J. exp Biol. 130, 203–217 (1987)
Printed in Great Britain © The Company of Biologists Limited 1987

THE ROLE OF DIVALENT CATIONS AND IONIC STRENGTH IN THE OSMOTIC SENSITIVITY OF GLUTAMATE OXIDATION IN OYSTER GILL MITOCHONDRIA

BY JAMES S. BALLANTYNE AND CHRISTOPHER D. MOYES*

Department of Zoology, University of Guelph, Guelph, Ontario, Canada, N1G 2W1

Accepted 24 February 1987

SUMMARY

The biochemical mechanism responsible for the osmotic sensitivity of glutamate oxidation in oyster (*Crassostrea virginica* Gmelin) gill mitochondria was examined. The relative roles of osmotically induced changes in matrix divalent cation concentration and ionic strength were determined. The calcium–magnesium ionophore A23187 inhibited glutamate oxidation in both low- (40 % of control rate) and high- (25 % of control rate) osmolarity media. Addition of $MgCl_2$ reversed A23187 inhibition, but at each $MgCl_2$ concentration (1–5 mmol l^{-1}) the rate of glutamate oxidation in the high-osmolarity medium was about 45 % that in the low-osmolarity medium. EDTA (4 mmol l^{-1}) stimulated glutamate oxidation at both osmolarities (more pronounced at high osmolarity), removing the dependency on assay medium osmolarity. The stimulation in response to EDTA was correlated with mitochondrial swelling, which required the presence of monovalent cations for maximal effect. These data suggest that osmotically induced changes in matrix $[Mg^{2+}]$ or $[Ca^{2+}]$ are not responsible for the osmotic sensitivity of glutamate oxidation by oyster gill mitochondria *in vitro*. Changes in extramitochondrial $[Mg^{2+}]$ may be involved in regulating the rate of glutamate oxidation *in vivo*, through effects on mitochondrial volume. The maximal rate of glutamate dehydrogenase was not very sensitive to the ionic strength of the assay medium. The rate of electron transport was highly dependent on ionic strength, with the maximal rate occurring in 50 mmol l^{-1} salt. The osmotic sensitivity of glutamate oxidation in oyster gill mitochondria is apparently due to the effects of changes in matrix ionic strength on the rate of electron transport.

INTRODUCTION

Regulation of mitochondrial volume *in vivo* is essential for the maintenance of controlled intermediary metabolism. Mitochondrial volume perturbations may occur in the tissues of osmoregulators that experience tissue osmotic stress as a result of various renal and nervous disorders (Pollock & Arieff, 1980). Mitochondrial volume

* Present address: Department of Zoology, University of British Columbia, Vancouver, BC, Canada, V6T 2A6.

Key words: mitochondria, magnesium, osmoconformer, bivalve mollusc, ionic strength.

changes also occur in the absence of tissue osmotic stress as a result of treatment with glucagon or alpha-adrenergic hormones (Halestrap, Quinlan, Armston & Whipps, 1985; Armston, Halestrap & Scott, 1982). The effects of these hormones on cellular processes such as oxidation (Armston *et al.* 1982) and gluconeogenesis (Quinlan, Thomas, Armston & Halestrap, 1983) have been attributed to mitochondrial volume changes occurring *in situ*.

