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Review

The regulation of the matrix volume of mammalian mitochondria in vivo and in vitro and its role in the control of mitochondrial metabolism

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Contents

Abbreviations: PP_i, inorganic pyrophosphate; ETF, electron-transferring flavoprotein; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; Mops, 4-morpholinepropane-sulphanic acid.

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I. Summary

The purpose of this article is to describe briefly the methods by which the intra-mitochondrial volume may be measured both in vitro and in situ, to summarise the mechanisms thought to regulate the mitochondrial volume and then to review in more detail the evidence that changes in the intra-mitochondrial volume play an important part in the regulation of liver mitochondrial metabolism by glucogenic hormones such as glucagon, adrenaline and vasopressin. It will be shown that these hormones cause an increase in matrix volume sufficient to produce significant activation of fatty acid oxidation, respiration and ATP production, pyruvate carboxylation, citrulline synthesis and glutamine hydrolysis. These are all processes activated by such hormones in vivo. I will go on to demonstrate that the increase in matrix volume is brought about by an increase in mitochondrial [PP_i]. This is able to stimulate K^+ entry into the matrix, perhaps through an interaction with the adenine nucleotide translocase. The rise in matrix [PP_i] is a consequence of an increase in cytosolic and hence mitochondrial [Ca^{2+}] which inhibits mitochondrial pyrophosphatase. In the final section of the review I provide evidence that changes in mitochondrial volume may be important in the responses of a variety of tissues to hormones and other stimuli. I write as a metabolist with a working knowledge of bioenergetics rather than the converse, and this will certainly be reflected in the approach taken. If I cause offence to any dedicated experts in the field of bioenergetics by my ignorance or lack of understanding of their studies I can only offer my apologies and ask to be corrected.

II. General introduction

The morphology of isolated mitochondria and those in situ has been studied in great detail using electron microscopy. The effects of changes in diet, energy status and osmotic support have been studied as well as differences observed between species and tissues [1–4]. It is well established that mammalian mitochondria are surrounded by two membranes, an outer membrane which is permeable to all molecules of about 6000 kDa or less and an inner membrane which is impermeable to all but a limited number of metabolites and ions [1,5,6]. The high permeability of the outer membrane is caused by the presence of porins, proteins which create a non specific channel through which molecules can cross the membrane [7,8]. Whilst it has been shown that the conductivity of porins can be voltage regulated [7–10], there is no evidence to suggest that the outer membrane exerts a permeability barrier to entry of metabolites or ions into the inter-membrane space under physiological conditions. This effectively precludes the possibility of regulating the volume of this compartment by any osmotically driven process. Rather it must be envisaged that its volume is determined in vivo by the protein concentration either side of the outer membrane. On isolation of mitochondria the osmotic pressure exerted by the protein between the inner and outer membranes is no longer balanced by extra-mitochondrial protein. This causes isolated mitochondria to be more rounded and to have a greater inter-membrane space than those in vivo, unless macromolecules such as dextran, heparin or polyvinyl-pyrrolidone are included in the medium [11–13].

In contrast to the outer membrane, the permeability of the inner membrane is limited to small polar molecules such as polyols of C_4 or less, lipid soluble compounds, and metabolites for which specific transport processes exist [14–16]. Thus, mitochondria are usually prepared in buffered solutions of low molecular weight impenetrant solutes such as sucrose or mannitol. The total osmolality of the buffer is similar to that of the cytosol (300 mosmolal) which is thought to maintain the matrix volume at a value close to that found in vivo [17,18]. Changing the osmolality of the osmotic support or entry of a permeant solute causes the matrix volume to change as water is taken up to equilibrate the osmotic pressure on either side of the membrane. Indeed it was shown many years ago that the intra-mitochondrial volume of de-energised mitochondria responds exactly as would be expected for a well-behaved osmometer [19–29]. Thus, the mitochondria expand and contract by the cristae folding and unfolding, the membrane supporting no apparent turgor pressure [1]. When the physical limit of swelling is approached the mitochondria become leaky and lose some of their osmotically active material [20,29,30].

The limited permeability of the inner membrane is important for two inter-related aspects of mitochondrial function. Firstly it allows the existence of a metabolic compartment within the cell quite distinct from the cytosol, although interconnected with it through the operation of specific transport mechanisms [15,16]. Such compartmentation of metabolism is an essential feature of metabolic control in higher organisms (see, for example, Ref. 31). Secondly the limited permeability of the inner membrane is essential if proton pumping by the respiratory chain is to generate a membrane potential and pH gradient across the membrane [32–34]. These two gradients not only participate in the production of ATP (according to the chemiosmotic hypothesis) but also influence the activity of specific transport systems for ions and metabolites [16,35]. This in turn allows the mitochondrial compartment to contain a different composition of permeant metabolites and ions to that of the cytosol [35,36]. One such ion, K^+, is the major intra-mitochondrial cation and through the operation of specific influx and efflux pathways the mitochondrial content of K^+ can be regulated. If movement of a

compensating anion such as phosphate also occurs there will be an imbalance of osmotic pressure across the membrane and this will cause a change in the intra-mitochondrial volume. This will be considered in detail in Section IV. In Section V I will demonstrate that such changes in the matrix volume do occur in response to hormonal stimulation of a cell and so influence a variety of mitochondrial processes which are essential in the overall action of the hormone on cellular metabolism. In Section VI I will describe how we believe such increases in mitochondrial volume are brought about and then in Section VII I will endeavour to integrate the conclusions of the previous sections into a scheme explaining the mechanism of action of hormones on liver mitochondrial metabolism. Finally, in Section VIII I will provide evidence that changes in the mitochondrial matrix volume may be significant in the regulation of mitochondrial metabolism in other tissues. However, it is necessary first of all to describe how the volume of the mitochondrial matrix may be measured.

III. Measurement of the intra-mitochondrial volume

An accurate measurement of the mitochondrial matrix volume is essential for calculation of the intra-mitochondrial concentration of metabolites, and for the estimation of membrane potential and pH gradient across the inner mitochondrial membrane [37–40]. Two approaches are possible. Firstly, the use of electron microscopy to visualise the mitochondria and secondly the measurement, either gravimetrically or isotopically, of the matrix water content. A third method, light-scattering, is available for measuring changes in the mitochondrial volume but it is unable to give absolute values unless first calibrated by another technique. Each method will be considered in turn, and their application to measurement of mitochondrial matrix volumes in both isolated mitochondria and in situ will be considered.

III-A. Electron microscopy

Estimates of the total matrix volume in both isolated mitochondria and in tissues can be made using electron microscopy of fixed material. Care must be exercised in extrapolating the data obtained in such two-dimensional images to draw conclusions about the matrix volume. It is possible that an increase in the area occupied by the matrix is due to a shape or conformational change of the mitochondria rather than the result of an increase in volume. Thus, for accurate determinations of the matrix volume the areas occupied by the matrix of many mitochondria in the population must be measured to ensure that mitochondria sectioned in all planes, conformations and orientations are represented. Statistically valid regimes for this have been devised [41–43] but the approach is both extremely time consuming and will give a value for the total intra-mitochondrial space. This may be smaller than the volume accessible to water and metabolites [22]. It is also difficult to assess what is truly matrix space when the arrangement of the cristae is complex [43]. In addition there is the risk that fixation of the material has changed the volume and conformation of the mitochondria although this is thought to be unlikely with most fixing techniques [1,44]. Nevertheless the results obtained when all the necessary precautions are taken do seem to agree with values obtained by other techniques [43] and provide additional information on the range of matrix volumes of individual mitochondria in the population. An indication of this range can also be determined using a Coulter particle counter although this probably measures the volume of the whole mitochondrion rather than that of the matrix [45]. To the author's limited knowledge morphological studies of mitochondrial structure in fixed tissues have not been made in sufficient detail to allow accurate calculation of matrix volumes (as opposed to the total volume occupied by the mitochondria). However, two dimensional images can give strong qualitative support for a change in matrix volume and examples of this will be presented in Section VIII.

III-B. Determination of matrix water content

III-B.1. Isolated mitochondria

In order to measure the matrix water content of isolated mitochondria three conditions must be met. Firstly, the mitochondria must be separated from the medium in which they are suspended without changing either their volume or their permeability properties. Secondly a method must be available for measuring the total water content of the mitochondria, and thirdly there must be a means of correcting this value for the water trapped between mitochondria and in the intermembrane space.

Separation of mitochondria is usually achieved by rapid centrifugation. However, Sitaramam and his colleagues have argued that under the influence of the gravitational field associated with this procedure the inner membrane becomes leaky to small polyols such as sucrose and mannitol [46–48]. The conclusions of these workers are not generally accepted and may reflect the lack of any precautions taken to prevent damage to the mitochondria by Ca^{2+} contaminating their media. It is well documented that treatment of mitochondria with Ca^{2+} can make them leaky to small molecules [49–51]. When Ca-chelating agents are present in the isolation medium entry of sucrose into the matrix during centrifugation is minimal, although a small leakage of mannitol into the matrix does occur during the time taken to isolate mitochondria [18]. Indeed this may account for the slightly larger matrix volume observed in such

TABLE I

Values of the mitochondrial and hepatocyte volumes measured under various experimental conditions

Measurement of ^3H_2O-[^{14}C]sucrose and ^3H_2O-[^{14}C]mannitol spaces was performed after sedimentation of cells or mitochondria either directly or through silicone oil as described in subsection III-B. Further details of the experimental protocol used may be found elsewhere [55a,55,63]. Values are presented as means ± S.E.M. of the number of experiments shown in parentheses. The statistical significance of the effect of oil (*) or experimental conditions a and b (+) was determined using a paired Student's *t*-test.

Experimental conditions	Spaces in μl per mg mitochondrial or cell protein			
	without silicone oil		with silicone oil	
	^3H_2O-[^{14}C]sucrose	^3H_2O-[^{14}C]mannitol	^3H_2O-[^{14}C]sucrose	^3H_2O-[^{14}C]mannitol
1. Mitochondria				
(a) Crude	2.33 ± 0.16 (5)	1.02 ± 0.05 (5)	–	
(b) Percoll treated	1.23 ± 0.04 (18) $^{++}$	0.97 ± 0.03 (18)	1.05 ± 0.05 (6) *	0.84 ± 0.03 (6) **
2. Hepatocytes				
(a) Control	1.66 ± 0.04 (3)	0.34 ± 0.03 (3)	073 ± 0.02 (3) **	−0.45 ± 0.06 (3) **
(b) 25 nM Vasopressin	–	0.40 ± 0.04 (3) $^{+}$	–	−0.52 ± 0.09 (3) **

* or $^{+}$: $P < 0.05$.
** or $^{++}$: $P < 0.01$.

mitochondria [18,50,51]. However, this entry of mannitol does not appear to be a consequence of centrifugation, since we have shown using a high pressure light scattering apparatus that at 20 °C neither sucrose nor mannitol enters the matrix of isolated mitochondria at a significant rate when pressures are applied in excess of 100 atmospheres, higher than those encountered during centrifugation [18]. When isolated mitochondria are incubated at 37 °C for more than 5 min significant leakage of mannitol, but not of sucrose, into the mitochondrial matrix can occur, probably as a result of a decline in membrane integrity [28].

Some workers centrifuge mitochondria through a silicone oil layer into $HClO_4$ when measuring the matrix volume (see Refs. 52–54). This has the advantage that it allows a more complete removal of the extra-matrix water from the pellet and prevents any mitochondrial metabolism occurring if measurement of mitochondrial metabolites is required. However, recent studies have indicated that this technique produces values for the matrix volume that are dependent on the mitochondrial protein concentration used, which is not the case when silicone oil is not used [54]. The reason for this discrepancy is not known, but we have shown that both in isolated hepatocytes [55] and to a lesser extent in mitochondria [55a] water is lost during passage of the particles through the oil, whilst the metabolites and extra-vesicular marker molecules such as sucrose, inulin or mannitol are not. This is apparent in the data of Table I.

Measurement of the total water content of the isolated mitochondrial pellet can be achieved gravimetrically [22,26,29] but is more usually performed using ^3H_2O [23,28,54]. It has been shown that [^{14}C]urea equilibrates with the same pool of water as does ^3H_2O and so can be used in its place [56,57]. This has some advantages in that ^3H_2O can readily evaporate or exchange with atmospheric water and thus precautions must be taken to avoid underestimating the true water content of the mitochondria [18,28,54,58]. Measurement of the extra-mitochondrial volume is usually achieved by measuring the amount of an extra-mitochondrial marker molecule present in the pellet. Sucrose, mannitol and Cl^- have been used most commonly for this purpose [22,24,28] although many other molecules have suitable properties. They should be able to cross the outer but not the inner mitochondrial membrane and preferably be uncharged to avoid binding to fixed charges on the membrane. Although chemical or enzymic analysis of the extra-mitochondrial marker can be performed [56,61], they are usually ^{14}C-labelled so that they may be measured in conjunction with ^3H_2O by dual label scintillation counting. The highest standards of dual label counting are essential if accurate matrix volumes are to be determined because only 10–15% of the total mitochondrial pellet volume will represent matrix water. Since this water is obtained by the subtraction of the extra-mitochondrial water space from the total water space, small errors in determining either will lead to large errors in the final result. Many years of measuring intra-mitochondrial volumes in this laboratory have led to a considerable refinement of the counting techniques used. It is essential to use unscratched glass vials with tight fitting lids to avoid evaporation of ^3H_2O. Exposure of the lids to fluorescent lighting or handling with plastic gloves can sometimes produce errors in counting. Plastic scintillation vials have consistently given inferior results. Some scintillation counters use one channel exclusively for detecting ^{14}C dpm and another channel for ^{3}H dpm which must be corrected for ^{14}C overspill. With such counters it is necessary to use a ^{3}H:^{14}C ratio of at least 10:1 and to keep quenching as low as possible. However, most modern scintillation counters use automatic

efficiency control and window settings which require correction for overspill of both ^{3}H counts into the ^{14}C channel and ^{14}C counts into the ^{3}H channel. This enables high efficiency of counting of both isotopes over a wide range of quench conditions and has the advantage of allowing the use of a ^{3}H:^{14}C ratio of 5:1 or less. In either case, quench curves must be regularly checked and care taken to ensure that the sample mixes into one clear phase with scintillation fluid. Minor perturbations in counting efficiency can be avoided by always counting pellet and corresponding supernatant samples sequentially.

