Measurement of the intramitochondrial volume in hepatocytes without cell disruption and its elevation by hormones and valinomycin

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1. Methods have been developed to measure the lysophospholipid content and matrix volume of liver cell mitochondria *in situ* in order to test the hypothesis that these parameters may be important in the hormonal control of mitochondrial function [Armston, Halestrap & Scott (1982) *Biochim. Biophys. Acta* **681**, 429–439]. 2. No change in the labelling of mitochondrial lysophospholipids with [^{32}P]P_i was detected after treatment of liver cells with glucagon, phenylephrine or vasopressin. Incorporation of [^{32}P]P_i into mitochondrial phosphatidylinositol was enhanced by phenylephrine and vasopressin. 3. Mitochondrial volumes were measured using rapid disruption of cells by sonication into $^3\text{H}_2\text{O}$ and [^{14}C]sucrose or without cell disruption using $^3\text{H}_2\text{O}$ and [^{14}C]mannitol. In control cells the two methods gave values of 1.09 and 0.40 $\mu\text{l}/\text{mg}$ of mitochondrial protein respectively, which represent 19 and 7% respectively of the total cell volume measured with $^3\text{H}_2\text{O}$ and inulin [^{14}C]carboxylic acid. 4. Both methods showed that glucagon, phenylephrine and 1 nM-valinomycin produced significant increases (13% and 26% using sucrose and mannitol respectively) in mitochondrial volume. The increase was coincident with the stimulation of gluconeogenesis from L-lactate and pyruvate and of mitochondrial respiratory chain activity. The effects of glucagon and phenylephrine were additive on both mitochondrial volume and respiratory chain activity, but not on gluconeogenesis. 5. Liver cells exposed to gluconeogenic hormones or low concentrations of valinomycin showed a decrease in light scattering at 520 nm correlating with the change in mitochondrial volume but without a change in whole-cell volume. The time course and hormone sensitivity of this response were similar to those for the hormonal stimulation of gluconeogenesis. The light-scattering response to glucagon, phenylephrine and vasopressin, but not to valinomycin, were greatly reduced or abolished in Ca^{2+} -free media.

Glucagon and adrenaline treatment of rats produces numerous changes in the properties of subsequently isolated liver mitochondria (see Armston *et al.*, 1982, and references therein). Many of these hormonal effects can be mimicked by increasing the volume of isolated mitochondria and reversed by aging mitochondria in the presence of low concentrations of Ca^{2+} , which activates endogenous phospholipase A₂ (Armston *et al.*, 1982). It has been suggested that glucagon stabilizes mitochondria against attack by phospholipase A₂ and that this may be the mechanism by which glucagon exerts its effect on so many mitochondrial par-

ameters (Siess *et al.*, 1981; Halestrap, 1981; Armston *et al.*, 1982). If this is the case it is possible that at least some of the effects of glucagon may be artefacts of phospholipase A₂ activity during mitochondrial preparation and so not of physiological importance (see Siess *et al.*, 1981; Armston *et al.*, 1982). Nevertheless there is evidence from work with isolated liver cells and perfused liver that mitochondrial function is stimulated by hormones under physiological conditions. Thus mitochondrial pyruvate transport and metabolism are stimulated (Thomas & Halestrap, 1981), glutaminase is activated (Joseph & McGivan, 1978), urea synthesis (which is limited by mitochondrial citrulline synthesis) is increased (Bryla *et al.*, 1977), oxygen consumption rises (Exton *et al.*, 1972; Parilla *et al.*, 1975), and mitochondrial ATP/ADP and NADH/

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NAD⁺ ratios are elevated (Bryla *et al.*, 1977; Siess *et al.*, 1977, 1978; Titheradge & Haynes, 1980b). All of these effects could be explained by either an increase in matrix volume or a drop in mitochondrial phospholipase A₂ activity *in vivo* (Halestrap, 1982; Armston *et al.*, 1982).

In the present paper we describe techniques for measuring changes in the lysophospholipid composition of the mitochondrial membrane and for estimating the mitochondrial volume *in situ*. We demonstrate that, whereas neither glucagon nor phenylephrine has an effect on the lysophospholipid composition of the membrane, both hormones cause an increase in the mitochondrial matrix volume.

Experimental

Materials

Rats. Male Wistar rats (300–400 g) were starved for 24 h but allowed free access to water before preparation of hepatocytes.

Chemicals. Unless stated otherwise the source of all chemicals, radiochemicals and biochemicals was the same as those given by Thomas & Halestrap (1981) and in the preceding paper (Halestrap & Quinlan, 1983). All phospholipid standards were obtained from Sigma Chemical Company, Poole, Dorset, U.K. The D-[1-¹⁴C]mannitol supplied by Amersham International contained about 1% [¹⁴C]-mannose. This was removed by incubation of the [¹⁴C]mannitol (final specific radioactivity 0.2 Ci/mol) in 20 mM-Tris/HCl (pH 7.4) containing 2 mM-MgCl₂, 2 mM-Mg ATP²⁻, 30 mM-mannitol, 5 μM-mannose and 3 units of hexokinase/ml at 37°C for 30 min. At this time 0.2 g of washed Dowex-50 (Cl⁻ form)/ml was added to absorb all hexose phosphates and adenine nucleotides and the mixture was centrifuged at 10000 g/min. The supernatant was kept; the resin was washed with an equal quantity of water and the washings were combined with the supernatant. The resulting solution was used directly in subsequent liver cell experiments. Analysis of the purified mannitol by t.l.c. on borate-treated cellulose acetate plates developed in pyridine/ethyl acetate/water (2:9:1, by vol.) showed that all contaminating [¹⁴C]mannose was removed by this treatment.

Methods

Preparation and incubation of liver cells. Hepatocytes were prepared essentially by the method of Berry & Friend (1969) as modified by Thomas & Halestrap (1981). After washing, cells were suspended in bicarbonate-buffered saline medium (Krebs & Henseleit, 1932) containing 20 mg of bovine serum albumin/ml and gassed with O₂/CO₂ (19:1) before storing on ice until use. In all experiments cells were incubated with shaking at 37°C in stoppered plastic vials (10 ml or 50 ml

capacity) under an atmosphere of O₂/CO₂ (19:1). Gassing with O₂/CO₂ (19:1) was repeated every 15 min during the incubation. L-Lactate (10 mM) and pyruvate (1 mM) were present as substrates for gluconeogenesis in all experiments and the endogenous fatty acids present on the albumin acted as an additional energy source. Rates of gluconeogenesis were estimated as described by Thomas & Halestrap (1981) using glucose oxidase to assay glucose. Glucose production was measured over a 15 min period and only after the cells had been incubated for at least 20 min. At this time rates of glucose output were constant (Thomas & Halestrap, 1981).

