Liver mitochondrial pyrophosphate concentration is increased by Ca^{2+} and regulates the intramitochondrial volume and adenine nucleotide content

Anne M. DAVIDSON and Andrew P. HALESTRAP

Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

1. The matrix pyrophosphate (PP_i) content of isolated energized rat liver mitochondria incubated in the presence of ATP, Mg^{2+} , P_i and respiratory substrate was about 100 pmol/mg of protein. 2. After incubation with sub-micromolar $[\text{Ca}^{2+}]$, this was increased by as much as 300 %. There was a correlation between the effects of Ca^{2+} on PP_i and on the increase in matrix volume reported previously [Halestrap, Quinlan, Whipps & Armston (1986) *Biochem. J.* 236, 779–787]. Half-maximal effects were seen at 0.3 μM - Ca^{2+} . 3. Coincident with these effects, the total adenine nucleotide content increased in a carboxyatractyloside-sensitive manner. 4. Incubation with 0.2–0.5 mM-butyrate induced similar but smaller effects on mitochondrial swelling and matrix PP_i and total adenine nucleotide content. Addition of butyrate after Ca^{2+} , or vice versa, caused Ca^{2+} -induced mitochondrial swelling to stop or reverse, while matrix PP_i increased 30-fold. 5. Addition of atractyloside or the omission of ATP from incubations greatly enhanced swelling induced by Ca^{2+} without increasing matrix PP_i . 6. Swelling of mitochondria incubated under de-energized conditions in iso-osmotic KSCN was progressively enhanced by the addition of increasing concentrations of PP_i (1–20 mM) or valinomycin. 7. In iso-osmotic potassium pyrophosphate swelling was slow initially, but accelerated with time. This acceleration was inhibited by ADP, whereas carboxyatractyloside induced rapid swelling. Swelling in other iso-osmotic PP_i salts showed that the rate of entry decreased in the order $\text{NH}_4^+ > \text{K}^+ > \text{Na}^+ > \text{Li}^+$, whereas choline, tetramethylammonium and Tris did not enter. It is suggested that the adenine nucleotide translocase transports small univalent cations when PP_i is bound and that PP_i can also be transported when the transporter is in the conformation induced by carboxyatractyloside. 8. It is concluded that Ca^{2+} and butyrate cause swelling of energized mitochondria through this effect of PP_i on K^+ permeability of the mitochondrial inner membrane. 9. Freeze-clamped livers from rats treated with glucagon or phenylephrine show 30–50 % increases in tissue PP_i . It is proposed that Ca^{2+} -mediated increases in mitochondrial PP_i are responsible for the increase in matrix volume and total adenine nucleotide content observed after hormone treatment.

INTRODUCTION

Rat liver mitochondria regulate their matrix volume through the operation of an electrogenic K^+ uniporter and a K^+/H^+ antiporter (Brierley, 1976; Garlid, 1979, 1980; Dordick *et al.*, 1980; Nakashima *et al.*, 1982; Brierley *et al.*, 1984). Previous studies in our laboratory have shown that sub-micromolar $[\text{Ca}^{2+}]$ can increase the electrogenic entry of K^+ and so cause mitochondrial swelling (Halestrap *et al.*, 1986). We believe this to be the mechanism by which hormones increase the matrix volume *in vivo* (Quinlan *et al.*, 1983). The effects of Ca^{2+} on mitochondrial swelling were found to be inhibited by the presence of ATP and activated by the presence of atractyloside (Halestrap *et al.*, 1986). This suggested that there might be some involvement of the adenine nucleotide transporter in the swelling process. Otto & Cook (1982) have reported that incubating liver mitochondria with high $[\text{Ca}^{2+}]$ in the presence of butyrate led to large accumulation of PP_i within the matrix. In studies on the production of inositol phosphate metabolites by a crude mitochondrial fraction, using high-voltage electrophoresis at pH 1.8, we sometimes detected an increase in PP_i in response to submicromolar $[\text{Ca}^{2+}]$ (Whipps *et al.*, 1987).

Several observations suggested that such an increase in $[\text{PP}_i]$ might be important in the ability of Ca^{2+} to increase

the matrix volume. Firstly, PP_i is known to be a substrate with a low affinity for the adenine nucleotide translocator (Krämer, 1980; Asimakis & Aprille, 1980; D'Souza & Wilson, 1982) and, as outlined above, this translocator has been implicated in the increase in matrix volume induced by Ca^{2+} . Secondly, it has been observed in several laboratories that the total adenine nucleotide content of liver mitochondria increases after hormone treatment (Bryla *et al.*, 1977; Siess *et al.*, 1977; Titheradge & Haynes, 1980; Aprille *et al.*, 1982; Soboll & Scholz, 1986). Since PP_i can exchange with adenine nucleotides on the ATP/ADP translocator, this observation would be consistent with a rise in mitochondrial $[\text{PP}_i]$ in response to hormones under the conditions known to cause an increase in mitochondrial matrix volume (Quinlan *et al.*, 1983). Thirdly, there are reports which suggest that the conformation of the adenine nucleotide transporter can influence the K^+ permeability of the mitochondrial inner membrane (Panov *et al.*, 1980; Jung & Brierley, 1981, 1984). Fourthly, it has been reported that butyrate, which is activated to butyryl-CoA in the mitochondria and generates intramitochondrial PP_i , affects the metabolism of hepatocytes in a similar manner to glucogenic hormones (Veech *et al.*, 1980).

In the present paper we investigate more fully the effects of Ca^{2+} and butyrate on mitochondrial $[\text{PP}_i]$, and provide evidence that it is an effect of PP_i on the adenine

nucleotide transporter that is responsible for the Ca^{2+} -mediated increase in K^+ permeability, matrix volume and total mitochondrial adenine nucleotides.

EXPERIMENTAL

Materials

Rats. Female Wistar rats (approx. 250 g body wt., fed *ad libitum* with Breeding Diet, from Oxoid, Basingstoke, Hants., U.K.) were used for the preparation of liver mitochondria, which was performed as described previously (Halestrap, 1975, 1978).

Chemicals. The sources of all chemicals, biochemicals and radiochemicals, except those listed below, were the same as given by Quinlan *et al.* (1983) and Halestrap *et al.* (1986). Pyrophosphate-dependent fructose-6-phosphate kinase (EC 2.7.1.90), D-fructose 6-phosphate, carboxyatractyloside and atractyloside were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Aldolase and a mixture of glycerol-3-phosphate dehydrogenase and triose-phosphate isomerase were obtained from Boehringer, Mannheim, Germany. Sodium n-butyrate was obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K. Solutions of PP_i were prepared freshly each day by neutralizing pyrophosphoric acid (Fluka, Buchs, Switzerland) with the relevant base.