The most usual extra-mitochondrial marker used is [^{14}C]sucrose, since this is thought to fufill all the necessary criteria and is relatively cheap. However, there are reports that some commercially available preparations have a highly charged contaminant that binds to mitochondria under conditions of low ionic strength and so leads to an overestimate of the extra-mitochondrial volume unless it is first removed by chromatographic purification [29]. Certainly the use of charged molecules as extra-mitochondrial markers is inadvisable, since at low ionic strength positively charged molecules such as choline will tend to bind to the mitochondrial membranes leading to an underestimate of matrix volume [28,59], whilst negatively charged molecules such as Cl^{-} may be excluded from the intermembrane space by the fixed negative charges [28]. Halestrap and Quinlan have also questioned whether sucrose is the most appropriate extra-mitochondrial marker and provided evidence that [^{14}C]mannitol might be preferable [28]. The values of the matrix volume obtained in this and other laboratories using mannitol were somewhat lower than those obtained using sucrose [18,28,52,54,61,62], but gave more consistent values when volumes were measured in media of different composition but the same osmolality [18]. Other workers have reported little difference between the values obtained using sucrose and mannitol [60], and in this laboratory recent determinations have found smaller differences between the values obtained with the two procedures than we observed previously. Investigating the cause of this discrepancy has led us to conclude that the purer the mitochondrial fraction the less difference between the values. This is shown in Table I. Thus, well-washed mitochondria that have been purified by Percoll density gradient centrifugation [63] have mitochondrial matrix volumes of about 1.2 μl/mg protein when measured using [^{14}C]sucrose as mitochondrial marker and about 1.0 μl/mg protein when [^{14}C]mannitol is used. However, in a crude unwashed mitochondrial fraction the values were 2.3 and 1.0 μl/mg protein, respectively. The likely explanation of this difference is that plasma membranes are present in crude mitochondrial fractions [63] and these may produce vesicles which are permeable to mannitol but not sucrose [57,64]. Contamination of mitochondria by lysosomes and peroxisomes may also contribute towards the differences. Thus, it is probable that mannitol and sucrose both give quite reliable values for the matrix volume in pure mitochondrial fractions, but mannitol may be preferable if there is any likelihood of contamination by other membrane vesicles.

III-B.2. Mitochondria in situ

Where a knowledge of the intra-mitochondrial matrix volume in situ is required, as when using rapid cell fractionation techniques to estimate the intra-mitochondrial concentrations of metabolites, it has usually been calculated from the values measured in isolated mitochondria and the mitochondrial content of the tissue [38,65]. However, an accurate measurement of the intra-mitochondrial matrix volume in situ is desirable. There have been attempts to measure the matrix volume using rapid cell disruption by digitonin and sheer force techniques and non-aqueous cell fractionation [66–68]. These approaches have not yielded very consistent data with values for the intra-mitochondrial matrix volume varying between 9 and 13% of the total cell water. Morphological techniques have suggested the total volume of the mitochondria, including the intermembrane space, is about 22% of the rat liver cell volume [69]. In order to obtain more accurate data for the mitochondrial matrix volume in isolated hepatocytes we have developed two approaches [63]. Firstly, we have rapidly disrupted hepatocytes by diluting the cell suspension into a small centrifuge tube containing EGTA sufficient to chelate the Ca^{2+} in the incubation buffer and also [^{14}C]sucrose and $^{3}H_2O$ for measurement of the mitochondrial matrix volume. The cells were then rapidly sonicated at low energy for 5 s and the released mitochondria sedimented within 60 s in a microcentrifuge. This technique of cell breakage was found to give mitochondria with good respiratory control ratios and in a good yield which could be accurately assessed by assay of citrate synthase in the mitochondrial pellet [64,70]. The value of the matrix space determined in this manner was between 1 and 1.4 μl per mg mitochondrial protein depending on the hormonal status of the cells (see subsection V-F) and represented about 20% of the total intra-cellular water. These values are similar to those found in conventionally isolated mitochondria incubated under energised conditions as might be expected. Some over-estimate of the intra-mitochondrial volume may occur as a result of contaminating vesicles such as lysosomes, peroxisomes, microsomes and plasma membranes [55a,63,70], but this should be relatively consistent within any experiment.

It can be argued that rapid cell disruption might perturb the mitochondria and so not truly reflect the situation in situ. To overcome this problem we developed a non-disruptive technique for determining the

matrix volume. For this we required a non-metabolisable and small molecular weight chemical that could cross both the plasma membrane and the outer mitochondrial membrane but not the inner mitochondrial membrane. We were able to demonstrate that [^{14}C]mannitol fulfilled these criteria [64], and this has recently been confirmed by others [57]. Thus, cells may be incubated with [^{14}C]mannitol and $^{3}H_2O$ for at least 30 min to allow mannitol to equilibrate across the membrane, and then the cells are rapidly centrifuged (6 s at 10000 × *g*) and the [^{14}C] and [^{3}H] in the pellet and supernatant determined. The intra-mitochondrial matrix volume is then determined from the $^{3}H_2O$-[^{14}C]mannitol space. This value is only a very small percentage of the total pellet water and thus great care must be taken in the dual label scintillation counting as outlined above. It should also be stressed that in our hands it is not possible to obtain accurate results if silicone oil filtration is used (see Table I), although Lund and Wiggins appear to use this technique successfully [57]. However, there is another significant difference between our own methodology and that of Lund and Wiggins. In our experiments we showed that the mannitol takes about 40 min to fully equilibrate with the cytosolic space. A 10 min preincubation was used by Lund and Wiggins which in our experiments would only give about 90% equilibration. This could lead to an over-estimate of the [^{14}C]mannitol-$^{3}H_2O$ space which compensates for the underestimate that the use of silicone oil filtration produces. Another potential source of error that must be recognised is that any intra-cellular marker such as [^{14}C]mannitol may be sequestered by pinocytotic vesicles, autophagic vacuoles and lysosomes in a time dependent manner [70a,b]. There is also the possibility of slow entry of the marker into the mitochondria [70a] although it is unlikely to be as rapid as that seen after prolonged incubation of isolated mitochondria where the integrity of the inner membrane deteriorates [28]. Both of these phenomena would lead to an under-estimate of the true intra-mitochondrial volume, whilst exclusion from some other intra-cellular compartment would have the opposite effect. Despite these reservations it is probable that the use of [^{14}C]mannitol should allow the detection of changes in the intra-mitochondrial volume, even if the absolute values are questionable.

In the original experiments performed in this laboratory the mitochondrial matrix space in situ was found to be only about 0.4–0.6 μl/mg mitochondrial protein, representing 7–10% of the total cell volume. This value is considerably lower than that obtained by the disruptive technique but similar to that measured in energised isolated mitochondria in parallel experiments. As outlined above in more recent experiments we have obtained larger values for the matrix space in isolated mitochondria using [^{14}C]mannitol and the same is true in intact hepatocytes (Table I). Thus, we now obtain values of 0.33–0.4 μl/mg cell protein which correspond to about 1.0 to 1.3 μl/mg mitochondrial protein. This is very similar to the results of Lund and Wiggins and to those obtained using [^{14}C]sucrose after rapid cell disuption. Our more recent data using [^{14}C]mannitol with isolated energised mitochondria are also consistent with these values (Table I). We have no certain explanation for the low values originally obtained in this laboratory, but the presence of a ^{14}C-labelled, metabolisable contaminant in the [^{14}C]mannitol is suspected even though precautions were taken to remove any residual mannose. This highlights the need for great caution in the choice and use of radio-labelled extra-mitochondrial markers as described above.

III-C. Light-scattering techniques

III-C.1. Isolated mitochondria

It has been known for many years that when mitochondria swell they show a decrease in light-scattering (see Refs. 14,20,29). This can be detected using a spectrophotometer or a more simple photometric device by measuring the decrease in absorbance at a suitable wavelength such as 520 nm (an isosbestic point for the cytochromes). The accepted explanation for this is that the swollen matrix has a lower refractive index than the condensed form, but the exact relationship between particle size and light-scattering is complex, especially when the diameter of the particle is similar to that of the wavelength of the incident light [71]. It is also well documented that conformational changes in the mitochondrial membrane or shape changes in the whole mitochondrion can influence light-scattering independently of changes in the matrix volume [29,72]. Thus, absolute measurements of matrix volume by this technique are not possible unless detailed calibration under well-defined conditions is first performed [26,29,73]. Nevertheless for measuring changes in mitochondrial volume with time light-scattering is the only practical technique. Indeed it was the method used in many of the pioneering studies on metabolite and ion transport into mitochondria [14,74]. Suspension of de-energised mitochondria in iso-osmotic solutions of the metabolite or ion concerned will cause swelling when there is net uptake of solute into the matrix. Accurate kinetics of transport are only possible if the light-scattering is calibrated to take into account changes that are not mediated by volume changes, and this has recently been attempted by Garlid and Beavis [75].

III-C.2. Mitochondria in situ

The use of light-scattering to measure changes in the matrix volume of isolated mitochondria is well accepted, but its use can be extended to the mitochondria in situ.

This possibility was first suggested by experiments of Hackenbrock et al. on the energy dependent conformational changes of mitochondria in intact ascites cells [76]. Thus, it was demonstrated that the same relationship between energy state, morphology of mitochondria and light-scattering could be observed with intact cells as with isolated mitochondria. We have used the same technique in isolated hepatocytes and correlated changes in light scattering induced by various treatments of the cell with measurements of mitochondrial volume but not with the whole cell volume [64]. Indeed the total intracellular volume could be changed by increasing the osmotic pressure of the extra-cellular medium without producing a significant change in light-scattering [64]. It is these observations which have led us to conclude that the mitochondrial matrix volume is under hormonal control and this will be considered in detail in subsection V-F.

IV. Regulation of the matrix volume of isolated mitochondria

The swelling of heart and liver mitochondria in various K^+ salts and the associated flux of $^{42}K^+$ across the inner membrane have been studied in the laboratories of Brierley and Garlid. This has led to the recognition of a K^+/H^+ antiporter which pumps K^+ out of the mitochondria and an electrogenic uniporter which mediates K^+ entry into the mitochondria [77–81]. It is thought that the operation of these two transporters in conjunction with the parallel movement of permeant anions such as phosphate is responsible for the regulation of the intra-mitochondrial K^+ content and, as an osmotic consequence, of the matrix volume. Under de-energised conditions increasing the matrix volume by a variety of means leads to an increase in the activity of the K^+/H^+ antiporter [78–83]. The resulting loss of K^+ and compensating anion allows the mitochondria to prevent excessive swelling by osmo-regulation. Similarly if K^+ entry into energised mitochondria is stimulated by addition of low concentrations of valinomycin, the mitochondria swell as expected. However, a compensatory stimulation of the K^+/H^+ antiporter occurs which prevents further swelling and a new equilibrium is established at an increased matrix volume [63,80,84]. Garlid has proposed that Mg^{2+} acts as a natural inhibitor of the K^+/H^+ antiporter whose concentration in the matrix decreases as the matrix volume increases and so allows stimulation of the antiporter [79,80]. Several laboratories have shown that treatment of mitochondria with A23187 and EDTA to deplete them of Mg^{2+} causes stimulation of K^+/H^+ fluxes [79–81,83,85–91]. However, it was pointed out by Brierley et al. that depletion of Mg^{2+} might cause non-specific effects on membrane permeability, since it is known that bound Mg^{2+} is important for membrane stability [81]. Indeed only when $[Mg^{2+}]$ is lowered with A23187 and EDTA to concentrations considerably less than assumed to occur under physiological conditions is appreciable stimulation of the antiporter observed [91]. This implies that $[Mg^{2+}]$ is unlikely to operate as a physiological regulator of the antiporter, although Garlid et al. do not accept this conclusion [100]. However, there are some instances where it is probable that K^+ permeability is enhanced by such non-specific membrane effects. For example K^+ permeability is increased when mitochondria are incubated with uncoupler or when the matrix NADH and adenine nucleotides are depleted [96,101,102]. Such conditions are known to activate mitochondrial phospholipase A_2 and so increase non-specific permeability of the inner membrane [103–107]. An alternative mechanism proposed to explain how the matrix volume can influence the operation of the K^+/H^+ antiporter is through the folding of the cristae or some other perturbation of the membrane structure [82,83]. This would seem quite probable, since many mitochondrial processes associated with the inner membrane are sensitive to small changes in matrix volume as will be described in subsection V-D.

The properties of the influx and efflux pathways for K^+ have been studied in some detail. The former has a K_m for K^+ of 6–12 mM and is activated by thiol reagents, phosphate and high pH [90–93]. In addition it shows activation by atractylate and adenine nucleotide depletion, and inhibition by ADP [90,94–97], which suggests some association with the adenine nucleotide transporter. This has recently been confirmed by work from this laboratory which will be presented in subsection VI-B. However, Diwan et al. have recently reported that a 53 kDa protein purified from liver mitochondrial membranes using a quinine affinity column acts as a K^+ ionophore when reconstituted into membrane vesicles [107a]. The activity of the K^+/H^+ antiporter is inhibited by quinine analogues and DCCD [87,92,98–100]. [^{14}C]DCCD labelling of a membrane protein of 82 kDa was reduced by addition of quinine or Mg^{2+} which has led Martin et al. to conclude that this protein is the antiporter protein [99]. However, it has been pointed out that neither quinine nor Mg^{2+} nor DCCD are very specific in their effects on the inner mitochondrial membrane [91,98] and final confirmation that this protein is the antiporter must await purification and reconstitution.

The regulation of the mitochondrial matrix volume through the operation of specific K^+ carriers depends on the parallel movement of anions such as phosphate. Under energised conditions this is thought to occur by movement of proton compensated pathways, since any electrogenic pathway would have the effect of forcing anions out of the matrix down the electrical gradient. However, under de-energised conditions there is evidence that a non-specific anion channel opens, espe-

cially when the matrix pH is high and [Mg^{2+}] low [108–110]. The anion channel is also inhibited by quinine and DCCD [109] and opens only at voltages of the opposite polarity to those found under physiological conditions [110]. Thus, it is hard to envisage how this channel could operate in the regulation of matrix volume except under pathological conditions where the mitochondria are de-energised [109].

