlling factor for oxidative phosphorylation in the intact cell.

INTRODUCTION

Although the K_m of oxygen for respiration is below $1 \mu\text{M}$ in isolated mitochondria (Bienfait *et al.*, 1975; de Groot *et al.*, 1985), oxygen delivery to the liver cell in the intact organ is critical due to intercellular and intracellular oxygen gradients. The intercellular oxygen gradients along the liver sinusoids can be greater than two orders of magnitude (Ji *et al.*, 1980; Sies, 1977). Within the liver cell oxygen gradients of up to 7 mmHg (0.93 kPa) exist towards the mitochondrial compartment, depending on the rate of oxygen uptake (Jones & Mason, 1978; de Groot & Noll, 1987). The need for sufficient oxygen delivery *in vivo* is met by the help of haemoglobin, which has not only a high buffering capacity for oxygen but also is supposed to facilitate diffusion of oxygen in the intercellular space (Scholander, 1960). In haemoglobin-free liver perfusion high $p\text{O}_2$ and high flow rate through the liver are supposed to compensate for lack of haemoglobin (Scholz, 1967; Lübbers, 1968). However, as indicated by lower mitochondrial ATP contents in haemoglobin-free perfused livers than in livers *in vivo* or isolated hepatocytes, oxygen delivery is critical in this system (Schwenke *et al.*, 1981). On the other hand, hepatocytes incubated in Erlenmeyer flasks gassed with O_2/CO_2 (19:1) in a shaking water bath as commonly used appear to be exposed to an unphysiologically high $p\text{O}_2$ and could therefore be subject to oxidative damage (Chance *et al.*, 1979).

Several fractionation techniques were developed to study bio-energetics at the subcellular level: (i) the

fractionation of tissue in non-aqueous solvents, and (ii) fractionation of isolated cells with digitonin or shearing forces (for a review see Zuurendonk *et al.*, 1979). Both types of methods, however, are not suitable for the study of subcellular adenylates during hypoxia in liver; the former since it is easily applicable only in intact tissue and the latter since too much time elapses during the centrifugation of lysed or broken cells to avoid phosphorylation of ADP. Therefore we tried to develop a fractionation technique using high pressure filtration of isolated cells which allows separation of mitochondrial and cytosolic compartments within a few seconds. Fractionation of cells by high pressure filtration has already successfully been applied to plant protoplasts (Lilley *et al.*, 1982). We show here that, with a high pressure filtration technique, fractionation of cells is fast enough to avoid rephosphorylation of ADP even under hypoxic conditions. With this method it is possible to study for the first time the dependence of subcellular adenine nucleotide contents on oxygen partial pressure in intact cells. Very low oxygen partial pressures during the incubation were maintained by using the oxystat system developed by Noll *et al.* (1986).

MATERIALS AND METHODS

Isolation of hepatocytes

Hepatocytes were isolated from male albino rats (Wistar strain) of 180–200 g, fed *ad libitum* with Altromin standard diet (Altromin, Lage, Germany) with free

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access to drinking water. The isolation procedure was according to Berry & Friend (1969) with slight modifications (Sies *et al.*, 1977).

Incubation of hepatocytes in Erlenmeyer flasks

The cells were incubated for 15 min in Krebs–Henseleit bicarbonate buffer containing 2% bovine serum albumin, 10 mM-glucose, 2.1 mM-lactate, 0.3 mM-pyruvate, 0.6 mM-DL-3-hydroxybutyrate, 0.3 mM-acetoacetate and 10 mM-Hepes at 37 °C in polyethylene 25 ml Erlenmeyer flasks in a shaking water bath (frequency 120 min⁻¹).

The protein concentration was about 34 mg/ml. The suspension was equilibrated with the following gas mixtures: (a) O₂/CO₂ (19:1), (b) N₂/CO₂ (19:1).

Incubation of hepatocytes in an oxystat system

Hepatocytes were incubated in the medium described above at a concentration of 15 mg of protein/ml in a feedback-controlled oxystat system (Noll *et al.*, 1986). A selected *p*O₂ between 0.1 and 100 mmHg was maintained constant by injecting appropriate amounts of oxygen-saturated medium into the reaction chamber using a motor burette. Oxygen was monitored by a Clark-type oxygen electrode. The oxystat system was under computer control which read the oxygen sensor, interacted with the motor burette and calculated the oxygen uptake. Samples for total extracts were collected with a 1 ml Hamilton syringe containing HClO₄ and samples for fractionation were collected with a 1 ml Hamilton syringe containing medium cooled to about -4 °C and equilibrated with oxygen at the same *p*O₂ as the incubation.