Methods

Measurement of the energized swelling of mitochondria. This was measured by using light-scattering at 520 nm as described previously (Quinlan *et al.*, 1983; Halestrap *et al.*, 1986). The incubation buffer contained 125 mM-KCl, 10 mM-Mops, 7 mM-Tris base, 0.5 mM-EGTA, 2.5 mM-potassium phosphate, 2.5 mM- MgCl_2 , 5 mM-Tris succinate and defatted, dialysed, bovine serum albumin (5 mg/ml) and was adjusted to pH 7.2 at 37 °C. In most incubations either 0.3 mM- or 1 mM- MgATP was also included, as indicated in the appropriate Figures and Tables. Mitochondria were added to a final concentration of about 4 mg/ml and thoroughly mixed before 3.5 ml portions were transferred to sample and reference cuvettes. The mitochondrial suspension was stirred vigorously under an atmosphere of 100% O_2 and maintained at 37 °C while A_{520} was monitored. After preincubation for 2 min, additions were made to the sample cuvettes as indicated in the Figures. Light-scattering was monitored for another 4–8 min before samples were taken for metabolite analysis.

Determination of metabolites. After incubation, mitochondria were first sedimented through silicone oil into HClO_4 as outlined by Halestrap & McGivan (1979). Duplicate 1 ml samples of mitochondrial suspension from both reference and sample cuvettes were carefully layered on top of 150 μl of a mixture of 65% (v/v) silicone oil (M.S. 550) and 35% (v/v) dinonyl phthalate, covering 100 μl of 10% (w/v) HClO_4 containing 20% (w/v) glycerol. Mitochondria were immediately sedimented by centrifugation for 60 s at 8000 g in an M.S.E. Micro Centaur bench centrifuge. The supernatant was removed and discarded, and the pellet was extracted in HClO_4 by vigorous vortex-mixing, followed by centrifugation (10000 g-min) to separate the aqueous and non-aqueous layers. Portions (80 μl) of HClO_4 extract were

removed with a micro syringe from each of the duplicate tubes and combined. Distilled water was added, and the samples were neutralized to pH 6.5–7.0 with 5 M-KOH containing 0.5 M-Mops and 20 mM-EDTA. A final volume of 1 ml containing approx. 2 mM-EDTA was attained. The EDTA was included to chelate Mg^{2+} and so to inhibit any remaining pyrophosphatase and adenylate kinase activity. The precipitated perchlorate was removed by centrifugation (10000 g min) after standing on ice for 30 min, and the neutralized extracts were stored at –70 °C before assay. Added PP_i showed no degradation when taken through this procedure. PP_i was determined by the method of Cook *et al.* (1978), but with 5 mM- MgCl_2 in the assay to compensate for EDTA in the extract. A sample (0.6–0.8 ml) was added to 3.6 ml of assay buffer divided in two cuvettes and the A_{340} was monitored in a split-beam spectrophotometer, with resolution at 340 nm of 10^{-4} unit, after addition of pyrophosphate-dependent phosphofructokinase to the sample cuvette. In some experiments the concentration of PP_i in the supernatant after sedimentation of the mitochondria was measured and was found to be $< 1 \mu\text{M}$. Thus correction of the PP_i content of the pellet for extramitochondrial PP_i contamination was unnecessary.

Adenine nucleotides were also determined in the neutralized HClO_4 extracts in some experiments. ATP was determined as described by Lamprecht & Trautschold (1974), and ADP and AMP were measured by the method of Jaworek *et al.* (1974). Contamination of the mitochondrial pellet with extramitochondrial adenine nucleotides was corrected for by inclusion of [^{14}C]sucrose (0.1 $\mu\text{Ci/ml}$) in the incubation medium. Assay of ^{14}C and adenine nucleotides in both pellet and supernatant fractions then allowed the necessary corrections to be made. It was found that about 45% of the ATP, 17% of the ADP and 8% of the AMP content of the pellet was extramitochondrial.

Determination of $[\text{Ca}^{2+}]$. The exact concentration of EGTA in the incubation medium containing mitochondria was determined by titration with standardized Ca^{2+} in the presence of 70 μM -Arsenazo III and Ruthenium Red (5 $\mu\text{g/ml}$), by using a double-beam spectrophotometer (670 nm–685 nm) as described by Halestrap *et al.* (1986). The concentration of free Ca^{2+} corresponding to a measured addition of CaCl_2 was then computed from the apparent dissociation constants of the ligands and metals in the buffer as described by Denton *et al.* (1978). Unless otherwise stated, all $[\text{Ca}^{2+}]$ values given are free concentrations of Ca^{2+} .

Measurement of mitochondrial volumes. In certain experiments, mitochondrial volumes were determined after completion of light-scattering measurements by addition of $^3\text{H}_2\text{O}$ (1 $\mu\text{Ci/ml}$) and [^{14}C]sucrose (0.1 $\mu\text{Ci/ml}$) as described by Halestrap *et al.* (1986). Further details are given in the legend to Fig. 3.

Swelling of de-energized mitochondria in iso-osmotic PP_i and thiocyanate solutions. Iso-osmotic solutions of KSCN (150 mM) and PP_i (80 mM, and neutralized with the appropriate base) were supplemented with 1 mM-EGTA, 10 mM-Mops, 7 mM-Tris and antimycin and rotenone (each 1 $\mu\text{g/ml}$). The final pH was 7.2. A 3.5 ml portion of this buffer was placed in the sample cuvette of a split-beam spectrophotometer, and a suitable back-off

Fig. 1. Effect of [Ca²⁺] on mitochondrial swelling and PP_i content

Mitochondria (4 mg of protein/ml) were incubated in both cuvettes of a split-beam spectrophotometer in standard KCl medium (see the Experimental section) containing 0.5 mM-EGTA and 1 mM-MgATP. Swelling was initiated by addition of 4–8 μ l of 250 mM-CaCl₂ to the experimental cuvette. Light-scattering was monitored for 4 min and the maximum sustained rate of swelling ($\blacktriangle$) was calculated as A_{520}/min . About 30 s elapsed between cessation of recording and centrifugation of the mitochondria for measurement of matrix PP_i ($\bullet$). [Ca²⁺] in the medium corresponding to measured additions of CaCl₂ was calculated as described in the Experimental section. Each point depicts the mean $\pm$ S.E.M. (error bar) of four to seven separate mitochondrial preparations.

grid of 1 $\text{\AA}$ unit was used for the reference cuvette. Mitochondria were added to the sample cuvette at a final protein concentration of 3 mg/ml and the change in A_{520} was monitored. The temperature was 30 °C. Other additions were made to the medium as indicated in the legend to Fig. 7.

