Characterization of a rapid cellular-fractionation technique for hepatocytes

Application in the measurement of mitochondrial membrane potential *in situ*

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A rapid cellular-fractionation technique [Hoek, Nicholls & Williamson (1980) *J. Biol. Chem.* **255**, 1458–1464] was further characterized by using hepatocytes. Of the mitochondrial marker-enzyme activity, 80% was routinely separated from 71–98% of the total cell activities of marker enzymes for plasma membranes, Golgi-membranes, endoplasmic reticulum, lysosomes and cytosol. The mitochondria were contaminated with 53% of cell nuclei. [^3H]Triphenylmethylphosphonium ion (TPMP $^+$) was added to hepatocytes in an attempt to measure cellular transmembrane electrical potentials. After rapid cell fractionation the electrical potential between mitochondria *in situ* and the incubation medium was found to be 202 mV. This value was slightly increased when hepatocytes were treated with oligomycin, but substantially decreased by oligomycin plus an uncoupler of oxidative phosphorylation. Although estimates of TPMP $^+$ binding were obtained, substantial difficulties prevented the accurate measurement of the electrical potential across the plasma membrane. It is concluded that TPMP $^+$ may be employed to demonstrate the integrity of mitochondria during the fractionation procedures. However, the cation is inadequate for the determination of the separate components of the electrical potential between the mitochondrial matrix and the incubation medium.

Methods that achieve substantial subcellular fractionation of cells within a very short period would be of widespread value for the analysis of hormone effects on intracellular compartments. One such method, described by Hoek *et al.* (1980), permits the isolation of a mitochondria-rich fraction from hepatocytes within seconds. This technique combined the effects of digitonin lysis of non-mitochondrial membranes (Zuurendonk & Tager, 1974) with mechanical disruption by an extrusion press (Tischler *et al.*, 1977). A particulate fraction, enriched in mitochondria, was separated from the cell extract by centrifugation through a layer of silicone oil in a specially constructed rotor (Scott *et al.*, 1980).

Murphy *et al.* (1980) used this technique to measure changes in mitochondrial Ca content after hormonal stimulation of hepatocytes. We wished

to undertake similar experiments [see the following paper (Shears & Kirk, 1984)], but we were concerned about two aspects of the technique. Firstly, neither Hoek *et al.* (1980) nor Murphy *et al.* (1980) reported on the extent to which the 'mitochondrial' fraction might be contaminated by nuclei, Golgi apparatus or plasma membranes, all of which may contain Ca (Bygrave, 1978). Our second criticism arises from the measurements of TPMP $^+$ uptake by hepatocytes and mitochondria-rich fractions and thus the estimation of cellular membrane potentials (Hoek *et al.*, 1980). These workers did not account for the possibility that albumin in their incubations might bind TPMP $^+$, as others have noted (Davis & Martin, 1982). In addition, Hoek *et al.* (1980) did not detect a significant proportion of TPMP $^+$ binding by isolated liver mitochondria, whereas Wilson & Forman (1982) indicate that as much as 66% of mitochondrial TPMP $^+$ may be bound. It is clear from other studies (Scott & Nicholls, 1980; Davis & Martin, 1982) that errors in the determination of TPMP $^+$ binding will lead to erroneous estimates of cellular membrane potentials. This is important, because the ability to

Abbreviations used: CCFP, carbonyl cyanide *p*-tri-fluoromethoxyphenylhydrazine; Tes, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulphonic acid; TPMP $^+$, triphenylmethylphosphonium ion; Ca and Ca $^{2+}$ are used to signify total calcium and ionized calcium respectively.

measure cellular transmembrane potentials would represent a valuable tool for the investigation of hormone effects on these parameters.

In the present paper we have further characterized the rapid fractionation technique of Hoek *et al.* (1980) with a view to assessing the importance of the criticisms outlined above.