Determination of mitochondrial volumes and respiratory chain activity after cell disruption by sonication. Hepatocytes (10–15 mg of protein/ml) prepared in low phosphate (0.575 mM) bicarbonate-buffered saline medium were incubated as described above for 20 min before adding hormones as required. After a further 5 min incubations were terminated by transferring 1 ml of cell suspension to a plastic 1.5 ml centrifuge tube containing 4 μmol of K⁺-EGTA, 1 μCi of ³H₂O and 0.1 μCi of [¹⁴C]-sucrose and immediately sonicating for 5 s on setting 3 with the micro probe of a sonicator (Dawe Instruments Ltd., type 7532A). The resulting cell homogenate was centrifuged at 10000 g for 1 min in an Eppendorf 5414 centrifuge and the supernatant was removed into HClO₄ at a final concentration of 2% (w/v). The pellet was resuspended in 250 μl of 10 mM-Tris/HCl (pH 7.4) and a portion was retained for assay of citrate synthase before adding HClO₄ to 2% (w/v) final. After removal of the precipitated protein by centrifugation equal volumes of pellet extract and supernatant were assayed for ³H and ¹⁴C (d.p.m.) in a scintillation counter with the precautions outlined by Armston *et al.* (1982) and Halestrap & Quinlan (1983). All samples were recycled four times and the average intramitochondrial volume was calculated for each sample by the method of Halestrap & McGivan (1979). At least four incubations were carried out for each condition. Correction was made for variation in the yield of mitochondrial protein obtained after sonication by assaying portions of each pellet for citrate synthase by the method of Srere *et al.* (1963). Citrate synthase is a very suitable mitochondrial marker since it is an extremely stable enzyme that is easy to assay and unequivocally exclusive to the mitochondria (Srere, 1972). In addition assays of citrate synthase were performed on mitochondrial preparations from the livers of rats of similar sex, age and weight, which allowed expression of the matrix volume in terms of μl/mg of mitochondrial protein. In 15 separate cell preparations the mean (±S.E.M.) percentage of the total cell protein that was mitochondrial was found to be 33.8 ± 1.4.

In conjunction with the measurement of mitochondrial volume ($^3\text{H}_2\text{O}/[^{14}\text{C}]\text{sucrose space}$) after sonication of the liver cells parallel experiments were performed to determine the respiratory chain activity of the mitochondria. The mitochondrial pellet (2–3 mg of protein) was resuspended in 2 ml of oxygen-electrode buffer containing 125 mM-KCl, 10 mM-Mops (4-morpholinepropanesulphonic acid), 7 mM-Tris, 1 mM-EGTA, 2.5 mM-potassium phosphate, 5 mM-succinate and 1 μg of rotenone/ml at pH 7.2. The O_2 uptake was measured at 25°C in a Clarke-type oxygen electrode before addition of 1 mM-ADP. After a steady rate was obtained, the rate on addition of 0.1 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was measured and then the rate after further addition of antimycin A (0.1 $\mu\text{g}/\text{ml}$) and ascorbate (10 mM) + *NNN'*-tetramethyl-*p*-phenylenediamine (0.3 mM) as described by Armston *et al.* (1982).

Determination of mitochondrial volumes in intact cells using $[^{14}\text{C}]\text{mannitol}$. In order to function as an extramitochondrial marker within the cell, mannitol must not be metabolized and must equilibrate across the plasma membrane but be excluded from the mitochondrial matrix. In the preceding paper (Halestrap & Quinlan, 1983) we demonstrated that mannitol does not cross the mitochondrial inner membrane. No enzyme capable of metabolizing D-mannitol in the liver has been described. However, in order to be certain whether or not D-mannitol was metabolized by isolated rat hepatocytes, several experimental approaches were used. First, it was shown that the hepatocytes produced no detectable glucose from D-mannitol. Secondly, cells were incubated with $[^{14}\text{C}]\text{mannitol}$ (0.2 $\mu\text{Ci}/\text{ml}$) for 1 h before being disrupted with 2% (w/v) HClO_4 . Passage of the HClO_4 supernatant (after neutralization with KOH) through a column of Dowex 50 (formate form) binds hexose phosphates but no significant binding of ^{14}C was observed. A parallel experiment with $[^{14}\text{C}]\text{glucose}$ gave substantial binding of ^{14}C radioactivity. Thirdly, the time course of uptake of $[^{14}\text{C}]\text{mannitol}$ into the cell was measured (as the $^3\text{H}_2\text{O}/[^{14}\text{C}]\text{mannitol space}$) over a 1 h incubation period and was shown to reach a constant value after 40 min (Fig. 1). This value was independent of mannitol concentration over the range 0.5–5 mM, and as described below was similar to the value of the mitochondrial volume obtained with isolated mitochondria. Neither of these observations would be expected if mannitol was being metabolized.

In order to measure the $^3\text{H}_2\text{O}/[^{14}\text{C}]\text{mannitol space}$ hepatocytes were incubated as described above with 0.5 mM-mannitol, 0.1 μCi of $[^{14}\text{C}]\text{mannitol}/\text{ml}$ and 1 μCi of $^3\text{H}_2\text{O}/\text{ml}$ for 35 min before addition of hormones, and after a further 5 min 1 ml of cell suspension was transferred to an Eppendorf

Fig. 1. The time course of $[^{14}\text{C}]\text{mannitol}$ entry into liver cells

Liver cells (about 12 mg of protein/ml) were incubated at 37°C with $^3\text{H}_2\text{O}$ and $[^{14}\text{C}]\text{mannitol}$ for the times shown, before measuring the $^3\text{H}_2\text{O}/[^{14}\text{C}]\text{mannitol space}$ (●) as described in the Experimental section. The values given are means of four observations on the same preparation of cells with the S.E.M. given as error bars (where these values are greater than the width of the symbol). Calculation of the space with respect to the mitochondrial protein (36.6% of the total cell protein) involved measurements of citrate synthase activity as described in the Experimental section. The additional value at zero time (■) represents the $^3\text{H}_2\text{O}/\text{inulin}/[^{14}\text{C}]\text{carboxylic acid space}$.

tube and centrifuged for 5 s at 10000 g. The pellet and supernatant were then treated identically to the mitochondria prepared by sonication except that the pellet was not assayed for citrate synthase. In parallel experiments citrate synthase was assayed in the total cell pellet to allow determination of the amount of mitochondrial protein (mg) present in the cell pellet and hence calculation of the $^3\text{H}_2\text{O}/[^{14}\text{C}]\text{mannitol space}$ as $\mu\text{l}/\text{mg}$ of mitochondrial protein.