Fractionation by high pressure filtration

Fig. 1 shows the apparatus used for high pressure filtration. It consisted of a fast coupling (polyethylene) which was connected to the gas bomb, the filtration chamber (Teflon, length 52 mm, inner diameter 10 mm) and a filter holder made from steel. Hepatocyte suspension (0.3 ml) was mixed with 0.7 ml of ice-cold incubation medium equilibrated with the gas mixture used for incubation (see above) and then quickly injected into the filtration chamber which was cooled to about -4 °C. By connecting the chamber to the gas bomb the suspension was pressed instantaneously through the filter combination at 350 kPa (3.45 atm) within 1–3 s into Eppendorf cups containing HClO₄ for metabolite extracts or phosphate buffer for marker enzymes. An aliquot of

0.5 ml was collected in the same way and deproteinized in HClO₄ without fractionation, by omitting the filters.

Three filters of 25 mm diameter were attached to the filter holder: (a) a membrane filter, pore size 10 µm and made from Teflon (Millipore), for cell disruption; (b) an absorption filter made from glassfibre (Sartorius); (c) a membrane filter, pore size 0.45 µm and made from polyvinylchloride (Millipore), to retain mitochondria. The Eppendorf cups collecting the filtrate were shaken in a rotation mixer (Eppendorf 33000) to assure fast mixing of the cytosolic fraction with HClO₄.

Assays

Determinations of activities of citrate synthase and lactate dehydrogenase as markers for mitochondrial and cytosolic compartments respectively and of adenine nucleotide contents were performed in total and cytosolic (filtrate) samples using enzymic analyses (Bergmeyer, 1970) with modifications (Soboll *et al.*, 1978). Protein was determined by the method of Lowry *et al.* (1951). Mitochondrial adenylate contents were obtained by subtracting cytosolic contents from contents in the whole cell.

Digitonin fractionation of hepatocytes (Zuurendonk & Tager, 1974)

Cell suspension (0.2 ml) from the same incubation used for high pressure filtration was mixed with fractionation medium containing 0.25 M-sucrose, 20 mM-Mops, 2 mM-EDTA and 2.4 mM-digitonin at -4 °C, equilibrated with the gas mixture used for incubation. After 30 s, 0.7 ml was centrifuged through a 0.5 ml silicone oil layer [AR 200 (Wacker Chemie)/SF 96/100 (General Electric), 8:1 v/v] into 0.12 ml of 1.88 M-HClO₄. The neutralized sediment was used for determination of mitochondrial adenine nucleotides, whereas 0.5 ml of the supernatant and 0.5 ml of the unfractionated cell suspension were deproteinized with HClO₄ and neutralized and used for cytosolic and total adenine nucleotide measurements, respectively.

Materials

All chemicals, including digitonin, were from Merck. Biochemicals and enzymes were from Boehringer-Mannheim and Sigma and were of the highest purity available. Bovine serum albumin (defatted, Fraction V, highest purity) was from Behring Werke.

RESULTS

High pressure filtration technique

Two fractions are obtained from the hepatocyte suspension after high pressure filtration: the filtrate and the filters. In both samples marker enzymes and protein were determined. The results are shown in Table 1; 50% of total lactate dehydrogenase and 7% of citrate synthase were found in the filtrate, assigning it to the cytosolic fraction. In contrast, the filters contained 70% of total citrate synthase and 30% of lactate dehydrogenase, indicating that most of the mitochondria were retained in the filters but also a considerable amount of the cytosolic fraction. Therefore mitochondrial metabolite contents cannot be obtained directly from the filters, but have to be calculated by subtraction of cytosolic from total contents. Taking together the amount of lactate dehydrogenase (citrate synthase) as well as protein content from

Fig. 1. Apparatus for high pressure filtration of hepatocytes

For details see the Materials and methods section.