RESULTS

Role of Ca²⁺ and PP_i in mitochondrial swelling

Previous work has demonstrated that exposure of rat liver mitochondria to Ca²⁺ results in a decrease in light-scattering, which corresponds to an increase in measured matrix volume (Halestrap *et al.*, 1986). Furthermore, these effects can be demonstrated at extramitochondrial [Ca²⁺] within the physiological cytoplasmic range. In Fig. 1 we demonstrate that the rate of mitochondrial swelling increases most sharply with increasing Ca²⁺ between 0.1 and 0.5 μ M, with little further increase between 0.5 and 2.0 μ M-Ca²⁺. A parallel increase in mitochondrial PP_i content was observed, which also responded most acutely to increasing [Ca²⁺] between 0.1 and 1.0 μ M-Ca²⁺. This sensitivity towards Ca²⁺ is very similar to that observed by Denton & McCormack (1985) and Hansford (1985) for the activation of mitochondrial dehydrogenases by Ca²⁺. Further increases in the rate of swelling and the mitochondrial PP_i content could be induced, but only when [Ca²⁺] was increased by an order of magnitude or more (Fig. 1). The experiments reported in this paper were performed in State 4, and with succinate (+ rotenone) as substrate to avoid possible interference from

parallel changes in the activity of the mitochondrial dehydrogenases. However, it was confirmed that similar effects were observed with glutamate+malate or 2-oxoglutarate as substrates and under State-3 conditions achieved by the addition of glucose and hexokinase (results not shown). Fig. 2 shows the time course of the increase in mitochondrial PP_i in relation to changes in light-scattering at an initial [Ca²⁺] of 8 μ M in the incubation medium. Most of the increase in PP_i took place in the first 4 min of incubation and thereafter reached a plateau, whereas light-scattering showed an initial rapid response due to a conformational change of the mitochondria (see Halestrap, 1987), followed by progressive swelling over an 8 min period. Basal mitochondrial PP_i content did not change in the same period. Similarly we investigated whether other agents that might be expected to increase intramitochondrial PP_i also induced swelling. Time courses for the increase in PP_i and decrease in light-scattering induced by 0.6 mM-butyrate are shown in Fig. 2.

As summarized in Table 1, the swelling induced by 0.6 mM-butyrate was accompanied by a 2-fold increase in mitochondrial PP_i content in either the presence or the absence of ATP. This may be explained by activation of butyrate to butyryl-CoA within the mitochondrial matrix (Aas & Bremer, 1968; Otto & Cook, 1982), which

Fig. 2. Time course of the changes in mitochondrial PP_i and light-scattering after addition of Ca²⁺ or butyrate

For the measurement of PP_i, mitochondria (4 mg/ml) were incubated at 37 °C (in an orbital water bath) in a sealed 70 ml plastic container containing 2.5 ml of KCl medium and 1 mM-MgATP. After 2 min preincubation, either 0.5 mM-CaCl₂ (corresponding to an initial [Ca²⁺] of 8 μ M) or 0.6 mM-butyrate was added and incubation continued for the required time before removal of samples for PP_i determination ($\bullet$) as described in Fig. 1. Time intervals were defined as the time elapsing between addition of Ca²⁺ or butyrate and commencement of centrifugation of the mitochondrial incubation. The data shown are from one of two experiments showing similar results. Light-scattering measurements were performed in parallel, as described in Fig. 3.

Table 1. Influence of ATP and carboxyatractyloside on swelling and pyrophosphate accumulation by mitochondria

Incubations were performed as described in the legend to Fig. 1 with either 0.6 mM-butyrate or 1 μ M- Ca^{2+} added to the experimental cuvette. When present, 10 μ M-carboxyatractyloside was added to both cuvettes. For butyrate addition, the total decrease in light-scattering (ΔA_{520}) was measured over the 4 min incubation, by which time changes were complete. For Ca^{2+} addition, the maximal rate of decrease in light-scattering (A_{520}/min) was measured to avoid damage to the mitochondria associated with prolonged and rapid swelling in the absence of ATP. All values are means $\pm$ S.E.M. of n observations. The statistical significances of the difference between experimental and control mitochondria, calculated from paired Student's t test, were * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, and between conditions with ATP present or absent † $P < 0.05$, ‡ $P < 0.01$; n.d., not determined.

Addition to experimental cuvette	n	PP_i in controls (pmol/mg)	Change in parameter induced by additions		
			ΔPP_i (pmol/mg)	$10^3 \times \text{Rate of swelling}$ ($\Delta A_{520}/\text{min}$)	$10^3 \times \text{Extent of swelling}$ (ΔA_{520})
Ca^{2+}	4	78 ± 7	$+74 \pm 9^*$	$15.28 \pm 1.8^{**}$	—
Butyrate	3	88 ± 10	$+79 \pm 3^{***}$	—	$9.12 \pm 1.19^*$
1 mM-ATP + butyrate	3	110 ± 13	$+115 \pm 9^{***}$	—	$5.81 \pm 0.83^{*\dagger}$
1 mM-ATP + Ca^{2+}	4	109 ± 7	$+147 \pm 20^{**\dagger}$	$2.64 \pm 0.21^{***\dagger\dagger}$	—
1 mM-ATP + carboxyatractyloside	4	109 ± 7	-15 ± 8	n.d.	—
1 mM-ATP + carboxyatractyloside + Ca^{2+}	4	109 ± 7	$+117 \pm 21^*$	$8.17 \pm 1.29^{**}$	—

Table 2. Effect of incubating liver mitochondria with Ca^{2+} or butyrate on their adenine nucleotide content

Mitochondria were incubated with or without 1 μ M- Ca^{2+} and/or 10 μ M-carboxyatractyloside or 0.6 mM-butyrate as described in the legend to Fig. 1. Samples were taken from the experimental cuvette (containing Ca^{2+} , carboxyatractyloside or butyrate) and reference cuvette (incubated control) for assay of adenine nucleotides. Where applicable, ATP was present in both cuvettes as indicated. Details of these assays and the correction factors applied to correct for carry-over of nucleotides from the incubation medium are given in the Experimental section. All values are expressed as means $\pm$ S.E.M. for the numbers of separate mitochondrial preparations shown (n). In a separate experiment, the loss of adenine nucleotides during control incubations was investigated. After a 5 min incubation period, the total adenine nucleotides decreased from 8.5 ± 0.30 to 7.72 ± 0.25 nmol/mg of protein ($n = 4$; $P < 0.01$ by paired Student's t test). The statistical significance of the effect of carboxyatractyloside (†), Ca^{2+} (*) and butyrate in the presence (‡) and absence (§) of ATP were determined by paired Student's t test. Three, two and one symbols represent $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively.