Light scattering by hepatocytes. The time course of light scattering by hepatocytes incubated in the cuvette chamber of a purpose-built split-beam spectrophotometer was continuously monitored as A_{520} using the system described by Halestrap (1982). After pre-incubating the cells (7–10 mg/ml) for 20 min 3.5 ml portions were transferred to each of two plastic cuvettes (1 cm light-path) maintained at 37°C. The cells were continuously stirred with an overhead stirrer and gassed with O_2/CO_2 (19:1). Under these conditions cells remained metabolically active for the duration of the experiment and gave rates of gluconeogenesis similar to those observed

under more conventional incubation conditions. After a steady baseline was obtained hormone additions were made to one cuvette and the light-scattering response was recorded. In some experiments total cell volume measurements of control and hormone-treated cells were made in conjunction with the monitoring of the hormonally induced changes in light scattering. Inulin [^{14}C]-carboxylic acid ($0.1\ \mu\text{Ci/ml}$ and $50\ \mu\text{g/ml}$) and $^3\text{H}_2\text{O}$ ($1\ \mu\text{Ci/ml}$) were added to both cuvettes 5–10 min before terminating the incubation by removing three samples each of 1 ml volume from each cuvette for measurement of the ' $^3\text{H}/^{14}\text{C}$ space' as described above. The mean cell volume ($\pm$ S.E.M. of nine observations on different preparations of cells) expressed as $\mu\text{l/mg}$ of cell protein was 1.89 ± 0.05 .

^{32}P P_i labelling and analysis of phospholipids. Cells ($7.5\ \text{mg/ml}$) were incubated for 1 h at 37°C in bicarbonate-buffered saline at pH 7.4 containing $0.5\ \text{mM}$ - ^{32}P P_i ($0.1\ \text{mCi/ml}$), albumin ($10\ \text{mg/ml}$), L-lactate ($10\ \text{mM}$) and pyruvate ($1\ \text{mM}$) with frequent gassing with O_2/CO_2 (19:1) as described by Vargas *et al.* (1982). Hormones were added 10 min before terminating cell incubations by transferring 1 ml of cell suspension to a 1.5 ml centrifuge tube and centrifuging for 5 s at $10000g$. The cells were resuspended in 1 ml of $300\ \text{mM}$ -sucrose/ $20\ \text{mM}$ -Tris/ $2\ \text{mM}$ -EGTA at pH 7.4 and 0°C and sonicated for $2 \times 5\ \text{s}$ as described above. The mitochondria were separated by centrifugation and further purified by Percoll density gradient centrifugation as described by Vargas *et al.* (1982). After washing the pellet with sucrose buffer and resuspending in $125\ \mu\text{l}$ of KCl ($2\ \text{M}$), phospholipids were extracted twice with $0.5\ \text{ml}$ of chloroform/methanol (2:1, v/v) containing $0.1\ \text{mg}$ of 2,6-dibutyl-*p*-cresol/ml, before being

dried under vacuum. The phospholipids were re-suspended in the same solvent and separated by t.l.c. on Merck 5735 silica-gel-coated plastic sheets in a two-dimensional system using chloroform/methanol/acetic acid/water (125:75:19:3, by vol.) and chloroform/methanol/aq. NH_3 (75:37:7, by vol.) in the first and second dimension respectively. Spots were located by autoradiography, cut out and the ^{32}P content was determined by liquid-scintillation counting. Identity of the spots was confirmed by using standard phospholipids visually detected with I_2 vapour.

Results

Hormone-induced changes in the ^{32}P P_i labelling pattern of the phospholipids of the mitochondrial membrane

Data are given in Table 1 that show that neither glucagon, phenylephrine nor vasopressin produced any changes in the ^{32}P P_i labelling of lysophosphatidylcholine or lysophosphatidylethanolamine of the mitochondrial membrane. Thus it would seem unlikely that there is any hormone-induced regulation of mitochondrial phospholipase A_2 within the intact liver cell. The only significant effects on mitochondrial phospholipid labelling with ^{32}P were seen on phosphatidylinositol, whose ^{32}P content rose by about 25% with phenylephrine and 250% with vasopressin. Similar results have been reported by Kirk & Michell (1981).

Measurement of mitochondrial volume after rapid cell disruption

Table 2 contains values for the mitochondrial volumes and respiratory chain activity measured

Table 1. *The effects of hormones on the ^{32}P P_i labelling of mitochondrial phospholipids in liver cells*
Liver cells were incubated for 1 h with ^{32}P P_i before addition of hormones and subsequent isolation of mitochondria and extraction of phospholipids. Further details of the techniques used are given in the Experimental section. Values given are means $\pm$ S.E.M. for the numbers of experiments shown, each experiment on a different preparation of cells and involving duplicate observations. Statistical significances were calculated by using paired Student's *t* test: * $P < 0.01$.