Tissues of osmoconformers can undergo dramatic changes in tissue osmolarity in response to variable ambient salinity, which would be expected to induce changes in mitochondrial volume. In a series of studies, we have examined the biochemical effects of changes in mitochondrial volume on oxidation of fatty acids, ketone bodies, Krebs cycle intermediates and pyruvate by mitochondria from several species of osmoconformers, including bivalve molluscs and elasmobranchs (Ballantyne & Storey, 1984; Ballantyne & Moon, 1985, 1986; Moyes, Moon & Ballantyne, 1986). Stimulation of mitochondrial oxidation occurs under hypotonic conditions; hypertonic conditions inhibit mitochondrial oxidation of various metabolites. Osmotic effects on mitochondrial oxidation *in vivo* would be expected to have profound effects on intermediary metabolism during osmotic stress. Mitochondrial oxidation of several osmotically important amino acids is enhanced by osmotic swelling; these include proline (Ballantyne & Storey, 1983, 1985), glutamate (Ballantyne & Moon, 1986), sarcosine (Moyes *et al.* 1986; Ballantyne, Moyes & Moon, 1986) and glycine (Ellis, Burcham, Paynter & Bishop, 1985; Moyes *et al.* 1986). These observations have led to the suggestion that osmotic effects on mitochondrial oxidation may be partly responsible for adjustment of organic osmolyte levels during osmotic stress.

Although it has been repeatedly shown that mitochondrial oxidation of osmotically active amino acids may be sensitive to assay medium osmolarity or, rather, tonicity, the biochemical mechanism by which these changes occur is not known. Osmotic effects on mitochondrial oxidation have been attributed to effects on substrate transport (Atsmon & Davis, 1967; Ballantyne & Storey, 1983; Moyes *et al.* 1986), oxidative phosphorylation (Campbell, Raison & Brady, 1975), and catabolic enzymes (Joseph, McGivan & Meijer, 1981; Ellis *et al.* 1985; Moyes *et al.* 1986). It has been suggested that the effector for this response is related to changes in the transmembrane pH gradient, matrix ionic strength, matrix concentrations of calcium or magnesium, as well as changes in membrane conformation occurring directly through stretching or indirectly through dilution of matrix magnesium (Joseph *et al.* 1981; Moyes *et al.* 1986). In the present study, we examine the nature of the effector responsible for the osmotic sensitivity of glutamate oxidation in mitochondria isolated from the gill of the American oyster, *Crassostrea virginica*. In another study (J. S. Ballantyne & C. D. Moyes, in preparation) we have shown that an optimal matrix milieu rather than an optimal mitochondrial volume is required for the maximal rates of glutamate oxidation. Glutamate oxidation is stimulated 2- to 3-fold by hypo-osmotic incubation of mitochondria from animals acclimated to full-strength sea water. A similar high rate of glutamate oxidation is observed when mitochondria from animals acclimated to dilute sea water are incubated in isosmotic media, where no swelling would be expected to occur. A high rate of glutamate oxidation in the

absence of swelling suggests that the rate is not dependent on mitochondrial volume *per se* but rather is dependent on some component of matrix milieu, which is affected by mitochondrial volume and acclimation osmolarity. Both osmotic swelling and acclimation to dilute sea water would be expected to cause similar changes in matrix divalent cation concentrations or ionic strength. Osmotically induced changes in matrix Mg^{2+} concentration may affect enzyme (Joseph *et al.* 1981) and transporter (Garlid, 1980; Kovacevic, Bajin & Pavlovic, 1980) activity in intact mitochondria. Effects of ionic strength on enzyme activity *in vitro* are also well-documented (Gilles, 1974; Sarkissian & Boatwright, 1974; Male & Storey, 1983; Batrel & Le Gal, 1984). We investigated the role of matrix divalent cation concentration and ionic strength in the osmotic sensitivity of glutamate oxidation in oyster gill mitochondria.

MATERIALS AND METHODS

Animals

Oysters (*Crassostrea virginica*), purchased from a local seafood supplier, were held in sea water at 10°C for 7–30 days in the Marine Biology Laboratory at the University of Guelph.

Mitochondrial isolation

Gill tissue from 3–6 animals was homogenized with three passes of a loosely fitting Potter–Elvehjem tissue grinder. The isolation medium consisted of 300 mmol l^{-1} sucrose, 50 mmol l^{-1} KCl, 50 mmol l^{-1} NaCl, 8 mmol l^{-1} EGTA, 1 % BSA (bovine serum albumin, essentially fatty-acid-free) in 30 mmol l^{-1} Hepes (pH 7·5 at 20°C). The homogenate was centrifuged for 10 min at 200 ***g***. The supernatant was centrifuged at 6560 ***g*** for 10 min. The mitochondrial pellet was rinsed twice then resuspended in isolation medium with only BSA omitted. BSA is known to bind several of the inhibitors used in the present study. The final mitochondrial protein concentration was 10–25 mg ml^{-1}.