Additions	Added ATP (mM)	n	Content (nmol/mg of mitochondrial protein)				$\frac{\text{ATP}}{\text{ADP}}$
			ATP	ADP	AMP	ATP + ADP + AMP	
None	1	8	2.59 ± 0.12	2.68 ± 0.09	2.30 ± 0.11	7.56 ± 0.2	0.97 ± 0.06
1 μ M- Ca^{2+}	1	8	$3.67 \pm 0.09^{***}$	$3.10 \pm 0.08^{***}$	$1.93 \pm 0.09^{***}$	$8.69 \pm 0.18^{***}$	$1.19 \pm 0.04^{**}$
10 μ M-carboxyatractyloside	1	8	$2.20 \pm 0.10^\dagger$	$3.19 \pm 0.21^\dagger$	$2.90 \pm 0.11^{\dagger\dagger\dagger}$	$8.30 \pm 0.25^\dagger$	$0.72 \pm 0.06^{\dagger\dagger}$
10 μ M-carboxyatractyloside + 1 μ M- Ca^{2+}	1	8	2.36 ± 0.12	3.21 ± 0.14	2.75 ± 0.11	8.33 ± 0.22	0.75 ± 0.05
0.6 mM-butyrate	1	5	1.31 ± 0.23	2.17 ± 0.45	$4.18 \pm 0.30^{\dagger\dagger}$	$7.78 \pm 0.32^\dagger$	0.72 ± 0.18
None	0	4	1.47 ± 0.13	1.46 ± 0.28	2.57 ± 0.18	5.50 ± 0.51	1.04 ± 0.27
1 μ M- Ca^{2+}	0	4	1.70 ± 0.25	1.39 ± 0.15	1.54 ± 0.23	4.62 ± 0.39	1.27 ± 0.25
0.6 mM-butyrate	0	4	$0.75 \pm 0.09^\S$	$0.55 \pm 0.23^\S$	$3.74 \pm 0.35^\S$	5.02 ± 0.32	1.20 ± 0.2

is reflected in a large increase in mitochondrial AMP (Table 2). Unlike the changes induced by Ca^{2+} , maximal increases in PP_i are observed 1 min after butyrate addition, concentrations falling again after 3 min, perhaps as a result of the build-up of AMP (Table 2). The increase in PP_i is accompanied by a rapid decrease in light-scattering, whereas light-scattering increases again as the PP_i decreases.

In Fig. 3 we demonstrate that the light-scattering

changes are accompanied by matrix volume changes, measured with $^3\text{H}_2\text{O}$ and [^{14}C]sucrose. Data are presented for addition of 0.3 μ M- Ca^{2+} and 0.6 mM-butyrate in the presence or absence of 1 mM-ATP, or 3 mM- PP_i in the absence of ATP. PP_i added externally is most likely to enter the matrix in exchange for adenine nucleotides on the adenine nucleotide translocase (Asimakis & Aprille, 1980; D'Souza & Wilson, 1982). However, this exchange is retarded in energized coupled mitochondria (D'Souza

Fig. 3. Light-scattering and matrix volume changes induced by Ca²⁺, butyrate and PP_i in the presence and absence of ATP

Incubations were carried out as described in the legend to Fig. 1 in the presence (continuous lines) or absence (broken lines) of 1 mM-MgATP. Additions to the experimental cuvette (final concn.) were as follows: traces i and ii, 0.6 mM-sodium butyrate; traces iii and iv, 0.37 mM-CaCl₂ ([Ca²⁺] = 0.3 μM); traces v and vi, 3 mM-PP_i. After termination of the light-scattering measurements, five 0.5 ml samples were taken from reference and experimental cuvettes for measurement of mitochondrial volumes by using ³H₂O and [¹⁴C]sucrose. The mean volume was 0.977 ± 0.013 μl/mg of protein (mean ± S.E.M. for five separate control incubations). The percentage increases in volume after the various additions are given alongside the appropriate traces (n.d., not determined).

& Wilson, 1982), and we could only detect swelling induced by external PP_i in the absence of competing ATP (Fig. 3). This contrasts with the effects seen in de-energized mitochondria, to be described in Fig. 7. The effects of ATP on swelling induced by butyrate and Ca²⁺ are discussed in the following section.

Further evidence that butyrate and Ca²⁺ might induce swelling by a common mechanism, namely an increase in matrix PP_i, is given in Fig. 4. A similar correlation is observed between PP_i and extent of swelling after 3.5 min whether swelling was induced by low [Ca²⁺] (within the cytoplasmic [Ca²⁺] range) or butyrate (< 0.5 mM). However, we show in Fig. 5 that when Ca²⁺ and butyrate were added together this correlation breaks down. Mitochondrial PP_i increased 30-fold, from 93 pmol/mg in control incubations to 3014 pmol/mg with Ca²⁺ followed by butyrate, and to 3869 pmol/mg when they were added in the reverse order. Concentrations of butyrate and Ca²⁺ used were 0.6 mM and 0.9 μM respectively. Even higher contents of PP_i, 10.6 nmol/mg, were measured by Otto & Cook (1982) in the presence of 10 mM-butyrate and 250 μM-Ca²⁺, and were presumed to be due to an increase in mitochondrial PP_i synthesis as a result of butyryl-CoA formation coupled to inhibition of endogenous pyrophosphatases by Ca²⁺. When this gross increase in mitochondrial PP_i occurred, swelling was

Fig. 4. Correlation between light-scattering and increase in PP_i induced by Ca²⁺ or butyrate

Mitochondria were incubated as described in the legend to Fig. 1, and the A₅₂₀ and increase in PP_i were measured. Decrease in light-scattering was measured for 3.5 min after additions to the experimental cuvette of either CaCl₂ (▲), to produce 0.29–0.88 μM [Ca²⁺], or 0.2–0.5 mM-butyrate (●).

Fig. 5. Combined effect of Ca²⁺ and butyrate on light-scattering

Incubations were carried out as described in the legend to Fig. 1. ATP (1 mM) was present in all cases, and 0.9 μM-Ca²⁺ or 0.6 mM-butyrate was added where indicated. Final concentrations of PP_i (pmol/mg of protein) are given alongside each trace. Mean PP_i concentration in controls was 93 ± 6 (mean ± S.E.M. for four separate observations).

inhibited, as shown in Fig. 5. Immediate inhibition, and even reversal, was seen when Ca²⁺ was added to mitochondria preincubated with butyrate, and after about 1 min when butyrate was added after Ca²⁺. A possible explanation may lie in the low solubility product (1.8×10^{-13}) of calcium pyrophosphate (Wiers, 1971; Veech *et al.*, 1980). High mitochondrial content of both Ca²⁺ and PP_i might therefore result in precipitation of calcium pyrophosphate and consequent lowering of free [PP_i], which we believe to be the important agent in inducing swelling.