Treatment	Number of experiments	Incorporation of ^{32}P P_i into phospholipid (% of that present in phosphatidylcholine + phosphatidylethanolamine)					
		Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidic acid	Phosphatidylinositol	Lyso-phosphatidylcholine	Lyso-phosphatidylethanolamine
Control	9	51.25 ± 2.91	48.84 ± 2.88	12.73 ± 1.28	14.69 ± 1.97	0.57 ± 0.06	0.55 ± 0.14
Glucagon ($0.1\ \mu\text{M}$)	9	51.54 ± 3.02	48.73 ± 3.12	12.99 ± 1.05	14.21 ± 1.47	0.61 ± 0.07	0.59 ± 0.10
Phenylephrine ($20\ \mu\text{M}$)	9	51.36 ± 3.19	48.63 ± 3.0	13.16 ± 0.91	$18.20 \pm 1.78^*$	0.56 ± 0.09	0.54 ± 0.10
Vasopressin ($25\ \text{nM}$)	5	50.23 ± 4.47	49.78 ± 4.97	14.10 ± 1.11	$48.14 \pm 6.98^*$	0.57 ± 0.11	0.59 ± 0.28

Table 2. *The effects of hormones on the mitochondrial volume and respiratory chain activity measured after rapid cell disruption*

Liver cells were incubated with L-lactate (10 mM) + pyruvate (1 mM) for 20 min before additions of hormone. After a further 10 min cells were disrupted by rapid sonication and mitochondrial volumes ($^3\text{H}_2\text{O}/[^{14}\text{C}]\text{sucrose space}$) were measured as described in the Experimental section. The rate of uncoupled succinate oxidation is expressed as a percentage of that in the presence of 10 mM-ascorbate + 0.3 mM-*NNN'*-tetramethylphenylenediamine (TMPD) to allow for differences in protein concentration (Armstrong *et al.*, 1982). Data are means $\pm$ S.E.M. of the number of experiments shown (*n*). Values in parentheses are experimental values as percentages of the respective control values. Statistical significances of experimental values versus control were calculated by paired Student's *t* test: $\dagger\dagger\dagger P < 0.01$; $\dagger\dagger P < 0.02$; $\dagger P < 0.05$.

Hormone added	Mitochondrial volume ($\mu\text{l}/\text{mg}$ of protein)		Rate of uncoupled succinate oxidation (% of rate of ascorbate + TMPD oxidation)	
	Control	Experimental	Control	Experimental
Glucagon (0.1 μM)	1.09 $\pm$ 0.05	1.24 $\pm$ 0.04 (114.0 $\pm$ 2.9 $\dagger\dagger\dagger$; <i>n</i> = 9)	54.8 $\pm$ 7.4	63.3 $\pm$ 7.7 (116.4 $\pm$ 2.6 $\dagger\dagger\dagger$; <i>n</i> = 5)
Phenylephrine (20 μM)	1.09 $\pm$ 0.05	1.22 $\pm$ 0.05 (112.3 $\pm$ 3.8 $\dagger\dagger$; <i>n</i> = 9)	54.8 $\pm$ 7.4	63.4 $\pm$ 10.1 (115.6 $\pm$ 4.9 $\dagger$; <i>n</i> = 5)
Glucagon + phenylephrine	1.12 $\pm$ 0.05	1.42 $\pm$ 0.10 (126.8 $\pm$ 6.4 $\dagger\dagger\dagger$; <i>n</i> = 8)	54.8 $\pm$ 7.4	73.2 $\pm$ 6.0 (129.2 $\pm$ 5.8 $\dagger\dagger\dagger$; <i>n</i> = 5)
Valinomycin (1 nM)	1.09 $\pm$ 0.05	1.29 $\pm$ 0.10 (117.0 $\pm$ 3.3 $\dagger$; <i>n</i> = 3)	56.4 $\pm$ 12.4	82.5 $\pm$ 11.4 (134.5 $\pm$ 7.7 $\dagger$; <i>n</i> = 3)

after rapid cell disruption by sonication. To ensure mitochondrial volumes were measured as rapidly after incubation of the cells as possible, cells were sonicated in the incubation medium to which EGTA had been added to chelate excess Ca^{2+} . The concentration of EGTA chosen was such that the free Ca^{2+} after sonication was about 0.1 μM , which is close to the cytoplasmic concentrations (Williamson *et al.*, 1981). The mitochondrial fraction isolated under these conditions was analysed for contamination by other cell components as described by Vargas *et al.* (1982). Contamination by cytoplasm and endoplasmic reticulum was less than 3% of the total cellular content of these fractions as determined using lactate dehydrogenase and cytochrome *P*-450. Acid phosphatase and 5'-nucleotidase contamination were 15 $\pm$ 1.5% and 25 $\pm$ 1.0% (means $\pm$ S.E.M. of three separate experiments) of the total cellular enzyme activity. Under these unusual isolation conditions mitochondria showed little or no swelling as judged by light-scattering observations made in parallel experiments. Furthermore, when assayed immediately after preparation the mitochondria showed normal rates of O_2 consumption and control ratios of at least 3 with succinate as respiratory substrate. The rate of uncoupled succinate oxidation was greater by about 15% when mitochondria were prepared from cells treated with glucagon (0.1 μM) or phenylephrine (20 μM) and by about 30% when both hormones were present together. Parallel measurements of the mitochondrial volume with $^3\text{H}_2\text{O}/[^{14}\text{C}]\text{sucrose}$ showed that this parameter was also increased by hormone treatment. In the absence of hormones the mean volume was 1.09 $\pm$ 0.05 $\mu\text{l}/\text{mg}$ of

mitochondrial protein and increased by 14, 12 and 27% when cells were treated respectively with glucagon, phenylephrine and both hormones together (Table 2). The rates of gluconeogenesis from L-lactate (10 mM) + pyruvate (1 mM) were similar under all three conditions, although exact values varied from day to day. Inspection of Table 3 shows that the mean stimulation was about 35–40% under all three conditions.

Valinomycin at very low concentrations has been shown to stimulate gluconeogenesis from L-lactate (Haynes *et al.*, 1974; Tolbert & Fain, 1974; Thomas & Halestrap, 1981). In Table 2 we demonstrate that concentrations that stimulate gluconeogenesis by about 20% (1 nM) also produced a 17% increase in mitochondrial volume and a 34% stimulation of uncoupled succinate oxidation. However, the exact sensitivity of cells to valinomycin varied from day to day.