Mitochondrial oxidation

One volume of mitochondrial suspension was added to nine volumes of reaction medium containing 150 mmol l^{-1} KCl, 150 mmol l^{-1} NaCl, 10 mmol l^{-1} KH_2PO_4 and a variable amount of sucrose in 30 mmol l^{-1} Hepes (pH 7·2 at 20°C). The standard low-osmolarity reaction medium contained no added sucrose (630 mosmol l^{-1}): the standard high-osmolarity reaction medium contained 450 mmol l^{-1} sucrose (1080 mosmol l^{-1}). The temperature of the mixture was held constant at 10°C. Oxygen consumption was measured using a Clark-type electrode. The rate of oxygen consumption in the absence of glutamate (+0·5 mmol l^{-1} ADP) was subtracted from the rate in the presence of glutamate (10 mmol l^{-1}) and ADP (0·5 mmol l^{-1}) to obtain the rate of glutamate oxidation. Respiratory control ratios for mitochondria isolated by this method are approximately 5. Permeability to NADH (2 mmol l^{-1}) is low under all experimental conditions (less than 2 % of the state 3 rate with glutamate as a substrate).

Effects of A23187, magnesium and CCCP

Mitochondria were given small volumes (<5 μl) of A23187 in absolute ethanol, 2 min after addition of ADP (0·5 mmol l^{-1}). After a 6-min incubation with the ionophore and ADP, mitochondria were given 10 mmol l^{-1} glutamate to determine the state 3 rate. Glutamate oxidation in the presence of the ionophore is expressed as a percentage of the rate in the absence of A23187, after subtracting the rate of oxidation of endogenous substrates (i.e. before the addition of glutamate). A concentration of 10 μg ml^{-1} A23187 was employed for subsequent studies to ensure a maximal effect under all experimental conditions. The effects of $MgCl_2$ on A23187 inhibition were examined as above with variable concentrations of $MgCl_2$ added immediately after addition of ADP, 2 min prior to the addition of A23187.

The effects of uncoupler on respiratory inhibition by A23187 and its alleviation by magnesium were also examined. CCCP (carbonyl cyanide-*m*-chlorophenylhydrazone), used at low concentrations, stimulates mitochondrial oxidation of glutamate but as the uncoupler concentration increases a reduction in oxidation rate is observed. The concentration of CCCP required to achieve the maximal rate was determined for each preparation of mitochondria (generally 6–10 μmol l^{-1}). The mitochondria were given only glutamate, followed by uncoupler added in small volumes until the maximal rate was achieved. In a separate assay on the same preparation, this concentration of CCCP was added as a preincubation at the same point at which ADP was added in parallel experiments. The effects of magnesium concentration on glutamate oxidation were determined in the presence and absence of CCCP. In each case, the rate prior to addition of glutamate was subtracted from the rate obtained after addition of glutamate (state 3 or uncoupled).

Effects of EDTA on mitochondrial volume and oxidation

Mitochondrial suspensions were given 4 mmol l^{-1} EDTA just prior to the addition of ADP. Glutamate was added after a 10-min preincubation with EDTA. EDTA was shown to stimulate oxidation rate at both osmolarities. Subsequent experiments were performed to determine if this effect was related to movement of monovalent cations. Mitochondria were isolated by the standard procedure, using isolation medium containing 50 mmol l^{-1} KCl and 50 mmol l^{-1} NaCl. The mitochondrial pellet was rinsed twice and resuspended in isolation medium with NaCl and KCl removed and the sucrose concentration elevated from 300 to 400 mmol l^{-1} to replace the chloride contribution to tonicity. Mitochondria were incubated in reaction media with KCl and NaCl removed, KH_2PO_4 replaced by $NH_4H_2PO_4$, and sucrose elevated and varied to alter osmolarity.