V. Changes in the mitochondrial matrix volume as a mechanism of hormone signal transduction across the liver mitochondrial membrane

V-A. Activation of liver mitochondrial metabolism by hormones in vivo

Glucagon, adrenaline and vasopressin all stimulate gluconeogenesis, urea synthesis, fatty acid oxidation and glutaminase activity in the perfused liver or isolated hepatocytes (reviewed in Refs. 107,111,112). Stimulation of gluconeogenesis by glucagon can be accounted for in part by an inhibition of pyruvate kinase and phosphofructokinase through cyclic AMP dependent phosphorylation mechanisms [112–118]. However, there is also considerable evidence for a stimulation of pyruvate carboxylation following glucagon treatment of hepatocytes [60,119–122]. In the case of adrenaline and vasopressin which act through phosphatidylinositol-4,5-bisphosphate breakdown and a rise in intracellular [Ca^{2+}] [123,124] effects on phosphofructokinase are not observed [112,113,116,118] and the effects on pyruvate kinase are small or absent [113,114,118,120,121,125]. Under these conditions pyruvate carboxylase is probably the major locus of the hormonal stimulation of gluconeogenesis [116,120–122]. Thus, a mechanism must be available for the hormonal message to cross the inner membrane.

Pyruvate carboxylase is activated by acetyl-CoA and this is thought to be the mechanism by which fatty acids stimulate gluconeogenesis from lactate and pyruvate in the liver (see Ref. 113), although other mechanisms may operate [113a]. Thus, it is possible that activation of pyruvate carboxylase is secondary to that of fatty acid oxidation. In the fed state glucagon can achieve this by cyclic AMP dependent phosphorylation and inhibition of acetyl-CoA carboxylase which leads to a decrease in cytosolic (malonyl-CoA). This metabolite acts as a powerful inhibitor of carnitine acyl transferase 1, which is thought to be the major rate controlling step in the oxidation of fatty acids. The activation of this enzyme through a decrease in malonyl-CoA concentrations probably accounts for the activation of fatty acid oxidation by glucagon in the fed state [126–128]. However, there are reasons to believe that this is not the only site of regulation of fatty acid oxidation by glucagon. Thus, glucagon stimulates fatty acid oxidation in the starved state where carnitine acyl transferase 1 is relatively insensitive to inhibition by malonyl-CoA and acetyl-CoA carboxylase is largely inactive [127–132], and also in biotin deficient rats where little if any acetyl-CoA carboxylase activity would be expected [133]. Furthermore, Ca-mobilising hormones such as phenylephrine and vasopressin can activate fatty acid oxidation [121,134–138] and yet these hormones either have no effect on acetyl-CoA carboxylase or activate the enzyme [134,139,140]. Thus, carnitine acyl transferase 1 is unlikely to be the locus of action of these hormones and this is confirmed by their ability to stimulate the oxidation of octanoate which enters the mitochondria in a carnitine independent fashion [136,137]. An intramitochondrial activation of fatty acid oxidation would appear the most likely explanation of these observations and this leads us back to a means of hormone signal transduction across the mitochondrial membrane.

Hormones which stimulate gluconeogenesis from such substrates as lactate, alanine and glutamine also stimulate urea synthesis under appropriate conditions. It is generally accepted that a major rate controlling step in this pathway is carbamoyl phosphate synthetase, an intra-mitochondrial enzyme [141,142]. Associated with activation of urea synthesis there is also an hormonal activation of glutaminase, another intra-mitochondrial enzyme [143–146]. The activation of these two enzymes of nitrogen metabolism ensures that NH_3 produced by amino acid catabolism is usually excreted as urea rather than as NH_4^+ (through the operation of kidney glutaminase) which is essential for pH homeostasis [147,148]. Once again their regulation requires hormone signal transduction across the mitochondrial inner membrane.

Hormonal stimulation of both gluconeogenesis and urea synthesis requires increased ATP utilisation and thus it is hardly surprising that the rate of oxygen consumption increases [136,149–157]. The conventional views of the regulation of respiration would imply that this must be achieved either by a decrease in proton motive force (which should be reflected in a decrease in mitochondrial $ATP/ADP \cdot P_i$) or by a rise in mitochondrial $NADH/NAD^+$ [158–162]. However, what is actually observed is an increase in mitochondrial $ATP/ADP \cdot P_i$ [130,131,142,163–167] and possibly in the membrane potential [118,168], whilst the $NADH/NAD^+$ ratio may increase transiently but returns to control levels or below even though respiration remains stimulated [121,153,169–172]. Furthermore, the concentrations of several mitochondrial metabolites such as 2-oxoglutarate, glutamate and succinyl-CoA decrease after addition of glucogenic hormones to hepatocytes [130,131,210,215]. These observations all imply that hormones are exerting significant effects on mitochondrial oxidative metabolism in vivo that cannot

be accounted for soley by changes in ATP utilisation or NADH production.

The evidence presented above suggest that hormones can have quite wide ranging effects on mitochondrial metabolism in vivo. This conclusion is strengthened by the observation that mitochondria prepared from rats treated with glucagon or adrenaline show changes in many aspects of their metabolism that persist following their isolation.

V-B. The effects of hormone treatment of rats on the behaviour of isolated liver mitochondria

In 1969 Adam and Haynes demonstrated that if rats were treated with glucagon, adrenaline or cortisol before isolation of liver mitochondria the rates of pyruvate carboxylation and decarboxylation were stimulated [173]. Since that time their observations have been confirmed in many laboratories [58,174–178] and it has also become apparent that a great many other aspects of mitochondrial function are influenced by the hormone treatment. These are summarised in Table II and have been reviewed in detail elsewhere [107,111,112]. Amongst the mitochondrial processes stimulated by hormone treatment are respiration, pyruvate carboxylation, citrulline synthesis and glutaminase activity all of which are stimulated by glucogenic hormones in vivo as outlined above. This suggests that whatever is happening to the mitochondria in situ to stimulate these processes may be stable during isolation of the mitochondria. If this were the case it would be a great asset in establishing how hormones influence mitochondrial function in vivo. Unfortunately, however, it is far from certain that the effects of hormone treatment that persist in the isolated mitochondria are the same as those effects occurring in vivo. Thus, the effects of hormone treatment that are observed in isolated mitochondria are very dependent on the composition of the isolation buffer [18,52,195,208–212] which has led some workers to suggest that at least some of the effects of hormone treatment are artefacts of the isolation procedure [195,207,208,211–213]. This view is strongly contested by others who have demonstrated that many of the effects of hormones are independent of the

TABLE II

Summary of the persistent effects of glucogenic hormones on the properties of isolated rat liver mitochondria

Effects are given as +(stimulation), −(inhibition), or N (none). A blank indicates no data are available in the literature. References are given in parentheses.

Parameter	cAMP-dependent hormones	Ca^{2+}-mobilising hormones
Pyruvate carboxylation	+(58, 173–178)	+(173, 174, 177–179)
Pyruvate decarboxylation	+(173, 174, 179, 180)	+(173, 174, 180)
Pyruvate transport	+(175, 177, 180)	+(180)
Uncoupled and State 3 oxidation of		
NADH-linked substrates	+(163, 165, 175, 181–188)	+(180, 188)
succinate	+(163, 165, 175, 181–188)	+(142, 165, 189)
acyl CoA	+(183, 190)	
duroquinol	+(183, 187)	
Transhydrogenase activity	+(184, 191)	
Succinate dehydrogenase	+(185–187) N (183)	N (186, 188)
Citrulline synthesis	+(142, 163, 192–196)	+(142, 188, 196)
N-acetylglutamate level	+(193–196)	
ATP/ADP ratio	+(130, 131, 142, 164, 165)	+(131, 142, 164, 188)
Total adenine nucleotide content	+(130, 131, 142, 164, 165, 197)	+(131, 142, 164, 188)
Phosphate content	+(197–199, 208)	
Magnesium content	+(182, 200) N (198)	
Potassium content	+(182)	
Respiration-driven ion accumulation	+(183, 189, 198, 199, 201, 202)	+(189)
ATP-driven ion accumulation	+(201)	
Na-dependent Ca efflux	+(203, 204)	
Uncoupler-dependent ATPase	+(164, 165, 201)	+(142, 164)
Adenine nucleotide transport	+(165) N (199)	
Retention time for accumulated Ca^{2+}	+(189, 198, 199)	+(189)
Calcium-induced massive swelling	−(58)	−(188)
Matrix volume	+(58, 64, 175, 205) N (18, 60, 209)	+(64)
Glutaminase activity	+(145, 206)	+(145)
Membrane potential	+(60, 182)	+(60, 188, 199)
pH gradient	+(180, 182, 199) N (54, 59)	+(189) N (60, 180, 188)
Leakage of adenine nucleotides	−(58)	
Endogenous phospholipase A_2 activity	−(207)	

mitochondrial isolation procedure [18,210]. Furthermore, hormonal stimulation of respiration, pyruvate carboxylation and citrulline synthesis can be demonstrated in liver cells permeabilised with fillipin [178], in crude liver homogenates [210] and in mitochondria produced from isolated hepatocytes by rapid cell disruption [70,178].

V-C. Mechanisms involved in the effects of hormones that persist in isolated liver mitochondria

Research in several laboratories has been directed towards establishing the basis of the persistent effects of hormone treatment and whether these effects are occurring in vivo. This work has been reviewed elsewhere [111,112,216,217] and only a brief summary will be given here. In this laboratory we have been unable to demonstrate any changes in protein phosphorylation of mitochondrial proteins that could account for the hormone effects [218]. Nor have we observed any significant changes in mitochondrial phospholipids in vivo [64]. However, when mitochondrial phospholipids were labelled with $^{32}P_i$ by treating rats with $^{32}P_i$ for 12 hours prior to hormone injection, we were able to show a decrease in the lysophospholipid content of the mitochondria from glucagon treated animals and a decrease in the activity of mitochondrial phospholipase A_2 towards endogenous substrates [207]. This data complemented our previous conclusions that many of the effects of glucagon treatment could be reversed by aging of mitochondria, a process known to involve mitochondrial phospholipase A_2 [58,183]. More recent studies on the effects of glucagon treatment on the sensitivity of the respiratory chain to inhibitors of electron flow have suggested that in addition to an effect on the lysophospholipid content of mitochondria, glucagon might also decrease lipid peroxidation [219]. Since lipid peroxides are extremely sensitive to attack by phospholipase A_2 [220] their decrease in response to glucagon may account for the reduced phospholipase A_2 activity. Whether such effects on lipid peroxidation occur in vivo is not known, but glucagon has been observed to cause a decrease in H_2O_2 production by liver cells, and this is a product of lipid peroxidation [221].

In Sections V-E and VII (summarised in Fig. 12) I will present evidence that the respiratory chain is activated in vivo in response to glucogenic hormones and that this is achieved by an increase in mitochondrial volume stimulating electron flow into the ubiquinone pool. It is known that restriction of electron flow through this region of the respiratory chain (as occurs naturally in hypoxia) can lead to the production of free radicals and hence the production of lipid peroxides [222,223]. Thus, if hormones are able to activate the respiratory chain in vivo they might cause a decrease in lipid peroxidation and thus account for the decreased damage by phospholipase A_2 during mitochondrial preparation. This would imply that the physiological effects of hormones on mitochondrial metabolism in situ might be reflected in the persistent effects seen in isolated mitochondria, but the mechanism by which they are achieved may be quite different.

Other explanations that have been put forward for the mechanism by which glucagon may exert its effects on mitochondrial metabolism are by an increase in the phosphate [208,209] or malate [224] content of mitochondria. In our own experiments we have shown that increasing the phosphate content of mitochondria by incubation with phosphate containing media can increase the matrix volume [18]. Furthermore, in some experiments we have been able to show that mitochondria isolated from glucagon treated animals have a slightly increased matrix volume [58,64,75,205], although this is not always observed [18,60,209]. A change in matrix volume cannot account for all the persistent effects of hormone treatment found in isolated mitochondria, especially those which also persist in sub-mitochondrial particles [184,207,219]. Nevertheless the detection of changes in matrix volume in some experiments and reports in the literature of the effects of valinomycin and osmolality on mitochondrial function led us to investigate whether changes in mitochondrial volume could be a mechanism of regulating mitochondrial function in vivo.

V-D. The effects of changing the matrix volume of mitochondria on their metabolism

In their original studies Adam and Haynes demonstrated that incubation of mitochondria in hypo-osmotic medium greatly stimulated their rate of pyruvate metabolism [173]. These data were confirmed in this and other laboratories [52,58] and other studies showed that a similar stimulation could be induced by addition of low concentrations (10^{-9} M) of valinomycin [225]. Many other aspects of mitochondrial metabolism are influenced by the mitochondrial matrix volume and these are summarised in Table III.

We have demonstrated that increasing the matrix volume not only stimulates pyruvate metabolism [58] but also increases the initial rate of citrulline synthesis [58] and the rate of uncoupled and ADP-stimulated oxidation of both succinate and those substrates generating intra-mitochondrial NADH [18,58]. In addition, work from McGivan's laboratory has demonstrated that glutaminase is activated by hypo-osmotic treatment of mitochondria [206,227,228]. It was suggested many years ago that uncoupler might inhibit the oxidation of palmitoyl carnitine by isolated brown fat mitochondria by depleting them of K^+ and so causing them to shrink [230,231]. Otto and Ontko have provided evidence that

TABLE III

The effects of increasing the mitochondrial matrix volume that are also observed in hepatocytes treated with hormones

1. Stimulation of ADP-stimulated oxidation of all respiratory substrates entering the respiratory chain before cytochrome *c*, but especially fatty acid oxidation.
2. Increase in the reduction state of cytochromes *b* and *c*.
3. Increase in ATP/ADP ratio.
4. Stimulation of pyruvate carboxylation.
5. Stimulation of citrulline synthesis.
6. Stimulation of glutaminase activity.