Measurement of intramitochondrial volume in situ using ^{14}C mannitol

In the Experimental section we provided evidence that ^{14}C mannitol could be used as an extra-mitochondrial marker in the intact cell just as in isolated mitochondria (Halestrap & Quinlan, 1983). In Table 3 results are presented to show that the hormonally induced increases in mitochondrial volume measured after cell disruption were also detected using ^{14}C mannitol in the intact hepatocyte. Use of citrate synthase as a measure of the mitochondrial content of the liver cells (see the Experimental section) allowed the expression of the $^3\text{H}_2\text{O}/[^{14}\text{C}]\text{mannitol}$ space of cells in terms of $\mu\text{l}/\text{mg}$ of mitochondrial protein. The values so obtained are

Table 3. *Effects of hormones on the mitochondrial volume of liver cells measured in situ using [^{14}C]mannitol*
Liver cells were incubated as described in the legend to Table 2 in the presence of $1\mu\text{Ci}$ of $^3\text{H}_2\text{O}/\text{ml}$ and $0.1\mu\text{Ci}$ of [^{14}C]mannitol/ml. Measurement of the $^3\text{H}_2\text{O}/[\text{C}]$ mannitol space and the rate of gluconeogenesis were carried out in parallel as described in the Experimental section. Data are means $\pm$ S.E.M. of the number of experiments shown. Values in parentheses are experimental values as percentages of the respective control values. Statistical significances of experimental values versus control were calculated by paired Student's *t* test: $\dagger\dagger\dagger P < 0.001$; $\dagger\dagger P < 0.01$; $\dagger P < 0.02$.

Hormone added	$^3\text{H}_2\text{O}/[\text{C}]$ mannitol space ($\mu\text{l}/\text{mg}$ of mitochondrial protein)		Rate of gluconeogenesis (nmol of glucose/mg of protein per min)		<i>n</i>
	Control	Experimental	Control	Experimental	
Glucagon ($0.1\mu\text{M}$)	0.40 ± 0.02	0.49 ± 0.03 (125.8 $\pm$ 5.2 $\dagger\dagger\dagger$)	4.78 ± 0.30	6.63 ± 0.41 (140.3 $\pm$ 6.2 $\dagger\dagger\dagger$)	15
Phenylephrine ($20\mu\text{M}$)	0.40 ± 0.02	0.50 ± 0.02 (128.3 $\pm$ 5.1 $\dagger\dagger\dagger$)	4.78 ± 0.30	6.58 ± 0.46 (138.9 $\pm$ 7.7 $\dagger\dagger\dagger$)	15
Glucagon + phenylephrine	0.44 ± 0.61	0.61 ± 0.03 (146.1 $\pm$ 10.1 $\dagger\dagger\dagger$)	4.99 ± 0.35	6.67 ± 0.62 (134.7 $\pm$ 6.7 $\dagger\dagger\dagger$)	12
Valinomycin (1 nM)	0.44 ± 0.03	0.49 ± 0.03 (113.8 $\pm$ 5.0 $\dagger$)	4.60 ± 0.33	5.52 ± 0.55 (119.2 $\pm$ 5.4 $\dagger\dagger$)	11

similar to the values obtained for isolated mitochondria in iso-osmotic medium reported in the preceding paper (Halestrap & Quinlan, 1983). In control cells the estimated mitochondrial volume was about $0.42\mu\text{l}/\text{mg}$ of cell protein and rose by about 25% with either glucagon or phenylephrine treatment of cells and by about 50% when both hormones were added together. With mitochondria isolated conventionally from rat liver incubated for 2 min at 37°C under State-4 conditions we obtained a value for the $^3\text{H}_2\text{O}/[\text{C}]$ mannitol space of $0.468 \pm 0.031\mu\text{l}/\text{mg}$ of protein (mean $\pm$ S.E.M. of three separate mitochondrial preparations). This compared with a $^3\text{H}_2\text{O}/[\text{C}]$ sucrose space of 1.331 ± 0.052 for the same mitochondria, a value similar to the values obtained in the sonication experiments of Table 2. It should be noted that the values obtained with isolated mitochondria obtained from the livers of decapitated rats should be regarded as hormone-treated, as is the case for other mitochondrial functions (A. E. Armston & A. P. Halestrap, unpublished work). A single experiment was performed to see whether vasopressin influenced the $^3\text{H}_2\text{O}/[\text{C}]$ mannitol space in the same manner as glucagon and phenylephrine. At 25 nM , vasopressin increased the space from 0.407 ± 0.032 to $0.631 \pm 0.031\mu\text{l}/\text{mg}$ of mitochondrial protein (values given as means $\pm$ S.E.M. of eight observations).

Light-scattering of hepatocytes

The complexities of measuring $^3\text{H}_2\text{O}/[\text{C}]$ mannitol spaces with accuracy have prevented the performance of detailed time courses and hormone dose-response curves using this technique. However, we discovered that hepatocytes incubated in a split-beam spectrophotometer produced a decrease in light scattering at 520 nm when treated with hormone. This is illustrated in Fig. 2. The change in light scattering could be detected at a variety of wavelengths and could not be accounted for by a change in light absorbed by some component. No

change in total cell volume was detected in these experiments when measured using $^3\text{H}_2\text{O}$ and inulin [^{14}C]carboxylic acid (Table 4). However, a similar

Fig. 2. *The effects of hormones on the light scattering by liver cells*

After pre-incubation for 20 min liver cells were transferred to a split-beam spectrophotometer and incubated at 37°C with constant stirring and gassing with O_2/CO_2 (19:1) as described in the Experimental section. Additions of hormones were made as indicated to the sample cuvette. Traces (vi) and (vii) were performed in the absence of Ca^{2+} but with addition of 0.15 mM EGTA 10 min before addition of the hormone. Abbreviations used: Gluc, glucagon; Phen, phenylephrine; Vaso, vasopressin. Corresponding measurements of total cell volume are given in Table 4. The traces shown are typical of at least five similar experiments.

light-scattering change induced by osmotic shock with sucrose (Fig. 3) gave a readily detectable volume change as shown in Table 4. Examination of the liver cells under the light microscope showed that hormone treatment gave no gross change in cell shape or morphology that would be capable of accounting for light-scattering changes. Nor did the presence of colchicine ($10\text{ }\mu\text{M}$) have an effect on the response, suggesting that microtubules were not involved in some intracellular morphological change that could produce an effect on light scattering. Thus it would appear possible that swelling of mitochondria within the cell is responsible for these changes in light scattering. This conclusion is made more tenable by the observation that glucagon and phenylephrine have additive effects on the light scattering, just as they do on the mitochondrial volume measured within the intact cell or after sonication (Fig. 2). Similarly, vasopressin exerts a larger effect on both parameters than do the other two hormones.