Table 1. Activities of marker enzymes, metabolite and protein content in the cytosolic fraction of hepatocytes after fractionation by high pressure filtration $n = 40 (\pm \text{S.E.M.})$

	Total	% of total	
		Cytosol	Filter
Citrate synthase (m-unit/mg of total protein)	37 ± 0.5	7.5 ± 0.2	69 ± 0.6
Lactate dehydrogenase (m-unit/mg of total protein)	1741 ± 36	54 ± 0.7	28 ± 0.1
Protein content (mg/ml)	8.2 ± 1.5	21 ± 0.5	56 ± 0.5
Glucose 6-phosphate (nmol/mg of total protein)	0.6 ± 0.04	51 ± 4.5	21 ± 2.0

Table 2. Subcellular contents of adenine nucleotides at different oxygen partial pressures in hepatocytes: comparison of digitonin and high pressure filtration fractionation techniques $n = 10-20 \pm \text{S.E.M.}$ Contents are in nmol/mg of cell protein after incubation in Erlenmeyer flasks.

	Digitonin fractionation			High pressure filtration fractionation		
	ATP	ADP	ATP/ADP	ATP	ADP	ATP/ADP
0 mmHg						
Total	9.7 ± 0.4	5.0 ± 0.2	2.0 ± 0.1	8.8 ± 0.4	4.1 ± 0.2	2.1 ± 0.1
Mitochondrial	2.2 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.1 ± 0.4	2.3 ± 0.1	0.4 ± 0.2
Cytosolic	8.7 ± 0.5	1.3 ± 0.1	6.8 ± 0.1	7.8 ± 0.6	1.8 ± 0.1	4.4 ± 0.2
670 mmHg						
Total	12.6 ± 1.2	1.8 ± 0.2	6.5 ± 0.8	11.8 ± 0.3	2.0 ± 0.1	6.1 ± 0.1
Mitochondrial	2.4 ± 0.5	1.1 ± 0.2	2.5 ± 0.3	2.6 ± 0.2	1.2 ± 0.1	2.5 ± 0.2
Cytosolic	9.8 ± 1.6	0.7 ± 0.2	14.3 ± 3.4	9.3 ± 0.3	0.8 ± 0.1	12.3 ± 0.4

filtrate+filters yields about 80% of the total. The residual 20% is retained in the filter chamber.

The 7% of citrate synthase activity found in the filtrate indicates slight mitochondrial contamination. This was in the range of contamination found with the digitonin fractionation technique (Zuurendonk & Tager, 1974). Only 20% of total protein could be detected in the cytosolic fraction, since insoluble proteins were retained by the filters. Therefore, to calculate cytosolic adenylate contents, 50% of total protein (according to 50% of lactate dehydrogenase activity/mg of total protein in the filtrate) was taken as cytosolic, assuming that soluble cytosolic marker enzymes and metabolites were retained in the filters and the fractionation chamber to the same extent. This assumption was confirmed by finding 50% of glucose 6-phosphate, a cytosolic marker metabolite, and 50% of lactate dehydrogenase in the filtrate (Table 1).

Cytosolic and mitochondrial ATP and ADP contents obtained by high pressure filtration of hepatocytes incubated in a shaking water bath were compared with adenylate contents measured after digitonin fractionation (Table 2). With O_2/CO_2 (19:1) subcellular adenine nucleotide contents and ATP/ADP ratios were rather similar with both techniques. However, at zero oxygen subcellular ATP and ATP/ADP ratios are higher

when obtained with the digitonin technique compared with high pressure filtration. Further, after digitonin fractionation a higher total ATP/ADP ratio of 3.7 is calculated from the sum of mitochondrial and cytosolic ATP and ADP contents, respectively, than the directly measured ratio of about 2.

Phosphorylation state of ATP at low oxygen pressure

By using the oxystat system developed by Noll *et al.* (1986) we studied the dependence of cellular phosphorylation state of ATP on oxygen partial pressure. Half-maximal rate of respiration is observed at a $p\text{O}_2$ of about 0.7 mmHg, whereas half-maximal ATP/ADP ratio and ATP content is reached at a $p\text{O}_2$ of 1.4 mmHg (Figs. 2 and 3). These values are lower by a factor of 3–5 than those found by Jones & Mason (1978), probably due to thorough mixing of oxygen with the hepatocyte suspension in our system. The cellular ATP/ADP ratios obtained in this system are much higher than the values normally found in hepatocyte suspensions incubated in a shaking water bath and gassed with O_2/CO_2 (19:1). Thus values of about 5 are already reached at 1 mmHg, whereas at 100 mmHg, which is considerably below the $p\text{O}_2$ of O_2/CO_2 (19:1), values of 10 or even higher were measured.

The subcellular ATP contents and ATP/ADP ratios

Fig. 2. Dependence of oxygen consumption and cellular ATP/ADP ratios of isolated hepatocytes on oxygen partial pressure

Hepatocytes were incubated in an oxystat system. For details see the Materials and methods section.