Experimental

Methods

Preparation and incubation of hepatocytes. Hepatocytes were prepared from fed male Wistar rats (200–250 g). The animals were anaesthetized with diethyl ether, and livers were perfused at 37°C (Berry & Friend, 1969) for 5 min at a flow rate of 30 ml/min with Ca²⁺-free Krebs–Ringer bicarbonate buffer (111 mM-NaCl/25 mM-NaHCO₃/4.7 mM-KCl/1.2 mM-KH₂PO₄/1.2 mM-MgSO₄; Krebs & Henseleit, 1932) containing 5 mM-Tes. The medium was continually gassed with O₂/CO₂ (19:1). The following were then added to the final concentrations indicated: CaCl₂ (2 mM), bovine serum albumin (2%, w/v) and collagenase (0.03–0.05%, w/v). The perfusion was continued for 25 min at a flow rate of 35–40 ml/min before excising the liver capsule into a plastic beaker. The isolated cells were washed three times at ambient temperature by suspension (10%, v/v) in the above-mentioned gassed Krebs–Ringer/Tes buffer followed by acceleration to 500 g for 5 s in a bench centrifuge. The hepatocytes were incubated at 37°C as 2 ml portions in stoppered plastic vials in the presence of Krebs–Ringer buffer/5 mM-Tes/10 mM-glucose/2% (w/v) bovine serum albumin/1.25 mM-CaCl₂. The cells were gassed every 15 min with O₂/CO₂ (19:1). Hepatocyte integrity was not significantly different from that previously described (Creba *et al.*, 1983). In addition, hepatocytes were found to release 5 ± 0.6%, 6 ± 0.7% and 8 ± 0.9% (*n* = 6) of their cellular lactate dehydrogenase activity after 0.25, 1 and 2 h respectively.

Separation of intact cells from the incubation medium. Samples (0.2–0.7 ml) of cell suspension were placed on 0.5 ml of silicone oil [*d* = 1.045; a 1:1 (v/v) mixture of AP100 and AR20; Wacker Chemicals Ltd., Walton-on-Thames, Surrey, U.K.] which was, unless otherwise stated, layered above 0.1 ml of 2 M-HClO₄ in 1.5 ml plastic centrifuge tubes. These samples were centrifuged at 12000 g for 20 s in a microcentrifuge (Jobling and Co., Stone, Staffordshire, U.K.). The extent of contamination of the lower phase with incubation medium was noted in parallel separations of hepatocytes incubated (for up to 10 min) with 0.2 μCi of ³H₂O/ml plus 0.04 μCi of [U-¹⁴C]-sucrose/ml. After assaying radioactivity (see below) in the two aqueous phases, total intracellular water was computed to be 0.86 ± 0.04 μl/mg dry

wt; the lower phase was contaminated by 0.75 ± 0.07 μl of extracellular medium/mg dry wt. (data from nine hepatocyte preparations).

Hepatocyte fractionation technique. This procedure combines the effects of digitonin lysis and mechanical disruption (Hoek *et al.*, 1980). Samples (0.9 ml) of the cell suspension were added to 0.18 ml of 70 mM-Tes/70 mM-EGTA/0.5 mM-Ruthenium Red and, unless otherwise indicated, 8.3 mg of digitonin/ml. The suspension was immediately drawn into a 1 ml plastic syringe via a 17 mm length of 0.1 mm-internal-diameter silicone tubing. The upward movement of the syringe plunger was driven by a source of compressed air. Within 5 s of mixing with digitonin, the suspension was forced through a 23-gauge needle with a pressure of 2.8 kg/cm², generated by the compressed air. The suspension was then delivered, by a 3.8 mm length of 0.5 mm-internal-diameter silicone tubing, into a specially constructed swing-out rotor (Scott *et al.*, 1980) rotating at full speed (14000 g), in a Hawksley Micro-haematocrit microcentrifuge (Hawksley and Sons, Lancing, Sussex, U.K.). The final volume of the extract was decreased to 0.9 ml by the 'dead' volume in the fractionation apparatus. This suspension was directed into two 1.5 ml centrifuge tubes. These contained, unless otherwise indicated, 0.1 ml of 2 M-HClO₄ below 0.5 ml of silicone oil [AP100/AR20, 1:1 (v/v)]. Centrifugation was usually continued for 20 s. Samples were removed from the lower aqueous phase by inserting a microsyringe through the oil.

Contamination of the lower phases with supernatant from above the oil was estimated to be 1.34 ± 0.03 μl/mg dry wt. (*n* = 7) in parallel fractionations of hepatocytes preincubated for up to 10 min in the presence of 0.2 μCi of ³H₂O/ml and 0.04 μCi of [U-¹⁴C]sucrose/ml.