Role of the adenine nucleotide translocator in mitochondrial swelling

In Fig. 3 and Table 1 we show that the presence of 1 mM-MgATP decreased the rate of Ca²⁺-induced swell-

ling by as much as 80 % (Table 1), despite the increase in PP_i being 2-fold greater in the presence of ATP. The presence of 1 mM-ATP also significantly decreased the extent of swelling induced by butyrate, although the effect was less marked than that with Ca^{2+} . The results

Fig. 6. Potentiation of Ca^{2+} -induced swelling by atractyloside

Mitochondria were incubated as described in Fig. 1 in the presence of 0.3 mM-MgATP, and light-scattering was monitored as A_{520} . Where indicated, $0.9 \mu\text{M}-Ca^{2+}$ and $5 \mu\text{g}$ of atractyloside/ml (Atrac.) were added. Mitochondrial matrix PP_i content (●) in experimental and reference cuvettes was determined after termination of recording, and PP_i content at the time of atractyloside addition was determined in a parallel experiment.

with butyrate must be interpreted with some caution, especially in the absence of added ATP, since large increases in mitochondrial AMP occur (see Table 2), which might themselves influence swelling, either directly through interaction with the adenine nucleotide translocase or by altering the mitochondrial $[Mg^{2+}]$. In addition, the time course of swelling and PP_i increase after addition of Ca^{2+} or butyrate are different, as shown in Fig. 2. This makes it difficult to compare the effects of ATP on the action of swelling agents by using data measured from different experiments (cf. Fig. 4, where all results were on the same mitochondrial preparation).

The ability of ATP to moderate Ca^{2+} -induced swelling is shared by ADP (Halestrap *et al.*, 1986). The protective effect of ATP could be removed by addition of either atractyloside or carboxyatractyloside, both inhibitors of the adenine nucleotide translocase. In Fig. 6 we show that the maximal rate of Ca^{2+} -induced swelling in the presence of 0.3 mM-MgATP (measured as A_{520}/min) was 4.4, and increased to 9.5 on addition of atractyloside without a corresponding change in matrix PP_i content. Table 1 includes similar data for carboxyatractyloside. This effect of these inhibitors appeared to be a potentiation of the Ca^{2+} -induced swelling, since addition of either inhibitor alone produced only a small degree of swelling ($1.00 \pm 0.07 A_{520}$ unit/min; mean $\pm$ S.E.M. for the response of three different mitochondrial preparations to atractyloside). This is consistent with the view that PP_i -induced swelling is maximal when the adenine nucleotide translocase is trapped in the 'C' conformation, as occurs when atractyloside is present or when external adenine nucleotides are absent (see Scherer & Klingenberg, 1974; Klingenberg, 1976; Halestrap *et al.*, 1985).

There is some evidence which suggests that when in the 'C' conformation the adenine nucleotide translocase can act as a K^+ channel (Panov *et al.*, 1980; Jung & Brierley,

Fig. 7. Swelling of de-energized mitochondria in iso-osmotic potassium pyrophosphate or thiocyanate

Swelling was initiated by addition of mitochondria (3 mg of protein/ml) to either 150 mM-KSCN (a) or 80 mM-KPP_i (b). Both media contained 10 mM-Mops, 7 mM-Tris, 1 mM-EGTA and $1 \mu\text{g}$ of both antimycin and rotenone/ml, and were adjusted to pH 7.2 with KOH. The temperature was 30 °C and additions were made as shown: PP_i (indicated by the concentration used), 0.5 mM-ADP (ADP), 10 μM -carboxyatractylate (CAT) and valinomycin at 1 μM -(VAL), 0.1 μM -(VAL*) or 0.5 μM -(VAL†). In (b), trace (i), 0.5 mM-ADP was present from the start of the incubation. The experiment shown is representative of at least two experiments. Data shown are from two different preparations of mitochondria, represented by traces (a)i–(a)v and (a)vi–(b)v respectively.

1981, 1984), and the data of Fig. 7 support this proposal, but implicate PP_i in the mechanism. The swelling of de-energized mitochondria in iso-osmotic KSCN (*a*) and KPPi (*b*) was studied in the absence and presence of ADP (which enhances the 'M' conformation) and carboxyatractyloside (which enhances the 'C' conformation). In the KSCN medium the rate of swelling can be taken as a measure of the K⁺-permeability, since the SCN⁻ ion is membrane-permeant. Under these conditions a slow rate of swelling was apparent, which was greatly accelerated by the addition of 1 μ M-valinomycin. In the absence of valinomycin, swelling was slightly inhibited by addition of 0.5 mM-ADP, but this could be reversed by addition of 10 μ M-carboxyatractyloside (Fig. 7*a*, trace vi). Increased rates of swelling could also be induced by addition of potassium pyrophosphate (1–20 mM), as shown in traces ii–v, and this swelling was not abolished by the presence of carboxyatractyloside (trace ii). The swelling induced by 20 mM-potassium pyrophosphate was of similar magnitude to that caused by 0.5 μ M-valinomycin. This clearly demonstrates that PP_i can greatly enhance the electrogenic permeability of the mitochondrial inner membrane towards K⁺. In Fig. 7(*b*) we demonstrate that, when incubated in iso-osmotic potassium pyrophosphate without further additions, mitochondria swelled slowly, but the swelling accelerated with time (trace ii) unless ADP was present (trace i). Swelling was greatly increased by addition of carboxyatractyloside whether or not ADP was present (traces i and iii), and there was no additional effect of 1 μ M-valinomycin (trace iii). Addition of valinomycin in the absence of carboxyatractyloside produced a moderate rate of swelling, which could be inhibited by ADP and stimulated by carboxyatractyloside (traces iii and iv). For mitochondria to swell in the potassium pyrophosphate medium, both K⁺ and PP_i must be able to enter. Thus the data of Fig. 7(*b*) suggest that in the 'C' conformation the adenine nucleotide translocator allows rapid electrogenic transport of PP_i as well as K⁺. The exact mechanism of the uptake of these ions must await further investigation. However, we have studied the specificity of the K⁺ channel by using iso-osmotic solutions of the PP_i salt of various cations. The PP_i salt of choline, tetramethylammonium and Tris did not support swelling induced by carboxyatractyloside, whereas rates of carboxyatractyloside-induced swelling in iso-osmotic PP_i salt of NH₄⁺, Na⁺ and Li⁺ (expressed as a percentage of the rate with the K⁺ salt) were 160, 89 and 37 % respectively. These results suggest that it is the diameter of the hydrated cation which is important for electrogenic transport, just as is observed for valinomycin and nonactin (Pressman, 1976). A similar conclusion was reached from the cation requirements for energized Ca²⁺-activated swelling (Halestrap *et al.*, 1986), as would be expected if this is mediated via an increased mitochondrial [PP_i].