Fig. 3. Cellular ATP and ADP contents at different pO_2 in isolated hepatocytes

Values are from the same experiments as in Fig. 2.

change in the same direction as the total ratios. They decrease from 12.8 in the cytosol (3.5 in the mitochondria) at 2 mmHg to 4.4 (0.4) at zero pO_2 (Tables 2 and 3).

DISCUSSION

High pressure filtration

The sum of mitochondrial+cytosolic ATP (ADP) obtained at 0 mmHg by digitonin fractionation yields 112% (58%) of total contents, respectively. This indicates that phosphorylation of ADP has occurred during fractionation, since digitonin fractionation of

Table 3. Subcellular contents of adenine nucleotides at low oxygen partial pressures in hepatocytes incubated in an oxystat system

High pressure filtration fractionation was used. Contents are in nmol/mg of cell protein; $n = 8-12 \pm \text{s.e.m.}$

	ATP	ADP	ATP/ADP
0.5 mmHg			
Total	7.9 ± 0.3	3.1 ± 0.2	2.6 ± 0.2
Mitochondrial	2.8 ± 0.2	2.3 ± 0.1	1.2 ± 0.1
Cytosolic	5.1 ± 0.4	0.8 ± 0.1	6.4 ± 0.3
2 mmHg			
Total	12.7 ± 0.4	2.0 ± 0.1	6.3 ± 0.4
Mitochondrial	5.0 ± 0.9	1.4 ± 0.2	3.5 ± 0.3
Cytosolic	7.9 ± 0.6	0.6 ± 0.1	12.8 ± 0.5

cells lasts 15–30 s and phosphorylation of ADP occurs still at a considerable rate at 0 °C (Heldt & Klingenberg, 1968). This is avoided by the shorter fractionation time with high pressure filtration (Table 2). The high pressure filtration technique, however, has the disadvantage that mitochondrial values are not obtained by direct measurement but by subtraction of cytosolic contents from the total contents. Therefore it is not possible to demonstrate directly that phosphorylation of ADP does not occur. On the other hand, total ATP/ADP ratios in liver cell extracts which were subjected to the same procedure as cells used for high pressure filtration, except that the filters were omitted from the chamber, yielded values identical to those obtained from direct $HClO_4$ extraction of the hepatocyte suspension (see the Materials and methods section). Thus phosphorylation of ADP during high pressure filtration seems to be improbable.

Phosphorylation state of ATP at very low oxygen pressure

From a comparison of ATP/ADP ratios obtained in hepatocytes incubated in Erlenmeyer flasks with those reached in the oxystat system, it appears that even in Erlenmeyer flasks large extracellular oxygen gradients are built up, whereas intracellular oxygen gradients seem to be very small. By simply shaking cell suspensions, aggregation of cells cannot be avoided and in addition unstirred layers of oxygen-depleted medium arise around the cell due to cellular oxygen consumption, which are removed by gently stirring and mixing the hepatocyte suspension with oxygen-saturated medium in the oxystat system.

Therefore it can be concluded that hepatocytes incubated in Erlenmeyer flasks are not exposed to an unphysiologically high oxygen pressure due to aggregation of cells and oxygen gradients within the suspension. A similar situation may be experienced by liver cells within the liver tissue. On the other hand, although the cytochromes of the respiratory chain appear to be fully oxidized *in vivo* (Sies, 1977), indicating saturation of the respiratory chain with oxygen, it has to be concluded that under physiological conditions cellular ATP synthesis is also determined by oxygen supply.

Subcellular contents of adenine nucleotides at low oxygen pressure

The subcellular distribution of ATP and ADP is not only dependent on the rate of ATP-synthesis and

breakdown, but also on adenine nucleotide translocation across the mitochondrial membrane connecting mitochondrial and cytosolic adenine nucleotide systems. Thus inhibition of adenine nucleotide transport leads to a decrease in cytosolic ATP/ADP ratio and an increase in the mitochondrial ratio. This has been demonstrated also for isolated hepatocytes (Soboll *et al.*, 1984). On the other hand, if adenine nucleotide transport exceeds the rate of ATP synthesis in mitochondrial oxidative phosphorylation, the mitochondrial ATP/ADP ratio should be rather low, whereas the cytosolic ratio then depends on the rate of adenine nucleotide supply and consumption. Our experiments show that if mitochondrial ATP synthesis is limited by oxygen supply, mitochondrial as well as cytosolic ATP contents and ATP/ADP ratios depend on pO_2 in a similar way. They decrease if oxygen is lowered. Therefore it can be suggested that the capacity of mitochondrial oxidative phosphorylation and adenine nucleotide transport are in the same order of magnitude and should be both subject to regulation. This is in agreement with kinetic studies on mitochondrial respiration and adenine nucleotide transport in isolated mitochondria (Klingenberg, 1976). A quantification of the contribution of the various steps in oxidative phosphorylation to their control by using the control theory developed by Kacser & Burns (1973) showed that the share in control is mainly divided between cytochrome oxidase, adenine nucleotide transport and extramitochondrial ATP-consuming processes (Groen *et al.*, 1982).