Estimation of the relative mitochondrial volume of hepatocytes. Hepatocytes were preincubated for 5 min in bicarbonate-buffered medium (Krebs & Henseleit, 1932) plus 5 mM-Tes, 10 mM-glucose, 1.25 mM-CaCl₂, 2% (w/v) bovine serum albumin, 0.2 μCi of ³H₂O/ml and 0.04 μCi of [U-¹⁴C]-sucrose/ml. The following were then added to the concentrations indicated: Ruthenium Red (0.08 mM), EGTA (12 mM), Tes (12 mM) and digitonin (0.15–0.2 mg/mg cell dry wt.). After a further 0.5–1 min, samples (0.5 ml) of the suspension were taken and the detergent-treated cells were centrifuged through silicone oil into 2 M-HClO₄. The residual supernatants always contained over 98% of the cell's lactate dehydrogenase and less than 2.5% of glutamate dehydrogenase. In four preparations of hepatocytes the sucrose-impermeable space was found to be 0.089 ± 0.01 μl/mg cell dry wt. This value represents 10% of cell volume, and was not significantly affected by phenylephrine, CCFP or

oligomycin. This result compares favourably with the relative mitochondrial volume of 12% found by Hoek *et al.* (1980).

Enzyme assays. Lactate dehydrogenase (EC 1.1.1.27) was assayed as described by Bergmeyer & Bernt (1974) in the presence of 1 μ M-rotenone. Glutamate dehydrogenase (EC 1.4.1.2) was measured as suggested by Schmidt (1974), except that the buffer was 50 mM-potassium phosphate, pH 7.4, as employed by McCarthy *et al.* (1980), and 1 μ M-rotenone was present. Aryl esterase (EC 3.1.1.2) and β -glucuronidase (EC 3.2.1.31) were assayed as described by Shephard & Hubscher (1969). Adenylate cyclase activity (EC 4.6.1.1) was determined by the method of Houslay *et al.* (1976); glucagon-stimulated activity could not be reliably measured, because of its inhibition by digitonin (Birnbaumer *et al.*, 1971; S. B. Shears & C. J. Kirk, unpublished work). Galactosyltransferase (EC 2.4.1.38) was measured by the method of Bretz & Stäubli (1977). DNA concentrations were determined (Burton, 1956) after extraction of DNA as described by Schneider (1957). Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard. All enzyme activities were assayed at 25°C, except adenylate cyclase (30°C) and galactosyltransferase and β -glucuronidase (37°C).

Enzyme activity in the incubation medium after cell fractionation (see above) was determined at least 30 min after the addition of Triton X-100 (0.5%, v/v, final concn.) to the supernatant and particulate material which remained above the silicone oil. [Triton was replaced by digitonin (1.4 mg/ml) for the assay of adenylate cyclase, since the former detergent inhibited this enzyme]. The total cellular activity of these enzymes was determined at least 30 min after the addition of 0.1 ml portions of the original cell suspension to 0.9 ml of bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing either 0.5% (v/v), Triton X-100 or, for adenylate cyclase assays, 1.4 mg of digitonin/ml. The medium also contained 12 mM-Tes, 12 mM-EGTA and 0.08 mM-Ruthenium Red, although these additions did not affect any of the measured enzyme activities (results not shown). Extracts were stored at 0–4°C before assaying for the various enzyme activities.

Enzyme activity in the mitochondria-rich fraction was assayed in samples where the aqueous phase below the silicone oil was 400 mM-sucrose rather than 2 M-HClO₄. Samples were stored at –20°C and the oil layer was poured away while maintaining the sucrose in a frozen state. Total enzyme recovery in the two phases above and below the silicone oil amounted to 95–108% of the activity in unfractionated cells. DNA recovery was 107%.

Assay of radioactivity. Aqueous samples were adjusted to 0.5 ml with water. A 5 ml portion of scintillant was added {xylene/Triton X-100 (2:1, v/v) plus 0.6% (w/v) PPO (diphenyloxazole) and 0.015% (w/v) POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]} and samples were counted for radioactivity in a liquid-scintillation spectrometer.

Assay of K⁺. Concentrations of K⁺ were determined by flame photometry in the presence of 5 mM-LiCl (Amoore *et al.*, 1958).

Isolation and incubation of liver mitochondria. Mitochondria were prepared by differential centrifugation in 0.25 M-sucrose (Shears & Bronk, 1979). The organelles were incubated at 30°C in a medium consisting of 120 mM-sucrose, 10 mM-sodium phosphate, pH 7.4, 5 mM-MgCl₂ and 5 mM-sodium succinate.