Ca^{2+} might increase palmitoyl carnitine oxidation in isolated liver mitochondria through an increase in the matrix volume [232]. More recent work in this laboratory has investigated the effects of increasing the mitochondrial matrix volume on the rates of fatty acid oxidation in isolated liver and heart mitochondria in more detail [61,229]. We have confirmed that β-oxidation is extremely sensitive to small changes in the matrix volume between 0.9 and 1.4 μl/mg protein [61]. This is shown in Fig. 1 where I also present data from our own and other laboratories on the volume sensitivity of glutaminase, respiration and pyruvate carboxylation. It is of considerable interest that all these processes are sensitive to an increase in matrix volume over the same range and has led us to ask three important questions. Firstly, how does the mitochondrial volume

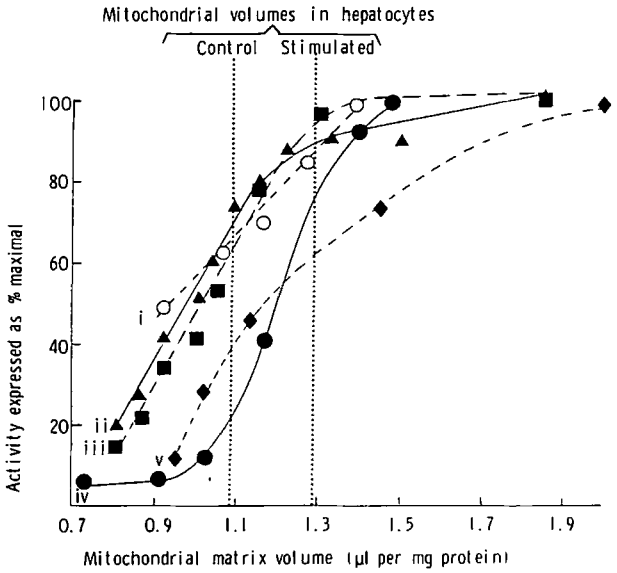

Fig. 1. The effects of changing the matrix volume on respiration, pyruvate carboxylation and glutaminase activity in isolated liver cells. Data for the rates of ADP stimulated oxidation of 5 mM glutamate + 1 mM malate (▲), 100 μM palmitoyl-carnitine (●) or 5 mM succinate + 1 μg/ml rotenone (■) were taken from Ref. 62. Data for the rate of pyruvate carboxylation (○) and glutaminase activity (◆) were taken from Ref. 52 and Ref. 228 respectively. In all cases mitochondrial volumes were altered by changing the osmolality of the incubation medium, and measured using ^3H_2O and [^{14}C]sucrose.

exert its effect on mitochondrial metabolism. Secondly, does the mitochondrial volume change in vivo within the range over which mitochondrial metabolism is sensitive to swelling. Thirdly, how could hormones regulate the intra-mitochondrial volume in vivo.

V-E. Mechanisms by which the matrix volume influences liver mitochondrial metabolism

Citrulline synthesis and pyruvate carboxylation both require a supply of ATP within the matrix. Since no extra-mitochondrial ATP is added in the experimental protocol we use to study these processes it must be generated by the respiratory chain. Thus, it is possible that the stimulation of pyruvate carboxylation and citrulline synthesis is secondary to the stimulation of the respiratory chain and we have provided evidence that this is the case. Increasing the matrix volume by incubating mitochondria in hypo-osmotic media increased the mitochondrial ATP/ADP ratio at the same time as stimulating pyruvate carboxylation and citrulline synthesis [58]. Both these processes have been shown to be activated by increases in mitochondrial ATP/ADP ratio under appropriate conditions [142,163,233–238]. This suggests that it may be the activation of the respiratory chain and subsequent increase in ATP/ADP ratio that is responsible for the activation of pyruvate carboxylation and citrulline synthesis. Conversely addition of low concentrations of a respiratory chain inhibitor such as amytal inhibited pyruvate carboxylation and citrulline synthesis by decreasing the ATP/ADP ratio as predicted [207]. We have also demonstrated that the respiratory chain exerts a strong influence on the rate of pyruvate carboxylation and gluconeogenesis in isolated hepatocytes [122,157]. Thus, the flux control coefficient of the respiratory chain for gluconeogenesis from L-lactate was found to be 0.61, indicating that it has a significant rate controlling influence on gluconeogenesis. In the presence of glucagon or phenylephrine the flux control coefficient dropped to 0.39 and 0.25, respectively [122], confirming that the respiratory chain is stimulated by these hormones and may play an important role in the regulation of gluconeogenesis (see Section VII).

The mechanism by which the respiratory chain can be regulated through changes in the matrix volume remains unclear. It is quite possible that there is a volume mediated effect on the fluidity of the inner mitochondrial membrane which is sensed by the respiratory chain. In halotolerant bacteria changes in the osmotic strength of the external medium are known to influence membrane fluidity [241], whilst changing the fluidity of the inner mitochondrial membrane by addition of benzyl alcohol, through the operation of phospholipase A_2 or by changes in diet can all influence the activity of the respiratory chain significantly

[58,183,239,240]. Furthermore, glutaminase is known to be associated with the inner membrane and its properties are greatly influenced by that association [228]. There have been recent reports that carbamoyl phosphate synthetase and ornithine transcarbamylase are also associated with the inner membrane which might provide an additional mechanism for controlling the rate of citrulline synthesis [242]. It is also possible that a change in membrane fluidity could affect the activity of the K^+/H^+ antiporter involved in osmo-regulation as described in Section IV. In this context it is of interest that membrane stretching can activate a Ca^{2+} channel in the choroid plexus epithelium membrane [243].

Another mechanism by which the matrix volume could influence the activity of membrane bound enzymes would be by the dilution of a specific activator or inhibitor of the enzyme. As outlined in Section IV, Mg^{2+} has been suggested to act as a physiological inhibitor of the K^+/H^+ antiporter and a similar suggestion has been made for the activation of glutaminase through increases in matrix volume [228]. However, using uncoupled mitochondria in the presence of A23187 and variable [Mg^{2+}] we were unable to find any significant inhibitory effect of Mg^{2+} on respiratory chain activity. Changing the matrix volume might also affect the folding of the cristae in such a way as to alter the local environment around membrane bound enzymes, but no studies on this possibility have been performed.

The location at which an increase in matrix volume stimulates electron flow through the respiratory chain and fatty acid oxidation has been investigated in this laboratory. The cytochrome spectra of mitochondria oxidising succinate or glutamate + malate under State 3 or uncoupled conditions have been compared in hyper-osmotic and iso-osmotic media. It was concluded that electron flow into the bc_1 complex was inhibited under hyper-osmotic conditions [183]. We have extended these observations by demonstrating that the effect of mitochondrial volume on the oxidation of both substrates was still apparent in the presence of ubiquinone-1 and ferricyanide [62]. Under these conditions ferricyanide acts as terminal electron acceptor without requiring electron flow through the bc_1 complex and cytochrome oxidase. Increasing the matrix volume was shown to stimulate the rate of oxidation of succinate and all substrates generating intra-mitochondrial NADH. This suggests that it is the oxidation of NADH itself that is stimulated rather than the production of NADH by the matrix dehydrogenases [58,61]. However, when duroquinol was used as respiratory substrate (donating electrons directly into the bc_1 complex), no effect of matrix volume on oxidation rates was observed [62]. Nor was there an effect of matrix volume on the oxidation of ascorbate in the presence of TMPD [58]. Thus, the evidence suggests that the effects of matrix volume must be associated with a locus on the reduced side of ubiquinone, leading to a regulation of electron flow into the ubiquinone pool.

The oxidation of palmitoyl-carnitine or short chain fatty acids not requiring carnitine to cross the inner membrane, was particularly sensitive to changes in the matrix volume [62]. In addition, with these substrates it was possible to identify the site of action of matrix volume more precisely by studying the redox state of the electron-transferring flavoprotein (ETF). This flavoprotein can be identified as a major contributor to the flavoprotein absorbance of liver mitochondria and its oxidation and reduction may be followed spectrophotometrically [62]. In hyper-osmotic media the flavoprotein remained highly reduced whilst when the matrix volume was increased, it was readily oxidised [62]. This implies that increasing the matrix volume stimulates the transfer of electrons from ETF to ubiquinone, a reaction which is catalysed by ETF dehydrogenase, an iron sulphur protein containing enzyme [244,245]. We do not have any information on how the activity of this enzyme responds to the matrix volume. However, since electron transfer from NADH dehydrogenase and succinate dehydrogenase also involves iron sulphur proteins, it seems likely that a similar mechanism may be responsible for the stimulation of NADH and succinate oxidation.

V-F. The effects of hormones on intra-mitochondrial volume in situ

We have used three different techniques (see Section III) to demonstrate that glucagon, Ca-mobilising

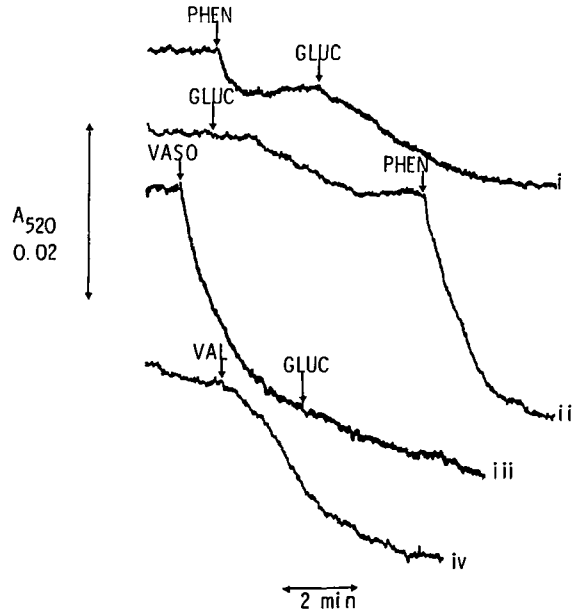

Fig. 2. The effects of hormones on the light-scattering of isolated rat hepatocytes. Hepatocytes from 24 h-starved rats were incubated with continuous stirring in a split-beam spectrophotometer at 37°C as described elsewhere [64]. Hormones were added as shown, 20 μM phenylephrine (PHEN), 0.1 μM glucagon (GLUC), 25 nM vasopressin (VASO) or 1 nM valinomycin (VAL).

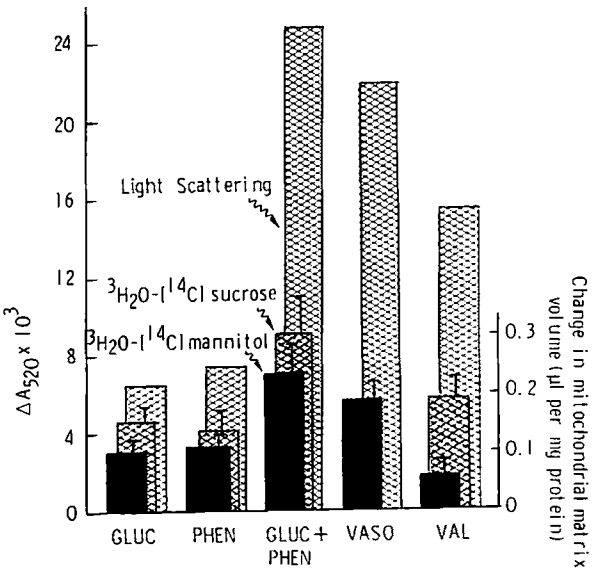

Fig. 3. Correlation between hormonally induced changes in light-scattering of hepatocytes and mitochondrial volumes measured in situ. Data are taken from Ref. 64 and Table I. Mitochondrial volumes were either measured in situ using [^{14}C]mannitol (solid shading) or with [^{14}C]sucrose after rapid cell disruption (stippled shading). Where error bars are given they represent the S.E.M. of 3–15 separate experiments.

hormones and valinomycin increase the matrix volume in situ over a range which will cause activation of mitochondrial metabolism. Rapid cell disruption into media containing [^{14}C]sucrose and ^3H_2O gave values for the intra-mitochondrial volume (in μl per mg mitochondrial protein) in control cells of 1.09 rising to 1.23 in the presence of glucagon or phenylephrine, 1.42 in the presence of both hormones together and 1.29 in the presence of 1 nM valinomycin. In parallel it was demonstrated that the hormones caused a decrease in light-scattering which was also greatest when both hormones were added together. This is illustrated in Fig. 2. Inspection of Fig. 1 shows that these small changes in matrix volume observed with hormone treatment may well be sufficient to account for many of the effects of hormones on mitochondrial metabolism in situ. Use of [^{14}C]mannitol and ^3H_2O in intact cells also demonstrated hormonally induced increases in the mitochondrial volume similar to those measured by the disruptive technique as summarised in Fig. 3. However, as discussed above and shown in Table I, more recent experiments with [^{14}C]mannitol and ^3H_2O have given larger intra-mitochondrial volumes both in situ and in vitro than our original experiments, but the effects of hormones are still apparent.

VI. The mechanism by which hormones increase the mitochondrial matrix volume

The ability of 1 nM valinomycin to increase the mitochondrial matrix volume in situ provides a plausible mechanism for the stimulation of gluconeogenesis by this antibiotic [120,225,226,246]. In addition to increasing the K^+ permeability of the plasma membrane and hence causing the membrane potential across this membrane to become more negative [246], the ionophore can have a direct effect on the inner mitochondrial membrane to increase its permeability to K^+. The slight delay before it exerts its mitochondrial effects probably represents the diffusion of the ionophore to the mitochondria [64]. However, in the case of hormones an intracellular signal must be generated which causes the increase in K^+ permeability and hence mitochondrial volume. In the case of the Ca^{2+} mobilising hormones such as phenylephrine and vasopressin an obvious candidate for this signal is Ca^{2+}.