The time courses of the scattering change induced by phenylephrine, vasopressin and glucagon are different (Fig. 2). Glucagon always required a lag phase of about 1 min before a light-scattering response was detected, whereas phenylephrine and vasopressin responded immediately. A more detailed analysis of the time course of phenylephrine confirmed this (Fig. 4). The magnitude of the response was similar with both glucagon and phenylephrine, but the maximum response was reached within 2 min with phenylephrine and 7 min with glucagon. If glucagon was added after phenylephrine the lag phase was greatly reduced or abolished and the maximal response developed more quickly. The response to vasopressin was usually

larger than that produced by phenylephrine and allowed little or no additional response to glucagon to be produced. Valinomycin (0.5 or 1 nM) produced a response similar to that observed with phenylephrine and vasopressin (Figs. 2 and 3), although there was a slight lag in onset of the effect (Fig. 4). As in the measurements of mitochondrial volume and rates of gluconeogenesis the response to valinomycin was somewhat variable from day to day and could be made very large. However, under these extreme conditions such an effect was accompanied by an inhibition of gluconeogenesis (results not shown). It is noteworthy that other workers have reported a lag of about 2 min before glucagon, but not noradrenaline or adrenaline, exerts its effects on mitochondrial pyruvate carboxylation, uncoupler-dependent ATPase activity and cellular glucose and urea output (Garrison & Haynes, 1975; Garrison & Borland, 1978; Titheradge *et al.*, 1979; Titheradge & Haynes, 1980b). We have confirmed the latter observation (results not shown), as have Siess & Wieland (1980). Detailed time courses of the effect of phenylephrine on the activation of glycogenolysis, Ca^{2+} fluxes and O_2 consumption in the perfused liver have demonstrated that these effects are exerted within 7–12 s from addition of hormone (Reinhart *et al.*, 1980, 1982a,b).

Table 4. *The effects of hormones and osmotic shock on the total intracellular volume of hepatocytes*

Hepatocytes were incubated in both cuvettes of a split-beam spectrophotometer as described in the legend to Fig. 2. $^3\text{H}_2\text{O}$ and inulin [^{14}C]carboxylic acid were added 5 min before cells were sedimented by centrifugation and the intracellular volume was measured as described in the Experimental section. Cells were centrifuged at the time of maximal light-scattering change. Values are means $\pm$ S.E.M. of four observations from a typical experiment. Statistical significances of experimental values compared with control values were assessed by using Student's *t* test; * $P < 0.001$.

Additions to sample cuvette	Intracellular volume ($\mu\text{l}/\text{mg}$ of cell protein)	
	Control	Experimental
Glucagon ($0.2\text{ }\mu\text{M}$)	1.86 ± 0.07	1.82 ± 0.06
Phenylephrine ($20\text{ }\mu\text{M}$)	1.85 ± 0.05	1.94 ± 0.06
Valinomycin (1 nM)	1.92 ± 0.03	1.89 ± 0.03
Sucrose (50 mM)	1.88 ± 0.03	$1.59 \pm 0.02^*$

Fig. 3. *The effects of valinomycin and osmotic shock on the light scattering by isolated liver cells*

The experimental procedure was identical with that given in the legend to Fig. 2. Corresponding measurements of cell volume are shown in Table 4. The traces shown are typical of at least five similar experiments.

A detailed dose-response curve of the light scattering towards hormones proved impossible because of the difficulty of distinguishing between drift and a hormone response at low hormone concentrations. However, the concentration of hormone required to produce half-maximal stimulation of glucose output under our conditions (1 nM-glucagon and 0.5 μ M-phenylephrine) produced half-maximal changes in light scattering as shown in Fig. 2. No light-scattering changes were observed with concentrations of hormones that gave no stimulation of gluconeogenesis, whereas the concentrations giving maximal responses in both cases were about the same (0.1 μ M-glucagon and 20 μ M-phenylephrine). At submaximal hormone concentrations the addition of both hormones together gave an additive effect on light scattering but not on gluconeogenesis, just as was observed at maximal hormone concentrations.

In Figs. 2 and 3 the effect of removing extra-

Fig. 4. Rapid time course of the effects of phenylephrine and valinomycin on the light scattering of liver cells. Experimental details are as given in the legend to Fig. 2. Ca^{2+} was present in both incubations.

cellular Ca^{2+} on the light-scattering response is demonstrated. The experiments were performed in Ca^{2+} -free medium to which 150 μ M-EGTA had been added 10 min before hormone addition. Little or no effect of glucagon, phenylephrine or vasopressin on light scattering is observed under these conditions, whereas valinomycin still exerted its effect. As reported by others (Kneer *et al.*, 1979) rates of gluconeogenesis were reduced under these conditions and the hormonal stimulation, especially by phenylephrine, was impaired (Table 5). Valinomycin sometimes slightly stimulated gluconeogenesis and sometimes was inhibitory.

In order to assess whether the light-scattering changes are of the right order of magnitude for the mitochondrial volume changes measured, the experiments of Fig. 5 were performed. Isolated mitochondria were incubated in the spectrophotometer at the same concentration (3–4 mg of protein/ml) as present in a cell incubation of about 8 mg of protein/ml used in whole-cell light-scattering experiments. $^3\text{H}_2\text{O}$ and ^{14}C sucrose were present to allow subsequent measurement of the matrix volume. Mitochondria were rapidly swollen by adding valinomycin (10 nM) or shrunk by adding 1 M-KCl to increase the osmolarity. Light-scattering changes and associated volume changes are shown in Fig. 5. It can be seen that the light-scattering changes accompanying a 10–15% change in mitochondrial volume are not dissimilar to those seen with phenylephrine or glucagon treatment of whole cells.

Conclusions

The data in the present paper indicate that glucagon, phenylephrine, vasopressin and valinomycin cause an elevation of mitochondrial volume *in situ* coincident with their stimulation of gluconeogenesis. We have used two methods to measure the mitochondrial volume and both detected changes in the volume of a similar magnitude. Rapid cell disruption yielded mitochondrial volumes of control cells of 1.09 $\mu\text{l}/\text{mg}$ of mitochondrial protein when

Table 5. The effects of Ca^{2+} on the stimulation of gluconeogenesis by various hormones

Cells were incubated with L-lactate (10 mM) and pyruvate (1 mM) and rates of gluconeogenesis determined as described in the Experimental section. Those experiments conducted in the absence of Ca^{2+} were supplemented with 0.15 mM-EGTA 5 min before hormone addition. Values are given as means $\pm$ S.E.M. of the numbers of observations shown and statistical significance of the experimental values compared with controls was judged using Student's *t* test: $\dagger\dagger\dagger P < 0.001$; $\dagger\dagger P < 0.01$; $\dagger P < 0.05$. Values in parentheses are percentages of control values.