Materials

All chemicals were of the highest grade commercially available. The following were purchased from Sigma, Poole, Dorset, U.K.: ADP, Alumina WN3, cyclic AMP, bovine serum albumin (Fraction V), CCFP, collagenase, digitonin, EGTA, oligomycin, rotenone, Ruthenium Red, TPMP⁺, valinomycin and all enzymes and enzyme substrates. Before use, the bovine serum albumin was dialysed against four changes of Ca²⁺-free incubation medium (Krebs & Henseleit, 1932), and stored in portions at –20°C. The concentration was assessed routinely by spectrophotometry, assuming $A_{280}^{1\%} = 6.6$ (Kolthoff *et al.*, 1965). Digitonin was dissolved in ethanol at 75°C and recrystallized by cooling to 0–4°C. Ruthenium Red was also recrystallized (Fletcher *et al.*, 1961).

All radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Triton X-100, PPO and POPOP were supplied by BDH, Poole, Dorset, U.K. The silicone oil was provided by Wacker Chemicals Ltd. The specially constructed swing-out rotor was purchased from Dr. D. G. Nicholls, Department of Psychiatry, University of Dundee, Ninewells Hospital, Dundee, Scotland, U.K. The cyclic AMP-binding protein was donated by Dr. G. Smith, Department of Medicine, Hope Hospital, Salford, Lancs., U.K.

Results and discussion

Profile of enzyme activity in the supernatants obtained after rapid cell fractionation: effects of digitonin concentration

During rapid cell fractionation the concentration of digitonin was a critical determinant of the pattern of enzyme activity recovered from the material remaining above the silicone oil, which includes a particulate fraction (Fig. 1). In the presence of 0.1–0.2 mg of digitonin/mg cell dry wt., 13–

Fig. 1. Recovery of lactate dehydrogenase, glutamate dehydrogenase and aryl esterase from above the silicone oil after rapid cell fractionation: the influence of digitonin concentration

Hepatocytes were incubated and fractionated as described under 'Methods'. Data are averages for duplicate fractionations at the indicated concentrations of digitonin, from eight preparations of hepatocytes. Total cellular activities of lactate dehydrogenase, glutamate dehydrogenase and aryl esterase were respectively 0.74 ± 0.01 , 0.50 ± 0.006 and 0.33 ± 0.05 μmol of product formed/min per mg cell dry wt. ($n = 8$). Oligomycin (5 mg/ml) and CCFP (10 μM) did not affect enzyme release when added to the incubations for up to 20 min before fractionation (results not shown).

24% of total cellular glutamate dehydrogenase activity was found to be present in this phase; in eight preparations of hepatocytes, $80 \pm 1\%$ of the glutamate dehydrogenase was centrifuged through the silicone oil. Within the same range of digitonin concentrations, over 95% of total cellular lactate dehydrogenase activity was recovered above the silicone oil. This indicates that less than 5% of intact cells contaminated the mitochondria-rich frac-

tions. The upper phase was also assayed for aryl-esterase, and at least 82% of the total cellular activity of this enzyme was recovered in this fraction. It therefore appears that the mitochondria-rich fractions will contain less than 18% of the cell's endoplasmic reticulum. If the concentration of digitonin was decreased to below 0.1 mg/mg cell dry wt., then less lactate dehydrogenase and aryl esterase activity was detected in the material remaining above the oil. This indicates that greater proportions of intact endoplasmic reticulum and/or intact cells were centrifuged through the silicone oil. When more than 0.2 mg of digitonin/mg cell dry wt. was employed, the proportion of broken mitochondria increased, as shown by the enhanced release of glutamate dehydrogenase (Fig. 1). It was concluded that 0.1–0.2 mg of digitonin/mg cell dry wt. was optimal for the separation of mitochondria from both intact cells and endoplasmic reticulum. In further experiments (not shown) we observed that 69% of the proportion of mitochondria which sedimented through the silicone oil did so within 5 s of their delivery into the centrifuge.

Hoek *et al.* (1980) were the first to combine the effects of digitonin lysis and mechanical disruption in order to achieve rapid fractionation of hepatocytes. At a digitonin concentration of 0.2 mg/mg cell dry wt., Hoek *et al.* (1980) separated 85% of intact mitochondria from 90% of intact cells, as judged by enzyme release. Therefore our fractionation data indicate a more extensive disruption of hepatocytes, but at the expense of slightly greater damage to the population of cell mitochondria. Murphy *et al.* (1980) used this rapid fractionation technique to separate 95% of cellular glutamate dehydrogenase activity from 85–95% of marker enzymes for cytosol and endoplasmic reticulum. We were unable to reproduce their data either by manipulating the concentration of digitonin (Fig. 1) or by modifying the output pressure through the extrusion needle (results not shown).