Changes in mitochondrial volume were monitored by measuring absorbance at 540 nm on a Varian DMS100 spectrophotometer. A reduction in absorbance of a mitochondrial suspension under these conditions indicates mitochondrial swelling. Mitochondrial suspensions were prepared as described for the studies on mitochondrial oxidation, using the standard media (containing KCl and NaCl). Mitochondria

(0·3 ml) were added to 2·7 ml of reaction medium precooled to 10°C in a 3·5 ml glass cuvette, with constant stirring.

Effects of ionic strength on the electron transport system and glutamate dehydrogenase

The effect of assay medium ionic strength on the electron transport system was determined using mitochondria broken by a combination of osmotic swelling and sonication. The mitochondrial pellet was washed and resuspended in 30 mmol l^{-1} Hepes (pH 7·5 at 20°C), followed by five high-intensity bursts of 10 s duration of a Megason Ultrasonic disintegrator, with chilling on ice for 20 s between bursts. One volume of mitochondria was added to four volumes of medium containing 30 mmol l^{-1} Hepes (pH 7·2 at 20°C) and variable concentrations of KCl + NaCl (equimolar), KCl, NaCl, potassium glutamate, sodium acetate or glycine. Oxygen consumption was determined as in previous experiments. As the maximum rate of oxygen consumption of this preparation was only about 50 % of the maximum rate achieved using intact mitochondria, it is likely that some disruption of the electron transport system occurred during preparation. Longer or shorter periods of sonication did not improve the rate of oxygen consumption of this preparation.

Glutamate dehydrogenase (GDH) activity was determined in the glutamate oxidizing direction at pH 8·6 and 10°C. The assay mixture contained 2 mmol l^{-1} NAD^+, 1 mmol l^{-1} ADP and was started with 40 mmol l^{-1} glutamate. These conditions have been shown to be optimal for GDH from other bivalve tissues (Moyes, Moon & Ballantyne, 1985). Mitochondria were prepared as before and resuspended in 30 mmol l^{-1} Hepes (pH 8·6 at 20°C). They were broken with a 10 s burst at high speed of a Brinkman Polytron. Membrane fragments were separated from the soluble fraction by two centrifugations of 1 min duration (15 000 ***g***), separated by 2 min of chilling on ice. Ionic strength was varied by changing KCl and NaCl concentrations (equimolar). Absorbance at 340 nm was monitored with a Varian DMS100 spectrophotometer.

Protein determination and biochemicals

Mitochondrial protein was determined by the biuret reaction (Gornall, Bardawill & David, 1949) using 10 % deoxycholate to solubilize the mitochondrial protein. All biochemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

RESULTS

Fig. 1 shows the effects of A23187 concentration on glutamate oxidation in the absence of magnesium. The ionophore had little effect on glutamate oxidation at low concentrations ($<0{\cdot}035\ \mu g\ ml^{-1}$) at either low or high osmolarity. Maximal inhibition occurred over the concentration range of $0{\cdot}1–10\ \mu g\ ml^{-1}$ A23187 at both osmolarities. A slightly greater inhibition was achieved at the high osmolarity (70 % inhibition at the high osmolarity *vs* 60 % at the low osmolarity). The effects of A23187 on the rate of glutamate oxidation are presented in Fig. 2. In the absence of

A23187, the rate of glutamate oxidation in the high-osmolarity medium was about 45 % that in the low-osmolarity medium. Addition of A23187 ($10\,\mu g\,ml^{-1}$) caused a marked decrease in the rate of glutamate oxidation at both osmolarities, but the rate of glutamate oxidation in the high-osmolarity medium remained at about 45 % of the