Treatment	Rate of gluconeogenesis (nmol of glucose/mg of protein per min)		n
	Ca^{2+} present (2.5 mM)	Ca^{2+} absent	
Control	5.82 ± 0.22	3.63 ± 0.25	10
Glucagon (0.1 μ M)	7.52 ± 0.28 (129.5 $\pm$ 2.9 $\dagger\dagger\dagger$)	4.23 ± 0.31 (117.4 $\pm$ 4.0 $\dagger\dagger$)	10
Phenylephrine (20 μ M)	8.18 ± 0.44 (140.9 $\pm$ 5.5 $\dagger\dagger\dagger$)	4.01 ± 0.38 (108.7 $\pm$ 5.0)	10
Valinomycin (1 nM)	6.76 ± 0.39 (112.5 $\pm$ 4.2 $\dagger$)	3.88 ± 0.23 (100.3 $\pm$ 6.0)	10
Control	5.35 ± 0.34	3.02 ± 0.14	3
Vasopressin (25 nM)	6.52 ± 0.20 (123.1 $\pm$ 5.9 $\dagger$)	3.18 ± 0.30 (104.6 $\pm$ 5.0)	3

Fig. 5. The light-scattering changes of isolated liver mitochondria accompanying alterations in the intramitochondrial volume

Mitochondria isolated conventionally from rat liver were incubated at 3–4 mg of protein/ml in a split-beam spectrophotometer. The buffer contained 125 mM-KCl, 10 mM-Mops, 7 mM-Tris base, 2 mM-potassium phosphate, 2 mM-MgCl₂, 0.5 mM-EGTA, 5 mM-succinate and 1 μg of rotenone/ml. The pH was 7.4 and the temperature 25°C. Oxygenation was maintained by constant stirring. The intramitochondrial volume was altered by the additions indicated, and at the time of maximal light-scattering response ³H₂O and [¹⁴C]sucrose were added to a final radioactivity of 1 μCi/ml and 0.1 μCi/ml respectively. Three 1 ml samples were then taken from each cuvette into centrifuge tubes for measurement of the intramitochondrial volume as described by Halestrap & Quinlan (1983). The values shown are expressed as μl/mg of mitochondrial protein (± S.E.M.).

measured using ³H₂O and [¹⁴C]sucrose. The values rose to about 1.23 μl/mg of mitochondrial protein when either glucagon- or phenylephrine-treated cells were used and to 1.42 μl/mg of mitochondrial protein when both hormones were present. The amount of mitochondrial protein as a percentage of the total cell protein was 33.8 ± 1.4% (mean ± S.E.M. of 15 observations), and the total cellular water was 1.89 ± 0.05 μl/mg of cell protein (mean ± S.E.M. of nine separate experiments). Thus using the ³H₂O/[¹⁴C]sucrose space to estimate the intramitochondrial volume about 20% of the total cell volume is intramitochondrial. However, when the ³H₂O/[¹⁴C]mannitol space was used without cell disruption the matrix volume was only 0.40 μl/mg of protein in control cells, rising to about 0.50 μl with glucagon or phenylephrine and 0.61 μl with both hormones. This represents about 7–10% of the total

cell volume. The discrepancy between the values for the matrix volume obtained with mannitol and sucrose as extramitochondrial markers *in situ* and after disruption of cells respectively is very similar to that observed in isolated mitochondria (Halestrap & Quinlan, 1983). Thus in State 4 at 37°C the mean values (± S.E.M. of nine observations) for the intramitochondrial volumes measured using sucrose and mannitol as extramitochondrial markers respectively were 1.33 ± 0.05 and 0.47 ± 0.03 μl/mg of protein.

The effects of hormones on the intramitochondrial volume would appear to be reflected in a change in the light scattering of the whole cell (Figs. 2–4). The decrease in light scattering observed correlates well with the change in mitochondrial volume detected and shows a similar time course and dose-response curve to the effects of hormones on gluconeogenesis from L-lactate plus pyruvate. Of particular interest is the loss or substantial reduction of hormone stimulation of gluconeogenesis and light-scattering changes seen in a Ca²⁺-depleted media. Valinomycin, a K⁺ ionophore, is still effective on light scattering in the absence of Ca²⁺, but not on gluconeogenesis (Fig. 3; Table 5). This may indicate a role for Ca²⁺ in the regulation of mitochondria volume, possibly through a Ca²⁺-activated K⁺ channel such as exists on many cell membranes (Lew & Ferreira, 1978). Valinomycin, by acting directly as a K⁺ channel, by-passes the need for Ca²⁺ to increase K⁺ permeability.

Previous work from this laboratory (Halestrap, 1981; Halestrap, 1982; Armston *et al.*, 1982) has demonstrated that many of the effects of hormone treatment of a rat on the properties of subsequently isolated liver mitochondria may be induced by an increase in the matrix volume or by a decrease in phospholipase A₂ activity. The data of Table 1 lend no support for phospholipase A₂ being inhibited *in situ*. However it does appear that an increase in matrix volume may be the physiological means of hormonal regulation of some aspects of mitochondrial function. In particular respiratory chain activity, pyruvate metabolism, citrulline synthesis and glutaminase activity, all of which are activated in the cell by glucagon and α-agonists, may be regulated in this way (Joseph & McGivan, 1978; Joseph *et al.*, 1980; Lacey *et al.*, 1981; Halestrap, 1982; Armston *et al.*, 1982).

The relationship between mitochondrial volume and the rate of gluconeogenesis is not a simple one, however, since the additive effects of glucagon and phenylephrine on the matrix volume are not matched by a similar additive effect on the rate of gluconeogenesis. Similarly vasopressin has a relatively larger effect on the light-scattering response than it does on gluconeogenesis. One possible explanation for this is the stimulatory effect of all three hormones (transiently in the case of glucagon) on pyruvate

dehydrogenase in liver mitochondria (Denton *et al.*, 1981). This could divert some pyruvate from pyruvate carboxylase through pyruvate dehydrogenase and so reduce the stimulation of gluconeogenesis observed. Another situation in which there is not a strict correlation between rates of gluconeogenesis and the light scattering of hepatocytes is in the absence of Ca^{2+} . Although the response to hormones of both parameters is greatly reduced or abolished, valinomycin still exerts its effect on light scattering, whereas the effect on gluconeogenesis is abolished. This indicates that changes in mitochondrial volume alone cannot account for all of the effects of hormones on gluconeogenesis.