Consequently, it appears that an important factor in regulating the overall rate of oxidative phosphorylation is the availability of its substrates, i.e. ADP, NADH and oxygen, whereas the capacities of oxidation and phosphorylation reactions seem to be of minor regulatory importance since they are of the same order of magnitude and high compared with the capacities of other cellular metabolic pathways.

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REFERENCES

- Bergmeyer, H. U. (1970) in *Methoden der Enzymatischen Analyse*, 2nd edn., Verlag Chemie, Weinheim

- Berry, M. N. & Friend, D. S. (1969) *J. Cell. Biol.* **43**, 506–520
 Bienfait, H. F., Jacobs, J. M. C. & Slater, E. C. (1975) *Biochim. Biophys. Acta* **376**, 446–457
 Chance, B., Sies, H. & Boveris, A. (1979) *Physiol. Rev.* **59**, 527–605
 Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., van der Meer, R. & Tager, J. M. (1982) *J. Biol. Chem.* **257**, 2754–2757
 de Groot, H., Noll, T. & Sies, H. (1985) *Arch. Biochem. Biophys.* **243**, 556–562
 de Groot, H. & Noll, T. (1987) *Biochem. Soc. Trans.* **15**, 363–365
 Heldt, H. W. & Klingenberg, M. (1968) *Eur. J. Biochem.* **4**, 1–8
 Ji, S., Lemasters, J. J. & Thurman, R. G. (1980) *FEBS Lett.* **113**, 37–41
 Jones, D. P. & Mason, H. S. (1978) *J. Biol. Chem.* **253**, 4874–4880
 Kacser, H. & Burns, J. A. (1973) in *Rate Control of Biological Processes* (Davies, D. D., ed.), pp. 65–104, Cambridge University Press, London
 Klingenberg, M. (1976) in *The Enzymes of Biological Membranes* (Martonosi, A. H., ed.), vol. 3, pp. 383–438, Plenum, New York and London
 Lilley, R. McC., Stitt, M., Mader, G. & Heldt, H. W. (1982) *Plant Physiol.* **70**, 965–970
 Lowry, O. H., Rosebrough, N. J., Farr, A. C. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
 Lübbers, D. W. (1968) in *Biochemie des Sauerstoffs* (Hess, B. & Staudinger, H. J., eds.), pp. 67–92, Springer Verlag, Berlin, Heidelberg and New York
 Noll, T., de Groot, H. & Wissemann, P. (1986) *Biochem. J.* **236**, 765–769
 Scholander, P. F. (1960) *Science* **131**, 585–590
 Scholz, R. (1965) in *Control of Energy Metabolism* (Chance, B., Estabrook, R. W. & Williamson, J. R., eds.), pp. 393–414, Academic Press, New York and London
 Schwenke, W. D., Soboll, S., Seitz, H. J. & Sies, H. (1981) *Biochem. J.* **200**, 405–408
 Sies, H. (1977) *Hoppe-Seyler's Z. Physiol. Chem.* **358**, 1021–1032
 Sies, H., Akerboom, T. P. M. & Tager, J. M. (1977) *Eur. J. Biochem.* **72**, 301–307
 Soboll, S., Seitz, H. J., Sies, H., Ziegler, B. & Scholz, R. (1984) *Biochem. J.* **220**, 371–376
 Soboll, S., Scholz, R. & Heldt, H. W. (1978) *Eur. J. Biochem.* **87**, 377–390
 Zuurendonk, P. F. & Tager, J. M. (1974) *Biochim. Biophys. Acta* **333**, 393–399
 Zuurendonk, P. F., Tischler, M. E., Akerboom, T. P. M., Van der Meer, R., Williamson, J. R. & Tager, J. M. (1979) *Methods Enzymol.* **55**, 207–223

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