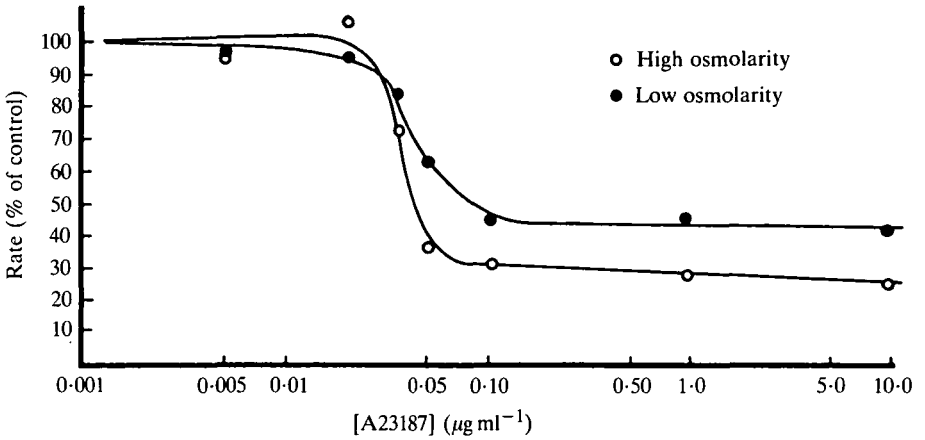

Fig. 1. Glutamate oxidation by oyster gill mitochondria in the presence of variable concentrations of A23187. Mitochondrial suspensions were preincubated for 6 min with A23187 ($10\,\mu g\,ml^{-1}$) before the addition of glutamate ($10\,mmol\,l^{-1}$). The rates are expressed as a percentage of the control rate determined in the absence of ionophore. Rates, each determined in the presence of ADP, are corrected for the oxidation of endogenous substrates.

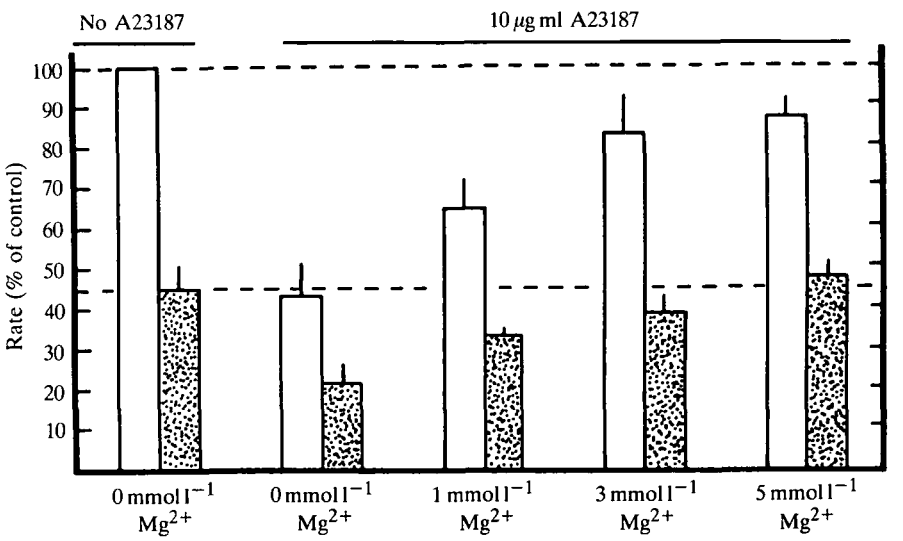

Fig. 2. The effect of magnesium on A23187-induced inhibition of mitochondrial glutamate oxidation. The rates are expressed as a percentage of the rate determined in the absence of A23187. Shaded bars represent the rates determined using the high-osmolarity medium. The magnesium concentrations reported refer to the concentration of $MgCl_2$ added to the cuvette. Rates are expressed as a percentage of the rate at low osmolarity in the absence of A23187 (mean ± S.E.) for five determinations.