Effects of Ca²⁺ on the adenine nucleotide content of liver mitochondria

PP_i can itself interact with and be transported by the adenine nucleotide translocase. Thus external PP_i has been shown to deplete de-energized rat liver mitochondria of adenine nucleotides by this means (Asimakis & Aprille, 1980; D'Souza & Wilson, 1982). Therefore one might predict that an expanded pool of mitochondrial PP_i exchanging with external adenine nucleotide, would lead to a net influx of the latter into mitochondria. In

Table 2 we show that there was a highly significant increase (15%) in total adenine nucleotide content in mitochondria exposed to 1 μ M-Ca²⁺, arising from significant increases in ATP (42%) and ADP (16%) but a fall in AMP of 19%. There was also a significant increase in ATP/ADP ratio of 22%. A similar but smaller effect was seen when mitochondria were incubated with 0.6 mM-butyrate, but under these conditions a larger proportion of the adenine nucleotides were present as AMP. During the 5 min incubation at 37 °C there was a small loss (10%) of adenine nucleotides in the control incubation when extramitochondrial ATP was present, which was prevented by the presence of carboxyatractyloside. However, this inhibitor also prevented the increase in adenine nucleotides induced by incubation with 1 μ M-Ca²⁺ and caused a significant increase in AMP (19% and 44% in the absence and presence of Ca²⁺ respectively). When incubated in the absence of ATP for 5 min, the mitochondrial adenine nucleotides decreased to about 65% of the initial value, and no increases were observed after treatment with Ca²⁺ or butyrate. These data suggest that adenine nucleotides can enter or leave the mitochondrial matrix by a carboxyatractyloside-sensitive pathway. The net influx of adenine nucleotides in response to incubation with Ca²⁺ occurred in the face of the greater concentrations of ATP and ADP than of PP_i, and the reported low transport affinity for PP_i compared with ATP in reconstituted vesicles (Krämer, 1985). However, since ATP, ADP and presumably PP_i are translocated as the free anion (Duszynski & Wojtczak, 1975; Krämer, 1980), and a high proportion is complexed with Mg²⁺ *in vivo*, caution must be taken when extrapolating *K_m* values based on total values to the situation *in vivo*. In addition, since the maximum translocation rate is high (Klingenberg, 1976; Stubbs *et al.*, 1978; Stubbs, 1981), substitution of PP_i for ATP in only a small fraction of the total exchange would result in a modest accumulation of adenine nucleotides in the matrix over a period of time.

DISCUSSION

Regulation of mitochondrial PP_i

The data presented in this paper demonstrate that there is a good correlation between the ability of both Ca²⁺ (0.1–1.0 μ M) and butyrate (0.2–0.5 mM) to increase intra-mitochondrial [PP_i] and the mitochondrial matrix volume (Figs. 1–4). Half-maximal activation by Ca²⁺ of both processes under incubation conditions that approximate to the physiological situation occurs at about 0.3 μ M extramitochondrial Ca²⁺. Thus they are thus sensitive to Ca²⁺ concentrations in the same range as the mitochondrial Ca²⁺-sensitive dehydrogenases (Denton & McCormack, 1985; Hansford, 1985) and poised to respond readily to the increase in Ca²⁺ that occurs upon hormone treatment of hepatocytes (Blackmore *et al.*, 1982; Charest *et al.*, 1983; Thomas *et al.*, 1984; Mauger *et al.*, 1984; Sistare *et al.*, 1985; Mauger & Claret, 1986; Poggioli *et al.*, 1986).

The mechanism by which Ca²⁺ increases mitochondrial [PP_i] is not known, but could result from either a stimulated production or an inhibited removal of PP_i. Although it has been reported by some workers (Irie *et al.*, 1970; Volk *et al.*, 1982) that Ca²⁺ can inhibit mitochondrial pyrophosphatases, the concentration used in their experiments was higher than expected within the

matrix. Further studies are required on the kinetics of mitochondrial pyrophosphatases under the conditions prevailing in the mitochondrial matrix. Stimulation of PP_i synthesis within the mitochondrial matrix could occur by two mechanisms. ATP-dependent activation of endogenous fatty acids produced by intramitochondrial phospholipase A_2 is one possibility. Ca^{2+} is known to stimulate this enzyme and so increase the concentrations of non-esterified fatty acids for such activation (see Halestrap, 1981; Armston & Halestrap, 1984). Although significant activation of phospholipase A_2 can occur at high Ca^{2+} loads and cause massive swelling of mitochondria (see e.g., Armston *et al.*, 1982; Armston & Halestrap, 1984), the process is inhibited by local anaesthetics, which were shown to have no effect on the swelling observed at low $[Ca^{2+}]$ (Halestrap *et al.*, 1986) or on changes in $[PP_i]$ (results not shown). Another potential source of PP_i is the proton-translocating pyrophosphatase best documented in bacteria (Baltscheffsky & Nyren, 1984), but also present in ox heart and rat liver mitochondria (Mansurova *et al.*, 1977; Volk & Baykov, 1984). The effects of physiological $[Ca^{2+}]$ on this enzyme have not been studied. It is probable that the final matrix $[PP_i]$ is determined by the activity of such a synthetic enzyme relative to the activity of any degradative pyrophosphatases (Irie *et al.*, 1970; Volk *et al.*, 1983) and the efflux of PP_i out of the mitochondria.