Employing the optimum concentration range for digitonin (0.1–0.2 mg/mg cell dry wt.) we analysed the distribution, after fractionation, of markers for Golgi membranes (galactosyltransferase), plasma membranes (adenylate cyclase), lysosomes (β -glucuronidase) and nuclei (DNA). Table 1 demonstrates that the rapid fractionation technique may be suitable for the measurement of Ca content of mitochondria, since these organelles were separated from over 70% of the cellular activities of markers for Golgi and plasma membranes. These results indicate that Ca in these fractions should contaminate mitochondrial Ca to only a minor extent. Table 1 also indicates that 53% of cell nuclei are associated with the mitochondria-rich fraction. Nuclei contain Ca (Bygrave, 1978), but it is likely that the nuclear pores would permit

Table 1. *Distribution of various intracellular markers after rapid cell fractionation*

Hepatocytes were fractionated in the presence of 0.1–0.2 mg of digitonin/mg cell dry wt., and the resultant supernatants were analysed. For incubation conditions, see legend to Fig. 1, except that protein release was determined in the absence of bovine serum albumin, which did not affect glutamate dehydrogenase or lactate dehydrogenase release. Data are means $\pm$ S.E.M. for the number of hepatocyte preparations in parentheses.

Marker	Activity or concentration in supernatant after fractionation	Total cellular activity or concentration	Release into supernatant (%)
Galactosyltransferase (3)	3.96 ± 0.34 pmol/min per mg dry wt.	5.32 ± 0.58 pmol/min per mg dry wt.	74
Adenylate cyclase (3)	1.70 ± 0.38 pmol/min per mg dry wt.	2.41 ± 0.45 pmol/min per mg dry wt.	71
β -Glucuronidase (5)	4.0 ± 0.71 nmol/min per mg dry wt.	5.2 ± 0.13 nmol/min per mg dry wt.	77
Protein (3)	0.47 ± 0.1 mg/mg dry wt.	0.79 ± 0.04 mg/mg dry wt.	59
DNA (4)	1.29 ± 0.26 μ g/mg dry wt.	2.77 ± 0.3 μ g/mg dry wt.	47

chelation of exchangeable Ca^{2+} by EGTA during fractionation. Hence only non-exchangeable nuclear Ca could contribute to the Ca content of the mitochondria-rich extract. An analysis of the maximum size of the non-exchangeable nuclear Ca pool may be achieved, since there is no known mechanisms by which it would be expected to be sensitive to uncouplers, in contrast with at least a proportion of the mitochondrial Ca pool (Nicholls, 1978). There is, however, a complication in that some mitochondrial Ca is retained in the presence of CCFP (Reed & Bygrave, 1974; S. B. Shears & C. J. Kirk, unpublished work). This matter is discussed further in the following paper (Shears & Kirk, 1984). Although Murphy *et al.* (1980) may have overestimated mitochondrial Ca content owing to nuclear contamination, nuclei have not, as yet, been implicated as a source of hormone-mobilized Ca. The usefulness of the fractionation technique for studying hormonal effects upon mitochondrial Ca depends upon there being no significant Ca redistribution during fractionation. This problem is critically assessed in the following paper (Shears & Kirk, 1984).

Table 1 also shows that, after cell fractionation, 77% of the total cell activity of β -glucuronidase was separated from the mitochondrial fraction. Although this enzyme has often been employed as a lysosomal marker, some 20% of total cell activity may be located in endoplasmic reticulum (Owens *et al.*, 1975). Nevertheless, our data indicate that, in our experiments, lysosomes were substantially separated from the mitochondria-rich fraction. Lysosomes have not been suggested to contain a significant proportion of cell Ca (e.g. Bygrave, 1978).