In addition to the physiological effects of hormones on mitochondrial function, it is possible that a change in membrane disposition involved in the mechanism of volume increase may also stabilize the mitochondria against endogenous phospholipase A_2 activity as previously suggested (Halestrap, 1981; Siess *et al.*, 1981; Armston *et al.*, 1982). Such a stabilization may result in changes in mitochondrial function developing during the preparation of mitochondria by conventional techniques (taking up to 1 h) that are not of physiological importance. Effects of hormones on the mitochondrial adenine nucleotide and phosphate content of mitochondria may fit into this category (Barritt *et al.*, 1978; Prpic *et al.*, 1978; Titheradge & Haynes, 1980a; Hensgens *et al.*, 1980) since these effects are not seen in rapidly isolated mitochondria (Siess *et al.*, 1977; Bryla *et al.*, 1977; Titheradge *et al.*, 1979; Titheradge & Haynes, 1980b) and are probably due to diminished leakage of adenine nucleotides and phosphate out of mitochondria from glucagon-treated rats during preparation (Armston *et al.*, 1982).

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References

- Armston, A. E., Halestrap, A. P. & Scott, R. D. (1982) *Biochim. Biophys. Acta* **681**, 429–439
- Barritt, G. J., Thorne, R. F. W. & Hughes, B. P. (1978) *Biochem. J.* **172**, 577–583
- Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520
- Bryla, J., Harris, E. J. & Plumb, J. A. (1977) *FEBS Lett.* **80**, 443–448
- Denton, R. M., McCormack, J. G. & Oviyasu, O. A. (1981) in *Short-Term Regulation of Liver Metabolism* (Hue, L. & Van de Werve, G., eds.), pp. 159–174. Elsevier/North-Holland Biomedical Press, Amsterdam
- Exton, J. H., Corbin, J. G. & Harper, S. C. (1972) *J. Biol. Chem.* **247**, 4996–5003
- Garrison, J. C. & Borland, M. K. (1978) *J. Biol. Chem.* **254**, 1129–1133
- Garrison, J. C. & Haynes, R. C. (1975) *J. Biol. Chem.* **250**, 2769–2777
- Halestrap, A. P. (1981) in *Short-Term Regulation of Liver Metabolism* (Hue, L. & Van de Werve, G., eds.), pp. 389–409, Elsevier/North-Holland Biomedical Press, Amsterdam
- Halestrap, A. P. (1982) *Biochem. J.* **204**, 37–47
- Halestrap, A. P. & McGivan, J. D. (1979) *Techniques in Metabolic Research* (Kornberg, H. L., Metcalfe, J. C., Northcote, D. H., Pogson, C. I. & Tipton, K. F., eds.), vol. B206, pp. 1–23, Elsevier/North-Holland Biomedical Press, Amsterdam
- Halestrap, A. P. & Quinlan, P. T. (1983) *Biochem. J.* **214**, 387–393
- Haynes, R. C., Garrison, J. C. & Yamazaki, R. K. (1974) *Mol. Pharmacol.* **10**, 381–388
- Hensgens, H. F. S. J., Verhoeven, A. J. & Meijer, A. J. (1980) *Eur. J. Biochem.* **107**, 197–205
- Joseph, S. K. & McGivan, J. D. (1978) *Biochim. Biophys. Acta* **563**, 16–28
- Joseph, S. K., McGivan, J. D. & Meijer, A. J. (1980) *Biochem. J.* **194**, 35–41
- Kirk, C. J. & Michell, R. H. (1981) *Biochem. J.* **194**, 155–163
- Kneer, N. M., Wagner, M. J. & Lardy, H. A. (1979) *J. Biol. Chem.* **254**, 12160–12168
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–36
- Lacey, J. H., Bradford, N. M., Joseph, S. K. & McGivan, J. D. (1981) *Biochem. J.* **194**, 19–33
- Lew, V. L. & Ferreira, H. G. (1978) *Curr. Top. Membr. Transp.* **10**, 217–277
- Prpic, V., Spencer, T. L. & Bygrave, F. L. (1978) *Biochem. J.* **176**, 705–716
- Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. (1980) *FEBS Lett.* **120**, 71–74
- Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. (1982a) *J. Biol. Chem.* **257**, 1906–1912
- Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. (1982b) *Biochem. J.* **208**, 619–630
- Siess, E. A. & Wieland, O. H. (1980) *Eur. J. Biochem.* **110**, 203–210
- Siess, E. A., Brocks, D. G., Lattke, H. K. & Wieland, O. H. (1977) *Biochem. J.* **172**, 517–521
- Siess, E. A., Brocks, D. G. & Wieland, O. H. (1978) *Biochem. J.* **172**, 517–521
- Siess, E. A., Fahimi, F. M. & Wieland, O. H. (1981) *Hoppe-Seyler's Z. Physiol. Chem.* **362**, 1643–1651
- Srere, P. A. (1972) *Curr. Top. Cell. Regul.* **5**, 229–283
- Srere, P. A., Barzil, H. & Gonen, L. (1963) *Acta Chem. Scand.* **17**, S129
- Thomas, A. P. & Halestrap, A. P. (1981) *Biochem. J.* **198**, 551–564
- Titheradge, M. A. & Haynes, R. C. (1980a) *J. Biol. Chem.* **255**, 1471–1477
- Titheradge, M. A. & Haynes, R. C. (1980b) *Arch. Biochem. Biophys.* **210**, 44–55
- Titheradge, M. A., Stinger, J. L. & Haynes, R. C. (1979) *Eur. J. Biochem.* **102**, 117–124
- Tolbert, M. E. M. & Fain, J. N. (1974) *J. Biol. Chem.* **249**, 1161–1166
- Vargas, A. M., Halestrap, A. P. & Denton, R. M. (1982) *Biochem. J.* **208**, 221–229
- Williamson, J. R., Cooper, R. H. & Hoek, J. B. (1981) *Biochim. Biophys. Acta* **639**, 243–295