Interaction between PP_i , the adenine nucleotide transporter and mitochondrial swelling

The correlation between $[PP_i]$ and swelling breaks down when both butyrate and Ca^{2+} are added together (Fig. 5). Under these conditions the $[PP_i]$ in the matrix rises more than 30-fold, but the swelling rapidly ceases. It is probable that this massive rise in PP_i is accompanied by precipitation of Ca_2PP_i or Mg_2PP_i , since the solubility product of both these complexes is very low (Wiers, 1971). Thus it is likely that uncomplexed PP_i is important in inducing swelling. It is known that it is the uncomplexed form of adenine nucleotides that bind to and are translocated by the adenine nucleotide translocase (Krämer, 1980; Klingenberg, 1981), and it would seem likely that the same is true for PP_i . The effects of adenine nucleotides and atractyloside/carboxyatractyloside on swelling induced by Ca^{2+} , butyrate or added PP_i (Figs. 5 and 6 and Tables 1 and 2 of the present paper; Halestrap *et al.*, 1986) implicate this carrier in the swelling process. Panov *et al.* (1980) have presented data that the conformation of the adenine nucleotide translocator that is stabilized by carboxyatractyloside may enhance the permeability of the mitochondrial inner membrane to K^+ . Jung & Brierley (1981, 1984) have also presented data that support a role for this translocator in regulating K^+ permeability in heart mitochondria. They showed that adenine nucleotide depletion enhanced K^+ permeability, whereas addition of ADP inhibited it. Our own data in Fig. 7 add further support for a role for the adenine nucleotide translocase in regulating K^+ entry into mitochondria and suggest that PP_i may bind to the translocator and induce permeability to K^+ and other small cations. In addition, we have shown that under de-energized conditions PP_i can cross the inner membrane by an electrogenic transport system that is enhanced by the presence of carboxyatractyloside, suggesting that perhaps the adenine nucleotide translocase in the 'C' conformation itself mediates transport.

Relationship to the effects of hormones *in vivo*

We believe that the data reported in the present paper support the following mechanism for the hormonal increase in mitochondrial matrix volume. A rise in cytoplasmic $[Ca^{2+}]$ allows uptake of Ca^{2+} into the mitochondria, as has been measured in several laboratories (Reinhart *et al.*, 1984; Shears & Kirk, 1984; McCormack, 1985; Assimacopoulos-Jeannet *et al.*, 1986). This then increases the mitochondrial $[PP_i]$, which interacts with the adenine nucleotide translocator, enabling it to transport K^+ into the mitochondria driven by the membrane potential. Uptake of an anion such as phosphate occurs to compensate the uptake of K^+ , and thus an osmotic imbalance causes the mitochondria to swell until the K^+/H^+ antiporter is stimulated sufficiently to balance the enhanced K^+ entry (Garlid, 1980; Dordick *et al.*, 1980; Nakashima *et al.*, 1982). An additional consequence of the PP_i binding to the adenine nucleotide translocator will be the uptake of adenine nucleotides into the mitochondria in exchange for PP_i , as occurs *in vitro* (Table 2) in response to Ca^{2+} . Haynes *et al.* (1986) have also reported that incubation of mitochondria with ATP in the presence of Ca^{2+} increases the mitochondrial adenine nucleotide content, but in a carboxyatractyloside-insensitive manner. Several significant differences in the experimental protocol of these workers should be noted. They used sucrose rather than KCl-based incubation media, incubated for longer times than in the present study and made no correction for extramitochondrial adenine nucleotides contaminating the mitochondrial pellet. The content of adenine nucleotides determined in their experiments was also considerably higher than those reported here, or values derived from rapid subcellular-fractionation techniques (Siess *et al.*, 1977; Titheradge & Haynes, 1980; Soboll & Scholz, 1986).

As outlined in the Introduction, increased mitochondrial total adenine nucleotides is a well-documented consequence of hormone action on the liver, and is thus readily explained by the current hypothesis. It is also significant that we have demonstrated that butyrate and other short-chain fatty acids which elevate cellular (and presumably mitochondrial) $[PP_i]$ (Veech *et al.*, 1980; Otto & Cook, 1982) caused a decrease in light-scattering of hepatocytes, consistent with an increase in mitochondrial matrix volume (Halestrap *et al.*, 1985). Furthermore, Veech *et al.* (1980) have reported that injection of rats with either short-chain fatty acids or PP_i has effects on liver metabolite concentrations similar to that of glucagon. We have freeze-clamped livers from control, glucagon- and phenylephrine-treated rats [see Armston & Halestrap (1984) for protocol] and measured the total PP_i content. The respective values (expressed as nmol/g wet wt. and means $\pm$ S.E.M. for the numbers of observations shown in parentheses) were 10.35 ± 0.40 (18), 14.34 ± 0.64 (9) and 12.27 ± 0.59 (17). These increases in PP_i were both highly significant by Student's *t* test ($P > 0.001$ and $P > 0.02$ respectively) and support our hypothesis. Measurement of the compartmentation of PP_i within the hepatocyte will indicate whether the hormonally induced changes are intramitochondrial as anticipated.

This work was supported by a grant from the Medical Research Council. We thank Ms. Anne Scott for carrying out some preliminary mitochondrial swelling experiments.