VI-A. The role of Ca^{2+}

Evidence for an important role for Ca^{2+} came from the observation that vasopressin and phenylephrine failed to elicit a light-scattering response of any magnitude when extra-cellular Ca^{2+} was removed just before hormone addition [64,84]. However, addition of Ca^{2+} back to the cells after vasopressin (but not glucagon) addition gave a large light-scattering response not observed in the absence of hormone [64]. It was also demonstrated that A23187 addition to hepatocytes produced a decrease in light-scattering concommitant with an increase in gluconeogenesis [84]. Further evidence for a role of Ca^{2+} came from studies of the effects of Ca^{2+} on isolated mitochondria [84]. Mitochondria were incubated under energised conditions in the presence of albumin, Mg^{2+}, phosphate, ATP and EGTA, and Ca^{2+} added to give a free [Ca^{2+}] of 0.1–2.0 μM, similar to the range of cytosolic Ca^{2+} concentrations observed in hepatocytes following hormone addition [246–257]. A decrease in light-scattering occurred with a time course similar to that seen in hepatocytes following hormone addition [258]. This is illustrated in Fig. 4. The half maximal response was seen at about 0.3 μM Ca^{2+} and it was shown to be associated with an uptake of K^+ and an increase in matrix volume [84,258]. It was prevented by the omission of phosphate from the medium or by addition of a respiratory chain inhibitor. Rb^+ could replace K^+ whilst Na^+, Li^+ and choline were progressively less effective. The presence of ruthenium red to block the electrogenic influx pathway for Ca^{2+} prevented the swelling unless high concentrations of Ca^{2+} were added. Thus, it appears that Ca^{2+} must enter the mitochondria in order to exert its effect. Ba^{2+}, Mn^{2+} and Sr^{2+} were unable to substitute for Ca^{2+}. Swelling occurred without uncoupling or any non-specific permeability increases of the inner membrane and was not affected by inhibitors of phospholipase A_2. Neither were inhibitors of the known ion transport mechanisms of the plasma membrane able to inhibit swelling at

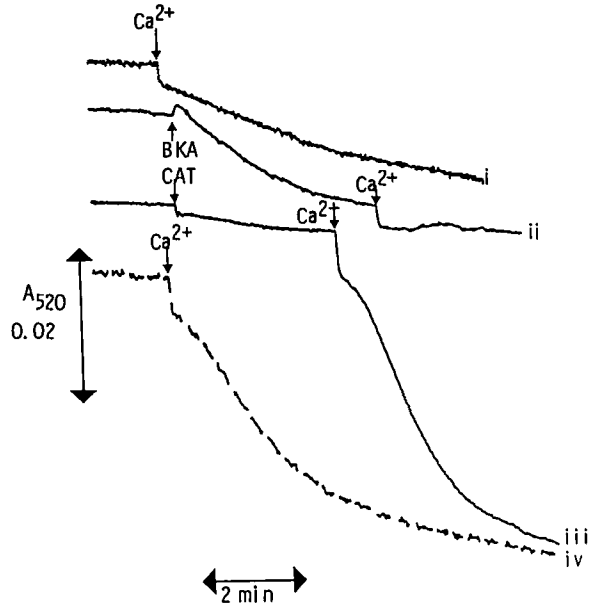

Fig. 4. The effects of ATP, bongkrekic acid and carboxyatractyloside on Ca^{2+}-induced swelling of isolated liver mitochondria. Isolated rat liver mitochondria were incubated at 37°C with continuous stirring in a split-beam spectrophotometer as described in Ref. 84. The buffered KCl medium contained 5 mM succinate, 2.5 mM phosphate, 2.5 mM Mg^{2+} and 0.5 mM EGTA. In addition, 1 mM Mg-ATP was present in traces i–iii (solid lines) and 10 μg/ml bongkrekic acid (BKA), 10 μM carboxyatractyloside (CAT) or Ca^{2+} (final $[Ca^{2+}]$, 0.4 μM) were added to the sample cuvette as indicated.

concentrations that would cause maximal inhibition of plasma membrane ion fluxes, although quinine was slightly inhibitory at 100 μM as has been described for the K^+ influx mechanism for isolated mitochondria (see Section IV). Swelling was enhanced if ATP was omitted from the buffer or if carboxyatractyloside was added, whilst the presence of bongkrekic acid inhibited swelling. In contrast addition of carboxyatractyloside in the absence of Ca^{2+} had no effect on swelling whilst bongkrekic acid stimulated swelling. This is illustrated in Fig. 4. These data suggest some involvement of the adenine nucleotide translocator in the Ca-stimulated entry of K^+ into mitochondria, and this is consistent with observations of others on the effects of adenine nucleotides and atractyloside on the K^+ permeability of mitochondria [94–97]. This is considered more fully in subsection VI-B.

Ca-mobilising hormones increase cytosolic $[Ca^{2+}]$ through 2 mechanisms (see Refs. 123,124,247–250). There is an initial and rapid release of Ca^{2+} from the endoplasmic reticulum as the result of a rise in inositol-1,4,5-trisphosphate derived from the hormone induced breakdown of phosphatidylinositol-4,5-bisphosphate. This rise in $[Ca^{2+}]$ is only transient unless extra-cellular Ca^{2+} is present since Ca^{2+} will be lost from the cell. However, there is a second phase of Ca^{2+} mobilisation which involves Ca^{2+} entry from outside and this leads to a net uptake of Ca^{2+} into the cell [250, 259–268]. More recent evidence suggests that within individual cells the increase in cytosolic $[Ca^{2+}]$ occurs as a sequence of transient pulses where Ca^{2+} rises to values of about 1 μM and then returns to basal, before rising again. The frequency of the pulses depends on the hormone concentration and there are differences between the pattern of Ca^{2+} transients seen with each hormone [257]. How these oscillations in cytosolic $[Ca^{2+}]$ are translated into changes in mitochondrial $[Ca^{2+}]$ is unclear, but both from direct measurements of mitochondrial $[Ca^{2+}]$ and from the observed activation of Ca-sensitve enzymes within the mitochondria it appears that there is an increase in mitochondrial $[Ca^{2+}]$ [268,270–275]. This sustained increase in cellular Ca^{2+} is greatly enhanced when glucagon (or cyclic AMP) are added before the Ca-mobilising hormone and under these conditions substantial net uptake of Ca^{2+} into the liver and their mitochondria can be observed [260–268].

It should be noted that the values in the literature for cytosolic $[Ca^{2+}]$ following hormone stimulation using Quin-2 fluorescence are largely restricted to the initial rise in $[Ca^{2+}]$. When incubations with hormone have been continued for longer, sustained rises in $[Ca^{2+}]$ are only observed when extracellular Ca^{2+} is present in the incubation media. Maintenance of this increase in $[Ca^{2+}]$ is only seen if the incubation medium contains HCO_3^- [255,269]. All our own experiments were performed in bicarbonate buffered media gassed with O_2/CO_2 and measurement of the NAD(P)H fluorescence (an indirect measure of intra-mitochondrial $[Ca^{2+}]$ under these conditions) indicated that there was a sustained increase in mitochondrial $[Ca^{2+}]$ following vasopressin or phenylephrine addition provided extracellular Ca^{2+} was present [157]. Like the effects of hormones on light-scattering the effect on NAD(P)H fluorescence was greater with vasopressin than with phenylephrine and greater still when glucagon was also present. Thus, our data imply that a sustained increase in mitochondrial $[Ca^{2+}]$ is responsible for the increase in the mitochondrial volume under these conditions.

However, the role of Ca^{2+} in the mechanism by which glucagon increases the mitochondrial volume is less clear. When added on its own glucagon does produce a rapid and significant increase in cytoplasmic $[Ca^{2+}]$ which can be mimmicked by addition of cyclic AMP or forskolin [261,269–271], although there may also be a second receptor for glucagon which stimulates phosphatidyl-4,5-bisphosphate breakdown [276]. However, comparison of the time courses of the light-scattering responses caused by glucagon and vasopressin or phenylephrine (Fig. 2) shows that the response to glucagon is delayed by 90 s or so whereas the response to the other hormones is almost immediate [64]. Yet the initial rise in cytosolic $[Ca^{2+}]$ in response to glucagon is rapid and is maximal within 30 s or so, not unlike the response to vasopressin. Net uptake of Ca^{2+} into the

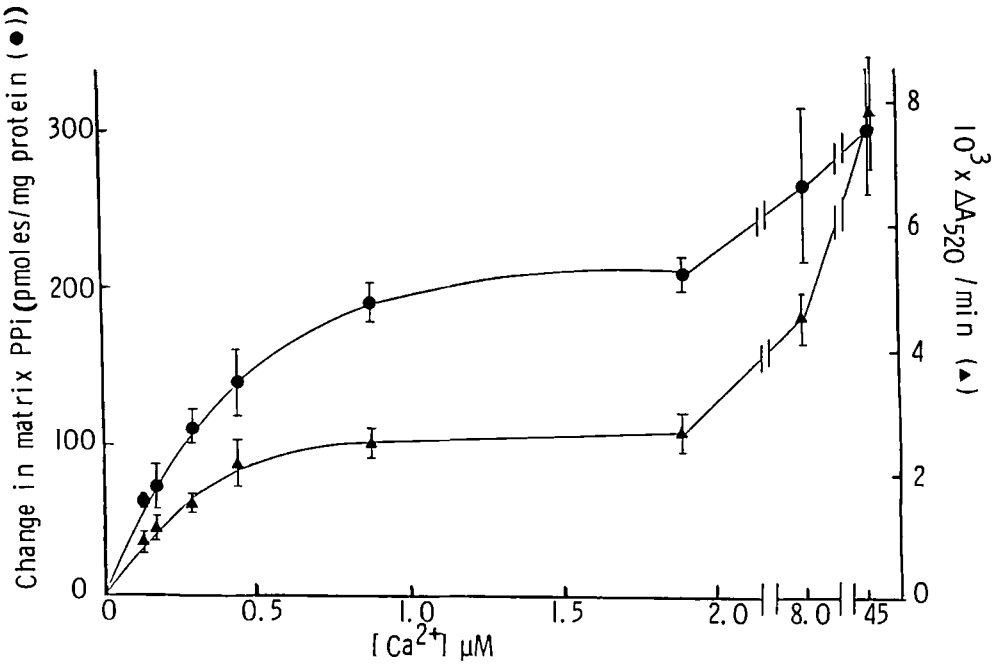

Fig. 5. The Ca^{2+} concentration dependence of the decrease in light scattering (▲) and matrix pyrophosphate content (●) of isolated liver mitochondria. Mitochondria were incubated under energised conditions in the presence of 5 mM succinate, 2.5 mM phosphate, 3.5 mM Mg^{2+}, 1 mM ATP, 0.5 mM EGTA and Ca^{2+} to give the [Ca^{2+}] shown. Details may be found elsewhere [258]. Error bars represent the S.E.M. of at least four separate experiments.

cell is not observed following glucagon treatment alone whereas it is with phenylephrine or vasopressin [84,262,263]. Thus, it would seem that the rise in cytosolic and therefore mitochondrial [Ca^{2+}] caused by glucagon added alone is not sufficient to cause an increase in mitochondrial volume. Rather glucagon may have an alternative cyclic AMP dependent mechanism by which it increases the matrix volume.

VI-B. The role of inorganic pyrophosphate (PP_i) in the regulation of the mitochondrial matrix volume

In the course of some experiments designed to study the effects of short chain fatty acids on the metabolism of hepatocytes we observed that butyrate, 4,5-pentenoate and valerate caused a decrease in light-scattering similar to that induced by hormones [111,217]. A similar response was apparent when isolated mitochondria were incubated with butyrate [258]. The activation of butyrate to butyryl-CoA occurs within the mitochondrial matrix and produces AMP and PP_i [277–279]. Indeed it had been demonstrated that in the presence of Ca^{2+} and butyrate liver mitochondria could produce large quantities of PP_i [278]. It is known that PP_i is a weak substrate for the adenine nucleotide translocator [280–282], and our earlier data had implied a role for this protein in the swelling process [84]. Thus, we decided to see whether Ca^{2+} might cause a rise in mitochondrial [PP_i] and that this might increase K^+ permeability by interacting with the adenine nucleotide translocator [258]. We were able to demonstrate a Ca^{2+}-induced rise in PP_i as shown in Fig. 5 and the response was half maximal at 0.3 μM just as was the light-scattering response. Furthermore, there was a close correlation between the time course of the Ca^{2+} or butyrate induced rise in PP_i and the decrease in

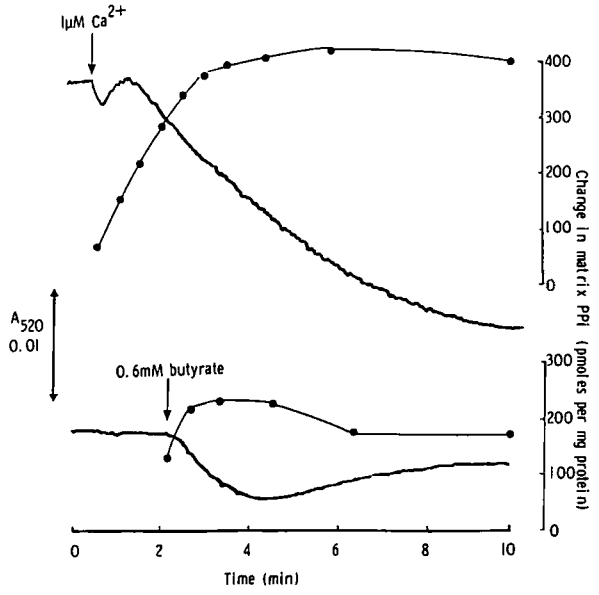

Fig. 6. Time course of the increase in matrix pyrophosphate occurring after addition of Ca^2 or butyrate to isolated liver mitochondria. Conditions are the same as those in Fig. 4 with Mg-ATP present at 0.5 mM. Data are taken from Ref. 258.

light-scattering (Fig. 6). Only when matrix PP_i was increased beyond about 500 pmol per mg protein (as in the presence of Ca^{2+} and butyrate) did the correlation break down. It is thought that this may be a consequence of the precipitation of $CaPP_i$ or $MgPP_i$ [283]. Addition of PP_i to energised mitochondria was also able to produce swelling provided that adenine nucleotides were not present [258].