Estimation of membrane potentials in hepatocytes

TPMP⁺ is a lipophilic cation that accumulates in cells in response to the cellular transmembrane electrical potentials (Hoek *et al.*, 1980; Scott & Nicholls, 1980; Davis & Martin, 1982). Hoek *et al.*

(1980) incubated hepatocytes with TPMP⁺ and attempted to determine the concentrations of TPMP⁺ in the mitochondrial ([TPMP⁺]_m), cytosolic ([TPMP⁺]_c) and extracellular ([TPMP⁺]_e) compartments. These determinations were inserted into the Nernst equation in order to estimate the transmembrane electrical potentials. As outlined in the introduction, we wanted to repeat these experiments as we had some reservations concerning the extent to which binding of TPMP⁺ might affect the results obtained. Hoek *et al.* (1980) defined total cell uptake of the cation in terms of an accumulation ratio (R_t), where:

$$R_t =$$

$$\frac{(\text{Intracellular amount of TPMP}^+)/(\text{cell volume})}{[\text{TPMP}^+]_e}$$

(1)

[TPMP⁺]_e and the intracellular amount of TPMP⁺ were determined in samples of incubation medium from which hepatocytes were removed by centrifugation through silicone oil. We confirmed the observation of Hoek *et al.* (1980) that, if hepatocytes were centrifuged into 2M-HClO₄, incomplete recoveries of TPMP⁺ were observed. Hoek *et al.* (1980) removed their cells into a mixture of 250 mM-sucrose plus 1% (w/v) Lubrol. However, we were unable to confirm that this medium would release all the TPMP⁺ accumulated by hepatocytes, even when the concentration of Lubrol (type PX or WX; Sigma) was increased to 5% (w/v). We were only able to release all the intracellular TPMP⁺ when cells were centrifuged into 0.2 ml of 400 mM-sucrose plus 2M-NaOH (results not shown). It is, therefore, difficult for us to compare our results with those of Hoek *et al.* (1980), because we cannot be confident that the latter workers had accounted for all their TPMP⁺ uptake.

For the determination of R_t (eqn. 1), we assumed that 28% of extracellular TPMP⁺ was bound. This

was the proportion of TPMP⁺ bound to 1 ml samples of bovine serum albumin dialysed at 37°C to equilibrium against bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 5 mM-Tes, 10 mM-glucose, 1.25 mM-CaCl₂ and 30 mM-[³H]TPMP⁺ (0.006 µCi/ml). It was subsequently noted that, in our hepatocytes, *R_t* attained a concentration-independent equilibrium value within 20 min when [TPMP⁺]_e ≤ 300 nM (results not shown).

We investigated the extent of TPMP⁺ binding in mitochondria prepared by classical differential centrifugation (Shears & Bronk, 1979). The trans-membrane distributions of TPMP⁺ were compared with those for K⁺ in the presence of valinomycin, assuming negligible binding of K⁺ (Scott & Nicholls, 1980; Wilson & Forman, 1982). Table 2 indicates that, between 0.08 and 2.3 mM extramitochondrial K⁺, the ratio between the trans-membrane distributions of K⁺ and TPMP⁺ was reasonably constant, averaging 0.62. This suggests that 38% of mitochondrial TPMP⁺ was bound. However, when extramitochondrial [K⁺] was raised so that TPMP⁺ accumulation by mitochondria was less than 100-fold above concentrations on the medium, 90% of TPMP⁺ was bound. In the latter case mitochondria would appear to lack respiratory control, since respiratory rates were not further enhanced by CCFP (results not shown).

Our observation that 38% of mitochondrial TPMP⁺ was bound (Table 2) is less than the value of 66% determined by Wilson & Forman (1982), but greater than in the study of Hoek *et al.* (1980), who suggested that mitochondrial TPMP⁺ binding was not significant. We have not investigated how this disagreement may be explained.

[TPMP⁺]_m was determined after rapid cell fractionation, and the ratio [TPMP⁺]_m/[TPMP⁺]_e was corrected for binding and inserted into the Nernst equation in order to estimate the electrical potential between the mitochondria *in situ* and the extracellular medium (ΔΨ_{m/e}):

$$\Delta\Psi_{m/e} = 61.5 \log \left(\frac{[\text{TPMP}^+]_m}{[\text{TPMP}^+]_e} \right) \text{ (mV)} \quad (2)$$

These data, and the determinations of *R_t* (eqn. 1), are described by Table 3. In our studies, *R_t* was over 700, substantially greater than the determinations of less than 200 obtained by Hoek *et al.* (1980). It is clear that these differences could arise both from our corrections for TPMP⁺ binding and from the failure of Hoek *et al.* (1980) to release all their intracellular TPMP⁺ with Lubrol (see above).