REFERENCES

- Aas, M. & Bremer, J. (1968) *Biochim. Biophys. Acta* **164**, 157–166
- Aprille, J. R., Nosek, M. T. & Brennan, W. A. (1982) *Biochem. Biophys. Res. Commun.* **108**, 834–839
- Armston, A. E. & Halestrap, A. P. (1984) *Biosci. Rep.* **4**, 903–908
- Armston, A. E., Halestrap, A. P. & Scott, R. D. (1982) *Biochim. Biophys. Acta* **681**, 429–439
- Asimakis, G. K. & Aprille, J. R. (1980) *FEBS Lett.* **117**, 157–160
- Assimakopoulos-Jeannet, F., McCormack, J. G. & Jeanrenaud, B. (1986) *J. Biol. Chem.* **261**, 8799–8804
- Baltscheffsky, M. & Nyren, P. (1984) in *Bioenergetics* (Ernster, L., ed.), pp. 187–206, Elsevier Science Publishers, Amsterdam
- Blackmore, P. F., Hughes, B. P., Shuman, E. A. & Exton, J. H. (1982) *J. Biol. Chem.* **257**, 190–197
- Brierley, G. P. (1976) *Mol. Cell. Biochem.* **10**, 41–62
- Brierley, G. P., Jurkowitz, M. S., Farooqui, T. & Jung, D. W. (1984) *J. Biol. Chem.* **259**, 14672–14678
- Bryla, J., Harris, E. J. & Plumb, J. A. (1977) *FEBS Lett.* **80**, 443–448
- Charest, R., Blackmore, P. F., Berthon, B. & Exton, J. H. (1983) *J. Biol. Chem.* **258**, 8769–8773
- Cook, G. A., O'Brien, W. E., Wood, H. G., King, M. T. & Veech, R. L. (1978) *Anal. Biochem.* **91**, 557–565
- Denton, R. M. & McCormack, J. G. (1985) *Am. J. Physiol.* **249**, E543–E554
- Denton, R. M., Richards, D. A. & Chin, J. G. (1978) *Biochem. J.* **176**, 899–906
- Dordick, R. S., Brierley, G. P. & Garlid, K. D. (1980) *J. Biol. Chem.* **255**, 10299–10303
- D'Souza, M. P. & Wilson, D. F. (1982) *Biochim. Biophys. Acta* **680**, 28–32
- Duszynski, J. & Wojtczak, L. (1975) *FEBS Lett.* **50**, 74–78
- Garlid, K. D. (1979) *Biochem. Biophys. Res. Commun.* **87**, 842–849
- Garlid, K. D. (1980) *J. Biol. Chem.* **255**, 11273–11279
- Halestrap, A. P. (1975) *Biochem. J.* **148**, 85–96
- Halestrap, A. P. (1978) *Biochem. J.* **172**, 389–398
- Halestrap, A. P. (1981) in *Short-Term Regulation of Liver Metabolism* (Hue, L. & Van de Werve, G., eds.), pp. 389–409, Elsevier/North-Holland, Amsterdam
- Halestrap, A. P. (1987) *Biochem. J.* **244**, 159–164
- Halestrap, A. P. & McGivan, J. D. (1979) in *Techniques in Metabolic Research* (Kornberg, H. L., Metcalfe, J. C., Northcote, D. H., Pogson, C. I. & Tipton, K. F., eds.), B206, pp. 1–23, Elsevier/North-Holland, Amsterdam
- Halestrap, A. P., Quinlan, P. T., Armston, A. E. & Whipps, D. E. (1985) in *Achievements and Perspectives in Mitochondrial Research*; Vol. 1, *Bioenergetics* (Quagliariello, E., Slater, E. C., Palmieri, F., Saccone, C. & Kroon, A. M., eds.), pp. 469–480, Elsevier Science Publishers, Amsterdam
- Halestrap, A. P., Quinlan, P. T., Whipps, D. E. & Armston, A. E. (1986) *Biochem. J.* **236**, 779–787
- Hansford, R. G. (1985) *Rev. Physiol. Biochem. Pharmacol.* **102**, 1–72
- Haynes, R. C., Picking, R. A. & Zaks, W. J. (1986) *J. Biol. Chem.* **261**, 16121–16125
- Irie, M., Yabuta, A., Kimura, K., Shindo, Y. & Tomito, K. (1970) *J. Biochem. (Tokyo)* **67**, 47–58
- Jaworek, D., Gruber, W. & Bergmeyer, H. W. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 4, pp. 2127–2131, Verlag Chemie, Weinheim, and Academic Press, New York
- Jung, D. W. & Brierley, G. P. (1981) *J. Biol. Chem.* **256**, 10490–10496
- Jung, D. W. & Brierley, G. P. (1984) *J. Biol. Chem.* **259**, 6904–6911
- Klingenberg, M. (1976) in *The Enzymes of Biological Membranes* (Martonosi, A., ed.), vol. 3, pp. 383–438, Plenum, New York
- Klingenberg, M. (1981) in *Mitochondria and Microsomes* (Lee, C. P., Schaty, G. & Dallner, G., eds.), pp. 293–316, Addison-Wesley, New York
- Křamer, R. (1980) *Biochim. Biophys. Acta* **592**, 615–620
- Křamer, R. (1985) *Biochem. Biophys. Res. Commun.* **127**, 129–135
- Lamprecht, W. & Trautschold, I. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 4, pp. 2101–2109, Verlag Chemie, Weinheim, and Academic Press, New York
- Mansurova, S. E., Shakhov, Y. A. & Kulaev, L. S. (1977) *FEBS Lett.* **74**, 31–34
- Mauger, J.-P. & Claret, M. (1986) *FEBS Lett.* **195**, 106–110
- Mauger, J.-P., Poggioli, J., Guesdon, F. & Claret, M. (1984) *Biochem. J.* **221**, 121–127
- McCormack, J. G. (1985) *Biochem. J.* **231**, 597–608
- Nakashima, R. A., Dordick, R. S. & Garlid, K. D. (1982) *J. Biol. Chem.* **257**, 12540–12545
- Otto, D. A. & Cook, G. A. (1982) *FEBS Lett.* **150**, 172–176
- Panov, A., Filippova, S. & Lyakhovich, V. (1980) *Arch. Biochem. Biophys.* **199**, 420–426
- Poggioli, J., Mauger, J.-P. & Claret, M. (1986) *Biochem. J.* **235**, 663–669
- Pressman, B. C. (1976) *Annu. Rev. Biochem.* **45**, 501–530
- Quinlan, P. T., Thomas, A. P., Armston, A. E. & Halestrap, A. P. (1983) *Biochem. J.* **214**, 395–404
- Reinhart, P. H., Van De Pol, E., Taylor, W. M. & Bygrave, F. L. (1984) *Biochem. J.* **218**, 415–420
- Scherer, B. & Klingenberg, M. (1974) *Biochemistry* **13**, 161–170
- Shears, S. B. & Kirk, C. J. (1984) *Biochem. J.* **219**, 383–389
- Siess, E. A., Brocks, G., Lattke, H. K. & Wieland, O. H. (1977) *Biochem. J.* **166**, 225–235
- Sistare, F. D., Picking, R. A. & Haynes, R. C. (1985) *J. Biol. Chem.* **260**, 12744–12747
- Soboll, S. & Scholz, R. (1986) *FEBS Lett.* **205**, 109–112
- Stubbs, M. (1981) in *Short-Term Regulation of Liver Metabolism* (Hue, L. & Van de Werve, G., eds.), pp. 411–425, Elsevier/North-Holland, Amsterdam
- Stubbs, M., Vignais, P. V. & Krebs, H. A. (1978) *Biochem. J.* **172**, 333–342
- Thomas, A. P., Alexander, J. & Williamson, J. R. (1984) *J. Biol. Chem.* **259**, 5574–5584
- Titheradge, M. A. & Haynes, R. C. (1980) *J. Biol. Chem.* **255**, 1471–1477
- Veech, R. L., Cook, G. A. & King, M. T. (1980) *FEBS Lett.* **117**, Suppl. K65–K72
- Volk, S. E. & Baykov, A. A. (1984) *Biochim. Biophys. Acta* **791**, 198–204
- Volk, S. E., Baykov, A. A., Duzhenko, V. S. & Avaeva, S. M. (1982) *Eur. J. Biochem.* **125**, 215–220
- Volk, S. E., Baykov, A. A., Kostenko, E. B. & Avaeva, S. H. (1983) *Biochim. Biophys. Acta* **744**, 127–134
- Whipps, D. E., Armston, A. E., Pryor, H. J. & Halestrap, A. P. (1987) *Biochem. J.* **241**, 835–845
- Wiers, B. H. (1971) *Inorg. Chem.* **10**, 2581–2584