In order to demonstrate an effect of PP_i on K^+ permeability directly and to explore further the involvement of the adenine nucleotide translocator, we have used the technique of swelling of de-energised mitochondria in iso-osmotic KSCN [14,75,258,284]. In this buffer swelling is limited by the entry of K^+ and can be greatly stimulated by the addition of valinomycin. Addition of ADP inhibited swelling and this effect was reversed by the addition of either carboxyatractyloside or bongkrekic acid as shown in Fig. 7a. Swelling was stimulated by addition of PP_i [258,284], 20 mM PP_i producing about the same rate of swelling as 0.5 μM valinomycin, a concentration ratio for their comparative efficacy of $4 \cdot 10^4$. In energised mitochondria exposed to 1 μM Ca^{2+}, matrix $[PP_i]$ increased by about 300 μM and swelling of the same order was induced by 0.5 nM valinomycin, a concentration ratio of $6 \cdot 10^4$. The similarity between these concentration ratios lends additional support to the PP_i induced K^+ permeability increase being responsible for the Ca^{2+} induced swelling. However, the effect of externally added PP_i was not inhibited by either carboxyatractyloside or bongkrekic acid, which argues against an involvement of the adenine nucleotide translocase when PP_i acts externally. In addition, as shown in Fig. 7b, the effects of PP_i could be mimicked by addition of *N*-(hydroxyethyl)-ethylenediamine-triacetic acid (HEDTA) a metal chelator whose Mg complex has a K_d of 43 μM at pH 7.2 similar to that of $MgPP_i$ (55 μM). It has been suggested that in energised mitochondria chelation of matrix Mg^{2+} causes their shrinkage through stimulation of the K^+/H^+ antiporter [80]. Since an increase in intra-mitochondrial $[PP_i]$ would chelate matrix Mg^{2+} and yet causes mitochondrial swelling, it is unlikely to be acting through an effect on the K^+/H^+ antiporter under energised conditions. Displacement of adenine nucleotides from the inner face of the adenine nucleotide translocase by matrix PP_i remains a possibility and would require that the carrier without adenine nucleotides bound allowed the electrogenic entry of K^+ into the mitochondria. However, the affinity of PP_i for the carrier is considerably less than that of ADP and ATP [282], which does not offer strong support for such a mechanism. An alternative explanation would be for PP_i to open another K^+ channel in the inner mitochondrial membrane such as the 53 kDa K^+-translocating protein recently isolated from mitochondrial inner membranes [99a] as described in Section IV.

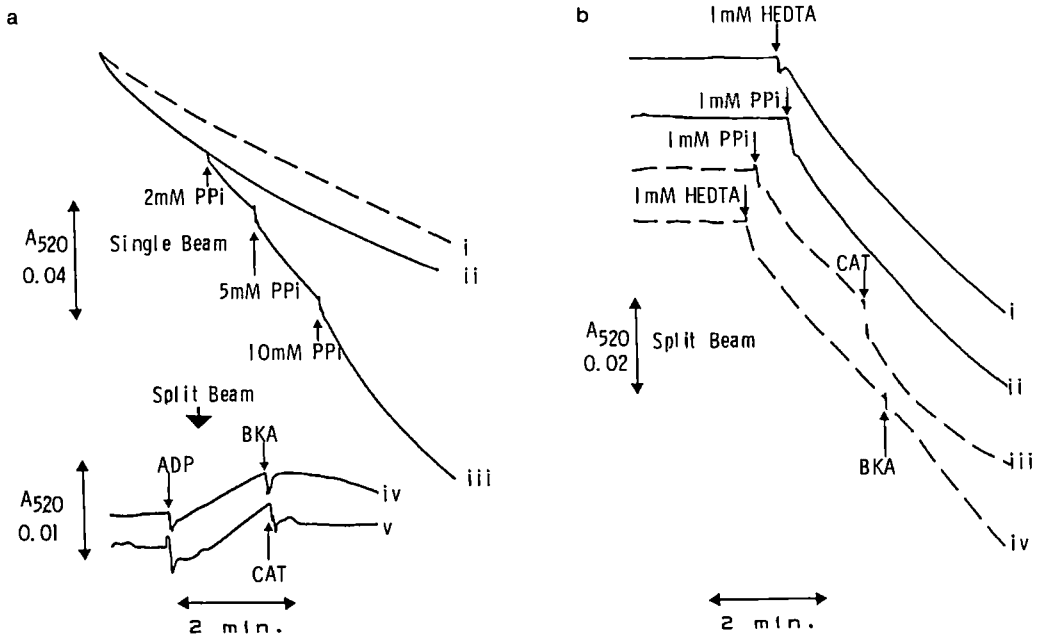

Fig. 7. The effects of substrates and inhibitors of the adenine nucleotide translocase and of Mg^{2+}-chelators on swelling of de-energised liver mitochondria in iso-osmotic KSCN. Liver mitochondria were rapidly mixed into 150 mM KSCN containing 10 mM Mops, 5 mM Tris and 1 μg/ml each of rotenone and antimycin at pH 7.2 to give a final concentration of 2 mg protein/ml, and transferred into both sample and reference cuvettes (a, traces iv, and v and b, traces i–iv) or just the sample cuvette (a, traces i–iii) of a split-beam spectrophotometer. In the latter case a suitable compensating filter was present in the reference cuvette to balance the absorbance signal. The temperature was 20 °C and A_{520} was monitored continuously. In those experiments represented by dashed traces 0.5 mM ADP was present in the buffer. Further additions were made to the sample cuvette as indicated: 10 μM carboxyatractyloside (CAT), 10 μg/ml bongkrekic acid (BKA), 0.5 mM ADP, and PP_i or *N*-(hydroxyethyl)-ethylenediamine-triacetic acid (HEDTA) at the concentrations shown. Further details may be found elsewhere [258, 284].

VI-C. The mechanism by which Ca^{2+} increases matrix PP_i

The concentration of PP_i within the mitochondrial matrix must represent a balance between its synthesis, degradation and transport out of the mitochondria. There are two potential sources of PP_i within the mitochondrial matrix. Firstly, it could be produced from the breakdown of ATP, perhaps as a consequence of the turnover of mitochondrial phospholipids by the operation of the Ca-activated phospholipase A_2 and subsequent re-esterification of the released fatty acids [105–107,207]. This involves activation of fatty acids to fatty acyl-CoA and consequently the production of both AMP and PP_i. However, we have shown that the inhibitor of mitochondrial phospholipase A_2, tetracaine, does not influence the ability of Ca^{2+} to increase the matrix volume and $[PP_i]$ [258]. Secondly there are reports of the presence of a PP_i-synthesising, proton-translocating pyrophosphatase which is membrane bound and driven by the protonmotive force [285,286]. Such an enzyme is well documented in bacteria where it plays a part in energy conservation [287] but is less well studied in mammalian mitochondria.

We have confirmed [258,284] that PP_i can enter or leave the mitochondria on the adenine nucleotide translocase in exchange for adenine nucleotides [280–282]. This may well account for the observed increase in total mitochondrial adenine nucleotides after incubation of mitochondria with micromolar $[Ca^{2+}]$ [258,288] and following treatment of rats or isolated hepatocytes with glucogenic hormones (see Table II for references). However, this would not be capable of allowing substantial net transport of PP_i out of the mitochondria. We have

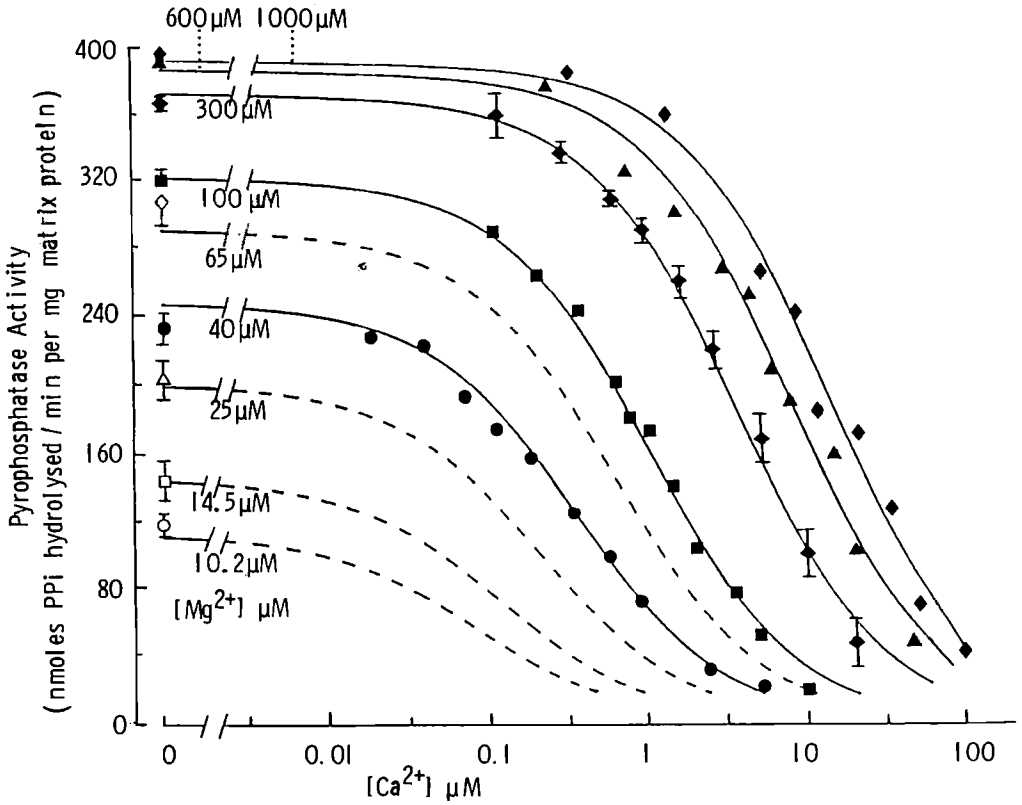

Fig. 8. The inhibition of mitochondrial matrix pyrophosphatase by Ca^{2+} at different concentrations of Mg^{2+}. Pyrophosphatase activity in a matrix fraction of liver mitochondria was assayed from time courses of PP_i hydrolysis [291] at the free concentration of Mg^{2+} and Ca^{2+} shown. Data points are for one preparation of enzyme unless error bars are given, where values are the means ± S.E.M. of values obtained with 3 separate preparations. The lines drawn are calculated by least squares regression analysis to the equation:

$$V = \frac{V_m}{\left\{1+\left(\frac{K_{m1}}{[MgPP_i]}\right)^{N_1}\right\}\left\{1+\left(\frac{[CaPP_i]}{K_{i1}}\right)^{N_1}\right\}\left\{1+\left(\frac{[PP_i]}{K_{i2}}\right)^{N_1}\right\}\left\{1+\left(\frac{K_{m2}}{[Mg^{2+}]}\right)^{N_2}\right\}}$$

where K_{m1}, K_{m2}, K_{i1} and K_{i2} are the K_m or K_i values for $MgPP_i$, Mg^{2+}, $CaPP_i$ and PP_i respectively, N_1 and N_2 are the Hill coefficients for $MgPP_i$ and Mg^{2+} respectively, and V_m is the V_{max} of the enzyme. The total $[PP_i]$ was 500 μM and concentrations of $MgPP_i$, $CaPP_i$ and free PP_i were calculated using a metal ligand binding program. K_m was set at 5 μM and the other parameter values (± S.E.) were calculated as K_{m2}, 23.1 ± 1.5 μM, K_{i1}, 0.067 ± 0.004 μM, K_{i2}, 506 ± 159 μM, N_1, 0.98 ± 0.03, N_2, 1.01 ± 0.07 and V_{max}, 394 ± 6 milli-units per mg matrix protein. At some $[Mg^{2+}]$ inhibition by Ca^{2+} was not studied and the theoretical curves are drawn as dashed lines.

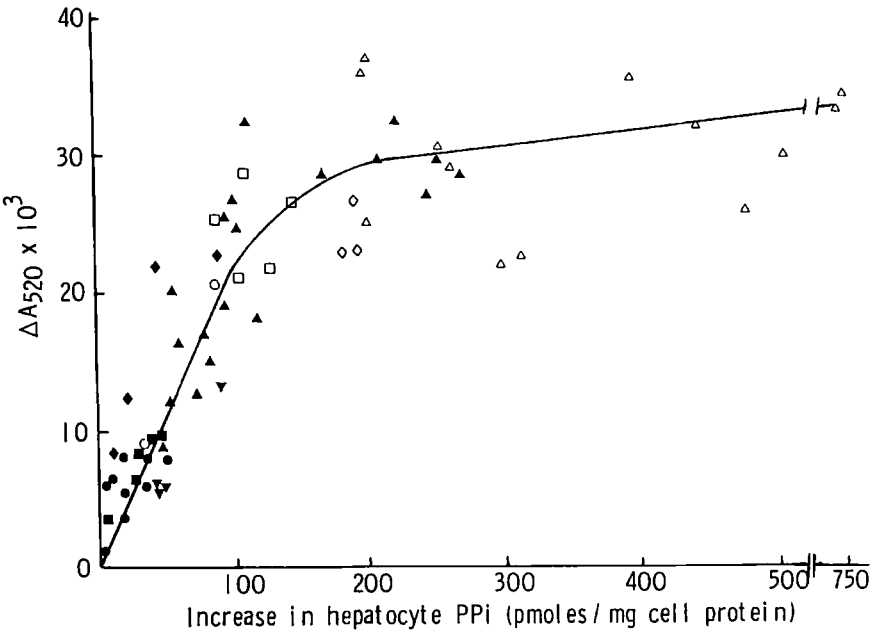

Fig. 9. Correlation between increase in hepatocyte pyrophosphate and the decrease in light-scattering caused by hormones. Hepatocytes were incubated in a spectrophotometer and the decrease in light-scattering following hormone addition monitored until completion. At this time samples of the cell suspension were taken for assay of PP_i after sedimenting cells through silicone oil into $HClO_4$. Details are given in Ref. 296. Hormones used were as follows: 0.1 μM glucagon (●), 20 μM phenylephrine (■), 25 nM vasopressin (▲), 10 μM ADP (◆), 0.6 mM butyrate (▼), 15 μM A23187 (○) and glucagon in the presence of phenylephrine (□), vasopressin (△) or ADP (◇).

demonstrated that the adenine nucleotide carrier can also catalyse an exchange of PP_i with P_i in the absence of adenine nucleotides which would allow net transport of PP_i across the membrane [284]. However, such a mechanism would not operate at a significant rate in the presence of physiological concentrations of adenine nucleotides, and so is unlikely to be important in the regulation of matrix [PP_i].