Our value for ΔΨ_{m/e} of 202 mV is slightly greater

Table 2. Comparisons between the ratios of intra-mitochondrial/extramitochondrial [K⁺] (*K_{in}⁺/K_{out}⁺*) and [TPMP⁺] (*TPMP_{in}⁺/TPMP_{out}⁺*) in the presence of valinomycin at several concentrations of K⁺

Mitochondria were incubated for 5 min as described under 'Methods', with the addition of 30 nM-TPMP⁺, 0.5 µM-valinomycin and K⁺ as indicated. *K_{in}⁺* and *K_{out}⁺* were determined after 0.5 ml samples were centrifuged at 12000*g* through 0.5 ml of silicone oil (*d* 1.045) into 0.2 ml of 2 M-HClO₄. In parallel incubations supplemented with 0.4 µCi of ³H₂O/ml and 0.04 µCi of [¹⁴C]sucrose/ml, mitochondrial volume was calculated. Further incubations were carried out with 0.1 µCi of [³H]TPMP⁺/ml and mitochondria were centrifuged through silicone oil into 0.2 ml of 400 mM-sucrose/2 M-NaOH, and TPMP⁺ uptake was determined. Results represent determinations in triplicate from two preparations of mitochondria.

<i>[K_{out}⁺] (mM)</i>	<i>K_{in}⁺ K_{out}⁺</i>	<i>TPMP_{in}⁺ TPMP_{out}⁺</i>	<i>K_{in}⁺ × TPMP_{out}⁺ K_{out}⁺ × TPMP_{in}⁺</i>
0.08	1311	1977	0.66
0.5	337	482	0.68
1.3	123	212	0.58
2.3	70	124	0.56
18.5	7.2	71.7	0.10

than that of 191 mV obtained by Hoek *et al.* (1980). It should be emphasized that although the mitochondria-rich fraction is contaminated with nuclei (Table 2), the latter appear not to possess a significant membrane potential (Hoek *et al.*, 1980). We therefore confirm that, during the rapid cellular fractionation, mitochondria retain a substantial electrical potential. Moreover, mitochondria prepared from hepatocytes treated with oligomycin and/or CCFP showed the expected changes in ΔΨ_{m/e}. Thus oligomycin increased ΔΨ_{m/e} by 10 mV (Table 3), as might be expected from its ability to inhibit the dissipation of H⁺ gradients through the mitochondrial ATPase. In addition, ΔΨ_{m/e} was decreased substantially by the addition of oligomycin plus CCFP to hepatocytes, which de-energizes their mitochondria. Although this procedure will inhibit mitochondrial ATP synthesis, it is unclear to what extent the electrical potential across the plasma membrane would be affected. This is because glucose in the medium would support ATP synthesis by glycolysis and, in any case, the Na⁺/K⁺-ATPase in the plasma membrane is not the sole origin of the membrane potential (Claret & Mazet, 1972).

Hoek *et al.* (1980) also used the rapid fractionation technique to derive values for the electrical potentials between the cytosol and extracellular medium (ΔΨ_{c/e}) and between the mitochondrial

Table 3. Estimation of R_t and $\Delta\Psi_{m/e}$ in hepatocytes

Portions of hepatocytes (2 ml) were incubated for 20 min as described under 'Methods', with the addition of 30 nM-TPMP⁺ and 0.1 μ Ci of [³H]TPMP⁺/ml. Either 0.01 ml of ethanol (control) or 0.01 ml of ethanolic solutions of oligomycin $\pm$ CCFP were then added. After a further 10 min, samples of cells were taken for the determination of R_t (see the text). Parallel samples of cells were fractionated with 0.1–0.2 mg digitonin/mg of cell dry wt. and [TPMP⁺] in the mitochondria-rich extract was determined; [TPMP⁺]_m was then estimated assuming the mitochondrial volume as determined under 'Methods'. This value was corrected for damaged mitochondria and residual intact cells by measuring the release of glutamate dehydrogenase and lactate dehydrogenase. Of the mitochondrial TPMP⁺, 38% was assumed to be bound (Table 2), except in the presence of CCFP (90% bound). Of the extracellular TPMP⁺, 28% was considered bound (see the text). Results are means $\pm$ S.E.M. for the numbers of hepatocyte preparations given in parentheses. Significance: * P < 0.05; ** P < 0.02; *** P < 0.001 (compared with control).