Table 1. *The effects of exogenous magnesium on the rate of glutamate oxidation at two osmolarities in the absence of A23187*

	$\frac{\text{Rate} + MgCl_2}{\text{Rate with no } MgCl_2}$
Low osmolarity	0·91 (0·04)
High osmolarity	0·81 (0·03)

Mean (S.E.) for five determinations.
$MgCl_2$ was given as a preincubation at 5 mmol l^{-1}.

rate in the low-osmolarity medium. Inhibition by the ionophore could be reduced in a concentration-dependent manner by preincubation with $MgCl_2$. The Mg^{2+} levels reported in Fig. 2 refer to the concentration of $MgCl_2$ added to the cuvette. No attempt was made to determine free Mg^{2+} concentrations. Maximal alleviation of inhibition was achieved when 3–5 mmol l^{-1} $MgCl_2$ was added to the cuvette. The rate of glutamate oxidation in medium of either osmolarity in the presence of the ionophore and 3 or 5 mmol l^{-1} $MgCl_2$ was not significantly different from the rate in the absence of the ionophore ($P > 0{\cdot}05$, Student's t-test). In a separate experiment, preincubation with 5 mmol l^{-1} $MgCl_2$ was shown to inhibit glutamate oxidation slightly at both the low (approximately 9 % inhibition) and high (approximately 19 % inhibition) osmolarity (Table 1).

Table 2 summarizes experiments performed to determine if the apparent Mg^{2+} requirement in mitochondria treated with A23187 was associated with a requirement for oxidative phosphorylation. Relatively little relief of inhibition of oxidation was observed when mitochondria were uncoupled by the addition of the appropriate amount of CCCP.

The effects of EDTA on glutamate oxidation are summarized in Fig. 3A. EDTA (4 mmol l^{-1}) stimulated glutamate oxidation at both osmolarities. At the low osmolarity, EDTA increased the rate of glutamate oxidation by approximately 35 %. At the high osmolarity, the rate of glutamate oxidation was doubled by EDTA. As a result of this differential stimulation, the effect of assay medium osmolarity on glutamate oxidation was removed in the presence of EDTA. Although EDTA binds calcium and magnesium with similar affinities, the addition of EGTA (a chelator with much greater affinity for calcium) had no effect on glutamate oxidation (data not

Table 2. *The effects of uncoupler on the apparent magnesium requirement for mitochondrial oxidation of glutamate in the presence of 10 μg ml^{-1} A23187*

	$\frac{\text{Rate with no } MgCl_2}{\text{Rate} + MgCl_2}$
CCCP	0·57 (0·05)
ADP	0·42 (0·04)

Mean (S.E.) for five determinations.
Assays were performed in the standard low-osmolarity medium.
$MgCl_2$ was given as a preincubation at 5 mmol l^{-1}.

shown), suggesting that magnesium is the critical divalent cation in the observed EDTA effect. A concentration of 0·8 mmol l^{-1} EGTA was present in all incubations as a result of its inclusion in the resuspension medium.

Fig. 3B demonstrates that potassium or sodium is apparently required to produce the response to EDTA. The KCl and NaCl in the reaction medium were replaced with sucrose, and osmolarity was varied with sucrose. While EDTA stimulates glutamate oxidation at each osmolarity, the effects are much less pronounced when NaCl and KCl are omitted from the media. Overall, the rates determined in the absence of NaCl and KCl are about 40 % lower than in the control assays (Fig. 3B), even though the tonicities were comparable. The activity of molluscan mitochondria is markedly improved by the presence of monovalent cations in the assay medium ($Na^+ > K^+ > choline^+$) (C. D. Moyes & J. S. Ballantyne, in preparation). EDTA (4 mmol l^{-1} as above) caused marked swelling of these mitochondria, as indicated by a decrease in absorbance at 540 nm (Fig. 4).