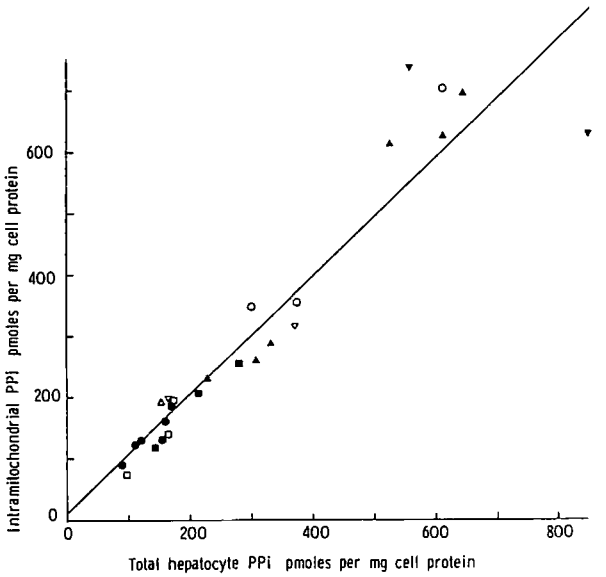

Fig. 10. The mitochondrial location of hepatocyte pyrophosphate. Hepatocytes were incubated in the absence (●) or presence of glucagon (□), phenylephrine (△), vasopressin (▽), A23187 (○), butyrate (■), glucagon + vasopressin (▼) or glucagon + phenylephrine (▲). The cells were either sedimented directly through silicone oil into $HClO_4$, or subject to rapid cell disruption using a combined shear force digitonin technique before sedimenting mitochondria through oil into $HClO_4$. Corrections were made for mitochondrial breakage using citrate synthase. Further details are given in Ref. 296.

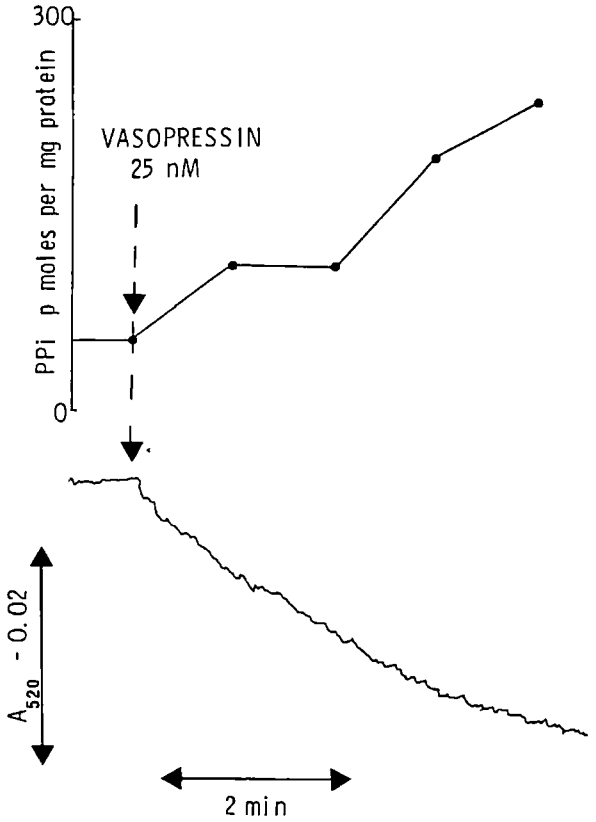

Fig. 11. The time course of the rise in hepatocyte pyrophosphate induced by vasopressin. The techniques used were the same as for Fig. 8, but incubations were terminated at the times shown.

Studies in both this and other laboratories have shown that when liver mitochondria are exposed to both butyrate and Ca^{2+} together, there is a massive increase in PP_i to values 20-fold above basal or more [258,278]. This is far greater than the sum of the effects of either agent on its own and suggests that Ca^{2+} may inhibit the breakdown of the PP_i that is produced by the activation of butyrate to butyryl-CoA. There appears to be significant pyrophosphatase activity associated with the mitochondrial matrix and this has been shown to be inhibited by Ca^{2+}, but only at concentrations far in excess of those found physiologically [289,290]. However, we have studied the effects of low concentrations of Ca^{2+} on the pyrophosphatase activity of a mitochondrial matrix fraction assayed under more physiological conditions [284,291] and results are shown in Fig. 8. The V_{max} activity of the enzyme is about 400 nmol PP_i hydrolysed per mg matrix protein at 37°C, and the apparent K_m for PP_i is less than 20 μM (see below). Ca^{2+} is a powerful inhibitor of the enzyme, the sensitivity to Ca^{2+} being decreased as the concentration of Mg^{2+}, which is essential for enzyme activity, is increased. We have successfully analysed our results using a model derived from that proposed for the yeast enzyme whose kinetics have been studied in detail [292]. The substrate for the enzyme is $MgPP_i$ for which the K_m is about 5 μM. Ca^{2+} acts primarily through formation of $CaPP_i$ which is a very strong competitive inhibitor with respect to $MgPP_i$ ($K_i < 0.1$ μM). Free PP_i acts as a very weak competitive inhibitor for the same site ($K_i \approx 500$ μM). There is an additional Mg^{2+} activating site on the enzyme ($K_{0.5} \approx 23$ μM) for which Ca^{2+} competes very poorly.

These results demonstrate that inhibition of mitochondrial matrix pyrophosphatase by Ca^{2+} probably accounts for the effects of Ca^{2+} on matrix $[PP_i]$. The effective K_i for Ca^{2+} inhibition of the enzyme at 0.3 mM $[Mg^{2+}]$, the probable matrix concentration [293], was 3.7 ± 0.4 μM (mean ± S.E.M. of three separate enzyme preparations). This is of the same order as the $K_{0.5}$ for Ca^{2+} activation of isocitrate dehydrogenase and of pyruvate dehydrogenase phosphate phosphatase, but significantly higher than that for 2-oxoglutarate dehydrogenases [294,295]. In contrast, the $K_{0.5}$ for Ca^{2+}-mediated increases in matrix volume and PP_i under physiological conditions is about 0.3 μM [258], similar to the $K_{0.5}$ for activation of 2-oxoglutarate and pyruvate dehydrogenases in intact mitochondria under such conditions [294,295]. This difference between the sensitivity of the isolated pyrophosphatase and the matrix PP_i content to $[Ca^{2+}]$ might be explained as follows. If the pyrophosphatase is not inhibited by a naturally occurring inhibitor, and the PP_i present in the matrix is free rather than present as an inert metal salt, the low K_m of the enzyme suggests that it must be working under V_{max} conditions. This would lead to a futile cycle of PP_i synthesis and breakdown which might account for a part of the State 4 rate of respiration. Possible endogenous inhibitors might be ATP, ADP and P_i, but we have shown that none of these have a significant effect on enzyme activity when present at 5 mM [291]. Thus, even the smallest inhibition of the enzyme will lead to a significant build up of PP_i until either further synthesis is inhibited or PP_i can be transported out of the matrix on the adenine nucleotide translocase.

VI-D. The subcellular location of PP_i in hepatocytes and its hormonal regulation

Freeze clamped livers of rats treated with glucagon or phenylephrine show a highly significant increase in tissue PP_i from a control value of about 10 nmol per g wet weight to about 14 and 12 nmol per g wet weight in the presence of the respective hormones [258]. If it is assumed that all this PP_i is mitochondrial, then from the known mitochondrial content of liver it can be calculated that the mitochondrial PP_i would be about 130 pmol per mg protein. This compares with a measured value of about 110 pmol per mg protein for isolated mitochondria [258] which suggests that much of the PP_i in the hepatocyte is intra-mitochondrial. Such a conclusion is not unexpected, since degradation of PP_i is regarded as an essential feature of those biosynthetic pathways in the cytosol which produce it. Treatment of isolated hepatocytes with hormones, other Ca^{2+}-mobilising agents such as A23187 and ADP, or with butyrate also caused an increase in whole cell PP_i and this correlated with the observed light scattering response (Ref. 296 and Fig. 9). Recently we have used a rapid sub-cellular fractionation technique to demonstrate directly that about 95% of cellular PP_i is intra-mitochondrial and that it is within this compartment that the hormonally induced increase in PP_i occurs as predicted (Ref. 296 and Fig. 10). The time course of the increase in PP_i and that of the light scattering induced by hormones are also similar (Fig. 11).

As predicted from the light scattering data discussed earlier the omission of Ca^{2+} from the medium inhibited the increase in PP_i just as it did the light scattering response [296]. Furthermore, addition of both glucagon and a Ca-mobilising hormone together caused a much larger increase in PP_i than does either hormone alone. Under these conditions it appeared that a point was reached at which the increase in PP_i was not accompanied by an increase in the light scattering response (Ref. 296 and Fig. 9). When both butyrate and vasopressin were added together the PP_i could reach levels as high as 800 pmol per mg cell protein which is equivalent to 2400 pmol per mg mitochondrial protein. This was

accompanied by an inhibition of the light scattering response much as was seen in isolated mitochondria incubated with both Ca^{2+} and butyrate [258,296].

Taken together the data we have obtained strongly support a role for a Ca^{2+} induced increase in mitochondrial PP_i being responsible for the increase in mitochondrial volume caused by addition of vasopressin and phenylephrine (alone or in combination with glucagon). With glucagon added alone the increase in matrix volume also appears to correlate with an increase in matrix PP_i but, as discussed above, it is unlikely that this is induced by Ca^{2+}. An alternative mechanism by which glucagon could increase the matrix PP_i would be through a change in the mitochondrial membrane (see subsection V-C) also causing an activation of the proton-translocating pyrophosphatase, and this possibility is under investigation.

VII. Summary of the mechanisms involved in the hormonal regulation of liver mitochondrial metabolism

In Fig. 12 I have attempted to draw together the information of Sections V and VI into a scheme

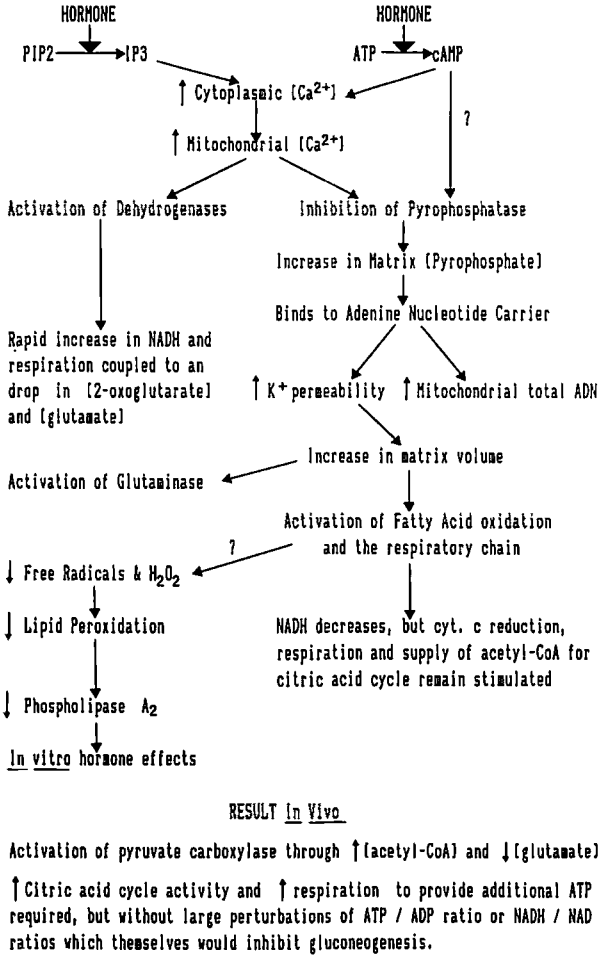

Fig. 12. A proposed scheme for the activation of mitochondrial metabolism by hormones in the liver. Increases and decreases in concentrations are indicated by ↑ and ↓, respectively.

summarising how we believe hormones acting at the cell surface can influence mitochondrial metabolism through changes in the matrix volume. Hormones that act through breakdown of phosphatidylinositol-4,5-bisphosphate such as vasopressin, angiotensin and phenylephrine cause a rise in cytoplasmic $[Ca^{2+}]$ which is relayed across the mitochondrial membrane to elevate mitochondrial $[Ca^{2+}]$. This rapidly activates the mitochondrial Ca-sensitive dehydrogenases [294,295] causing a rise in mitochondrial NADH/NAD^+ ratio, which can be detected by an increase in NAD(P)H fluorescence [157,169–171] and an increase in β-hydroxybutyrate/acetoacetate ratio [121,153,172]. There is also an equally rapid increase in the rate of respiration and the reduction state of cytochrome *c* [149–157]. Data illustrating the effects of vasopressin addition to hepatocytes on their NAD(P)H fluorescence, cytochrome *c* reduction state and light-scattering are shown in Fig. 13 where comparison is made with the effects of Ca^{2+} addition on isolated liver mitochondria. It should be noted that the responses are very similar including the transience of the increase in NAD(P)H fluorescence which decreases again with the same time course as the increase in mitochondrial volume detected by the decrease in light-scattering [157]. Such a transient change is also seen with phenylephrine [121,157,169–171] and yet in all cases cytochrome *c* remains more reduced [157] and the rate of respiration stimulated [149–156]. This may be explained by a Ca-mediated increase in matrix $[PP_i]$ and mitochondrial volume causing stimulation of the respiratory chain and increasing electron flow from NADH to cytochrome *c*. Further evidence for such a mechanism is provided by the effects of valinomycin on intact cells or isolated mitochondria (Fig. 13). Here there is no rapid Ca-induced increase in NADH and cytochrome *c* reduction, but rather a slow oxidation of NADH and reduction of cytochrome *c* whose time courses parallel those for the decrease in light-scattering. This is exactly what would be expected for a stimulation of electron flow between NADH and cytochrome *c* mediated by an increase in mitochondrial matrix volume [157].