		$\frac{[\text{TPMP}^+]_m}{[\text{TPMP}^+]_e}$	$\Delta\Psi_{m/e}$ (mV)	R_t
Control	(5)	1951 $\pm$ 80	202 $\pm$ 1.3	704 $\pm$ 27
+ Oligomycin (5 mg/ml)	(4)	2848 $\pm$ 255**	212 $\pm$ 2.6**	885 $\pm$ 55*
+ Oligomycin (5 mg/ml) and CCFP (50 μ M)	(4)	10.6 $\pm$ 1.5***	63 $\pm$ 4.3***	100 $\pm$ 19***

and the cytosol ($\Delta\Psi_{m/c}$), where:

$$\Delta\Psi_{m/c} = \Delta\Psi_{m/e} - \Delta\Psi_{c/e} \quad (3)$$

Hoek *et al.* (1980) suggested that the value of R_t obtained from de-energized hepatocytes (' R_b ', = 100 in Table 3) was the sum of intracellular binding plus TPMP⁺ uptake by non-mitochondrial organelles. They went on to propose the following relationship:

$$R_t - R_b = V_c \frac{[\text{TPMP}^+]_c}{[\text{TPMP}^+]_e} + V_m \frac{[\text{TPMP}^+]_m}{[\text{TPMP}^+]_e} \quad (4)$$

where V_c and V_m represent fractional volumes of cytosolic and mitochondrial compartments (0.9 and 0.1 respectively, see under 'Methods'). The ratio $[\text{TPMP}^+]_c/[\text{TPMP}^+]_e$ was then inserted into the Nernst equation in order to estimate $\Delta\Psi_{c/e}$:

$$\Delta\Psi_{c/e} = 61.5 \log \left(\frac{[\text{TPMP}^+]_c}{[\text{TPMP}^+]_e} \right) \quad (5)$$

We have used our data (Table 3) uncorrected for mitochondrial TPMP⁺ binding (Hoek *et al.*, 1980), in these equations to estimate values for $\Delta\Psi_{c/e}$ and $\Delta\Psi_{m/c}$ in control hepatocytes of 154 and 48 mV respectively. The very low values of $\Delta\Psi_{m/c}$ result from estimates of $\Delta\Psi_{c/e}$ that are substantially above the accepted values of 30–40 mV indicated by microelectrode measurements (Friedmann *et al.*, 1971; Claret & Mazet, 1972), ³⁶Cl[−] uptake (Baur *et al.*, 1975) and [¹⁴C]thiocyanate exclusion (Hoek *et al.*, 1980). In view of this discrepancy, we employed an alternative correction for cytosolic TPMP⁺ binding. This correction presumes a constant proportion of binding, as in isolated coupled mitochondria (Table 2) and in synaptosomes (Scott & Nicholls, 1980) and cells from epididymal fatpads (Davis *et al.*, 1981). The proportion of cyto-

solic TPMP⁺ binding in the latter studies was estimated by comparing R_t for TPMP⁺ (eqn. 1) with R_t for Rb⁺ in the presence of 150 mM-K⁺ plus 0.5 μ M-valinomycin.

In our hepatocytes, using K⁺ instead of Rb⁺, we obtained a ratio for R_t (K⁺)/ R_t (TPMP⁺) of 0.0271 $\pm$ 0.003 (n = 3). This result indicates that 97.3% of cytosolic TPMP⁺ was bound. When our data are now inserted into eqns. (1)–(5) [ignoring R_b (eqn. 4) and correcting for proportionate TPMP⁺ binding], we obtained values for $\Delta\Psi_{c/e}$ and $\Delta\Psi_{m/c}$ of 66 and 136 mV respectively. The latter value for $\Delta\Psi_{c/e}$ still exceeds the 30–40 mV obtained by various other techniques (see above), which would set $\Delta\Psi_{m/c}$ to 160–170 mV. Indeed, three factors suggest that these determinations of $\Delta\Psi_{c/e}$ may also be incorrect. Firstly, since de-energization of mitochondria appears to modify the extent of TPMP⁺ binding (Table 2), we cannot rule out the possibility that de-energization of cells with valinomycin plus K⁺ has a similar effect. In this case, determinations of cytosolic binding in de-energized cells may overestimate the extent of binding when membrane potentials are at normal levels. Secondly, we cannot dismiss the possibility that non-mitochondrial organelles may accumulate TPMP⁺. Thirdly, if the intracellular activity coefficient for TPMP⁺ was significantly different from the extracellular activity coefficient, an erroneous estimation of membrane potential would occur. Hence, we conclude that TPMP⁺ cannot be reliably employed to determine $\Delta\Psi_{m/c}$ and $\Delta\Psi_{c/e}$ using the rapid fractionation technique of Hoek *et al.* (1980).

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