Glucagon also causes a rise in cytosolic $[Ca^{2+}]$ as described in subsection VI-D and this is accompanied by an activation of the Ca-sensitive dehydrogenases [294,295] and an increase in NAD(P)H fluorescence [121,157,169–171]. This rise in NAD(P)H fluorescence is more transient than with vasopressin or phenylephrine and may lead to the mitochondrial NADH becoming more oxidised than in the absence of hormones [121,157,298]. However, once again both the rate of respiration and the reduction state of cytochrome *c* remain elevated, consistent with a stimulation of the respiratory chain mediated by an increase in matrix volume. As with vasopressin and phenylephrine this is thought to be brought about by an increase in

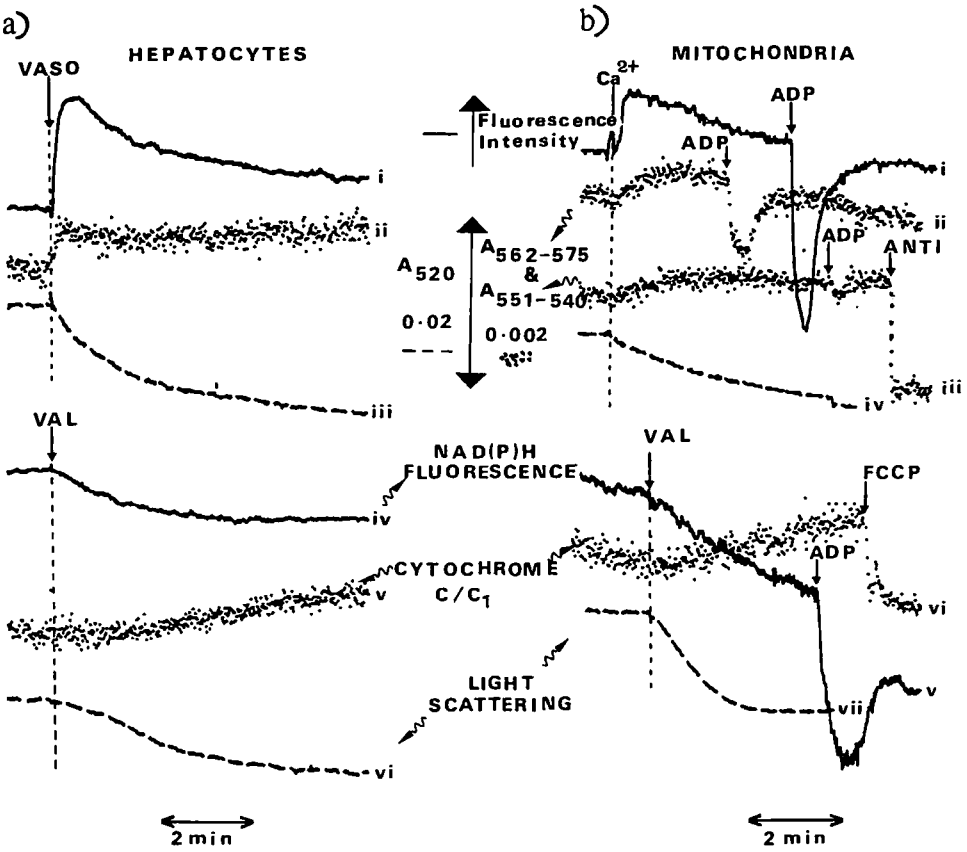

Fig. 13. Comparison of the effects of vasopressin or valinomycin on NAD(P) fluorescence, cytochrome *c* reduction and light scattering by hepatocytes with those of Ca^{2+} and valinomycin on isolated mitochondria. Data are taken from Ref. 157. Additions made to hepatocytes (a) were 25 nM vasopressin (VASO) or 1 nM valinomycin (VAL) and to mitochondria (b) 0.5 μM free [Ca^{2+}], 1 nM valinomycin (VAL), 0.4 mM ADP, 1 μg/ml antimycin (ANTI) or 1 μM carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP). In trace (ii) of (b) changes in the redox state of cytochrome b_{562} caused by Ca^{2+} are included for comparison with the data for cytochrome *c*.

matrix [PP_i] but, as discussed in subsection VI-D, it may not be dependent on a rise in matrix [Ca^{2+}].

With both classes of hormones the stimulation of respiration seen in the perfused liver is biphasic [121,153,155,172,299] and this is illustrated in Fig. 14. There is an initial rapid increase in the rate of respiration which follows the time course of the increase in NAD(P)H fluorescence, then a slight decrease and finally another increase in rate. This latter phase follows the same time course as the increase in mitochondrial matrix volume, and thus may represent a volume-mediated stimulation of the respiratory chain. Such a two phase mechanism for the stimulation of respiration offers distinct advantages for the cell. It allows the respiratory chain to synthesise ATP at a faster rate to fuel the increased rates of gluconeogenesis and citrulline synthesis, but it does so without decreasing ATP/ADP or NAD^+/NADH ratios, both of which can be inhibitory for gluconeogenesis (see Refs. 157,300). Indeed mitochondrial ATP/ADP ratios often increase [130,131,142,163–167], which may be important for the activation of pyruvate carboxylation [233–237]. Furthermore, the increase in matrix volume is able to stimulate fatty acid oxidation [61,232] and hence increase production of acetyl-CoA to supply the stimulated citric acid cycle and to activate pyruvate carboxylase. There is therefore a concerted stimulation of both the production and utilisation of NADH and ATP to

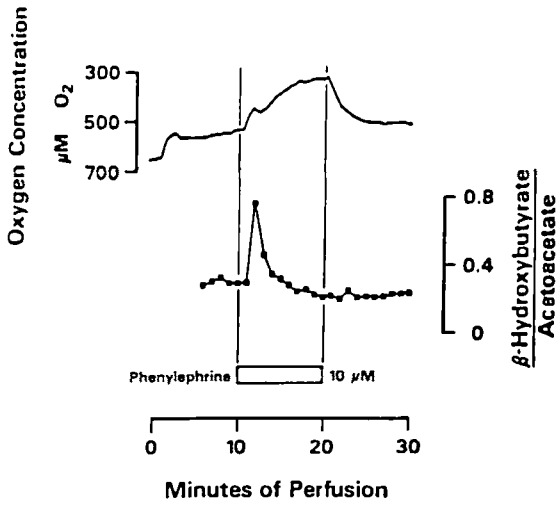

Fig. 14. The effects of phenylephrine on oxygen consumption and β-hydroxy-butyrate/acetoacetate ratios in the perfused rat liver. Data are reproduced from Ref. 153 with permission.

supply the increased requirements of the cell with minimal disturbance of their concentration. The concentrations of glutamate and 2-oxoglutarate are decreased however as a result of the stimulation of 2-oxoglutarate dehydrogenase [130,131,172,301–304], but this is desirable for glutamate acts as an inhibitor of pyruvate carboxylase [304]. Thus, a decrease in its concentration may be important in the hormonal stimulation of this enzyme.

Further evidence for an increase in matrix volume playing a vital role in the stimulation of fatty acid oxidation, flux through the citric acid cycle, respiration and the rate of gluconeogenesis comes from studies using mild respiratory chain inhibitors [62]. We have demonstrated that the rate of respiration, fatty acid oxidation and gluconeogenesis are all very sensitive to inhibition by such agents, yet without changes in tissue ATP concentrations. Such experiments also allowed us to demonstrate that the flux control coefficient of the respiratory chain towards gluconeogenesis is very high (0.61), but is decreased after glucagon or phenylephrine treatment to 0.39 and 0.25, respectively, as a result of the stimulation of the respiratory chain. These conclusions do not necessarily conflict with those of Groen et al. [305] who conclude that the majority of flux control is exerted at the level of pyruvate carboxylase and pyruvate kinase, since the effects on gluconeogenesis of modulating the respiratory chain may be mediated through an effect on either or both of these enzymes. At present the exact mechanisms involved are unclear [62,306,307].

The increase in respiratory chain activity is also important to provide the additional ATP required for the stimulated rate of urea synthesis observed in the presence of hormones. Although it is thought by many workers that primary stimulation of this pathway is mediated through increases in *N*-acetylglutamate concentrations [193–196], these changes occur much more slowly than the rapid increases in urea synthesis observed following phenylephrine treatment [303]. In addition to the effects of the intra-mitochondrial volume on respiration and consequently on metabolism that is dependent on respiration, the increase in matrix volume is responsible for the observed activation of mitochondrial glutaminase [143–146]. This enzyme is important in acid base balance to ensure that the amide group of any glutamine reaching the liver is converted into urea rather than utilized in the kidney for the excretion of NH_4^+ [146–148].

VIII. Regulation of the mitochondrial volume in other tissues and in pathological states

VIII-A. The liver in disease

Electron microscopic analysis of the liver of humans and animals in various diseased or drug induced states has revealed the existence of giant mitochondria [308]. Whilst such mitochondria do not necessarily have increased matrix volumes per mg mitochondrial protein, and indeed no measurements of this parameter have been made, it would seem quite probable that this is so. Two situations are of particular interest. Firstly, alcohol induced damage to liver [309]. It is known that alcohol can cause a rise in cytosolic $[Ca^{2+}]$ which might be expected to increase mitochondrial $[PP_i]$ and matrix volumes in its own right. However, in addition to this a major product of alcohol metabolism is acetate [311] and this can be metabolised by liver mitochondria with the generation of PP_i [312]. Since we know that butyrate and Ca^{2+} can produce massive increases in PP_i in isolated mitochondria it is possible that alcohol abuse might do the same and that this might account for the large mitochondria observed. Secondly such giant mitochondria are sometimes observed in diabetes or prolonged starvation [308]. Under these conditions ketosis occurs and acetate is produced by hydrolysis of acetyl-CoA [313]. This might also be expected to yield high levels of PP_i and it is therefore of interest that Cohen has observed that about 25% of the livers of diabetic rats show a large PP_i NMR signal [314]. In preliminary experiments using freeze clamped livers of two diabetic and three control rats we found that the diabetic rats showed increases of 60% and 1300% in their total tissue PP_i when compared to the control rats. The very high value found in one corresponds to the levels of PP_i detected by Cohen in some of her diabetic rats.

VIII-B. Brown fat

Upon cold adaption or stimulation with adrenaline the rate of oxygen consumption by brown fat increases dramatically [315]. This is usually thought to be due to an increase in the concentration of the uncoupling protein (thermogenin) or its activation by fatty-acid displacement of guanine or adenine nucleotides [316]. However, it is also well documented from electron microscopic studies of brown fat that activation of respiration is accompanied by increases in mitochondrial volume [317–319]. More recently evidence has been presented that a 1 h exposure to noradrenaline or to the cold increased the mitochondrial matrix volume measured using ^3H_2O from almost undetectable levels to about 1 μl/mg protein. This was accompanied by an unmasking of existing thermogenin which could be mimicked in control mitochondria by swelling [319]. It is also known that the oxidation of fatty acids and other substrates by brown fat mitochondria is negligble under conditions of matrix condensation [230,231] Thus, the data suggest that increases in the mitochondrial volume of brown fat mitochondria may be an essential process in the stimulation of thermogenesis. Measurements of PP_i in brown fat have yet to be made.

VIII-C. Secretory tissues

A wide variety of exocrine glands, when stimulated to secrete, form watery vacuoles [320]. Morphological studies on the rat parotid, lacrimal and sweat glands have shown that formation of these vacuoles is also accompanied by an increase in the size of the mitochondria which is dependent on the presence of extracellular $[Ca^{2+}]$ [320,321]. Whether this is important for secretion or whether it involves a rise in PP_i is not known, but secretion is usually accompanied by an increase in respiration.

VIII-D. The heart

The volume of isolated rat heart mitochondria can be increased by exposure to similar Ca^{2+} concentrations as cause liver mitochondria to swell [229]. Such increases in volume are also able to stimulate the respiratory chain of heart mitochondria and especially the rate of β-oxidation of fatty acids in a similar manner to that observed in liver [229]. However, there are no available data for the mitochondrial volume in the heart nor any studies on whether it might be regulated under physiological conditions. It is known that reperfusion after ischaemia does cause considerable ultrastructural changes to the mitochondria including a Ca^{2+}_cdependent swelling [322], but this is a highly pathological and potentially lethal situation.

Nevertheless, there are reasons to believe that increases in mitochondrial volume may be an important response of the heart to stimulatory hormones such as adrenaline or to increased workload. This would allow the heart to increase its rate of respiration without greatly perturbing its ATP/ADP ratio or NADH/NAD^+ ratio just as was argued for the liver. Under conditions of incubation thought to mimic the situation in vivo the mitochondrial volume of isolated heart mitochondria is in the range over which fatty acid oxidation and respiration are extremely sensitive to Ca^{2+}-mediated changes in volume. It is also known that the increased respiration under these conditions is accompanied by a Ca^{2+} activation of mitochondrial Ca^{2+} sensitive dehydrogenases. This can be abolished by the addition of Ruthenium Red to prevent entry of Ca^{2+} into the mitochondria [286,323]. Recent experiments using hearts perfused with glucose have demonstrated that the surface NAD(P)H fluorescence increases under stimulated conditions confirming that activation of mitochondrial dehydrogenases is occurring [324]. Since it has not been possible to demonstrate significant changes in the ATP/ADP · P_i ratio under these conditions [324–326], it has been suggested that the increase in NADH may be responsible for the increased rate of respiration [327]. Nevertheless, it would seem physiologically desirable to complement the activation of the mitochondrial Ca-sensitive dehydrogenases which produce NADH with a volume mediated stimulation of its oxidation. Such a mechanism would avoid stimulation of respiration being accompanied by large increases in NADH which would have the undesirable effect of increasing lactate output by the heart. When fatty acids rather than glucose are the respiratory substrate the increase in matrix volume may play an additional role. Stimulation of flux through the citric acid cycle cannot be achieved by Ca^{2+} activation of 2-oxoglutarate and isocitrate dehydrogenases alone, since an increased supply of acetyl-CoA is also required. When glucose is the respiratory substrate this is achieved by Ca^{2+} activation of pyruvate dehydrogenase [286,323]. However, when fatty acids are the main respiratory fuel an alternative mechanism must be available. It would seem appropriate that this should also be a Ca^{2+} dependent mechanism, and a Ca^{2+}-mediated increase in the matrix volume leading to stimulation of β-oxidation of fatty acids would provide such a mechanism.

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