GDH was found to be relatively insensitive to changes in ionic strength (Fig. 5). No reduction in activity was noted at very low ionic strength: little effect of ionic strength was noted up to 85 mmol l^{-1} NaCl + 85 mmol l^{-1} KCl. At the highest ionic strength tested, GDH activity was 60 % of the maximum activity.

Ionic strength strongly affected the activity of the electron transport system (Fig. 5). The maximum rate of electron transport occurs at a low ionic strength (25 mmol l^{-1} NaCl + 25 mmol l^{-1} KCl). Below and above this ionic strength, the rate of electron transport is sharply reduced. Between the range of 85 mmol l^{-1}

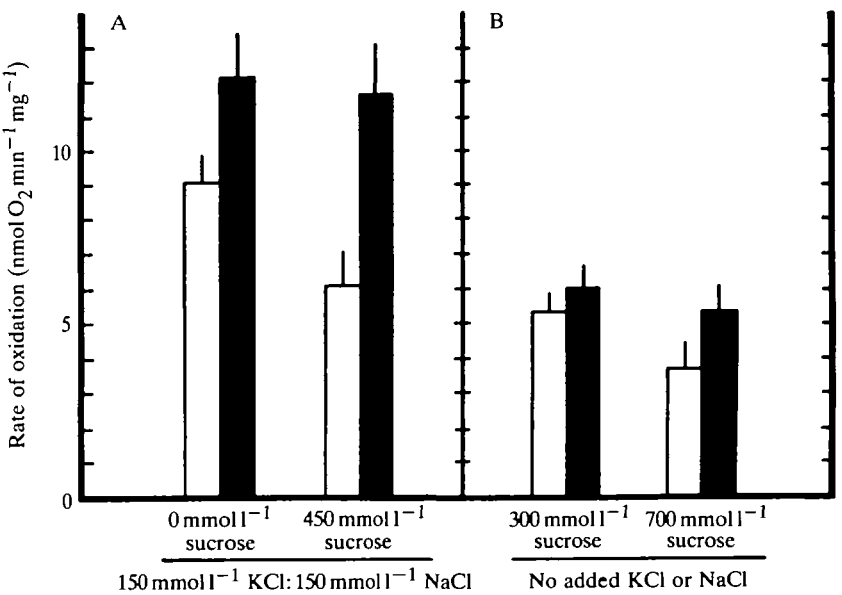

Fig. 3. Effects of EDTA on the oxidation of glutamate at two osmolarities in the presence (A) and absence (B) of added KCl and NaCl. Shaded bars represent the rates determined using mitochondrial preparations preincubated with 4 mmol l^{-1} EDTA. The rates (as mean ± S.E.) were determined on six (A) and five (B) preparations.

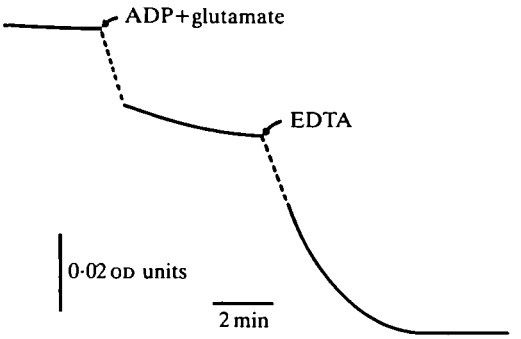

Fig. 4. The effects of EDTA on mitochondrial volume, using absorbance at 540 nm as an indicator of volume changes. Mitochondria were incubated in the high-osmolarity medium. Additions to the cuvette are indicated on the figure (0·5 mmol l^{-1} ADP, 10 mmol l^{-1} glutamate, 4 mmol l^{-1} EDTA). After additions to the cuvette, several seconds were required before the absorbance returned to the scale (broken lines). This experiment was repeated on four preparations, and a typical tracing is redrawn for this figure. Random fluctuations in absorbance (a maximum of 0·005 optical density units) have not been redrawn.

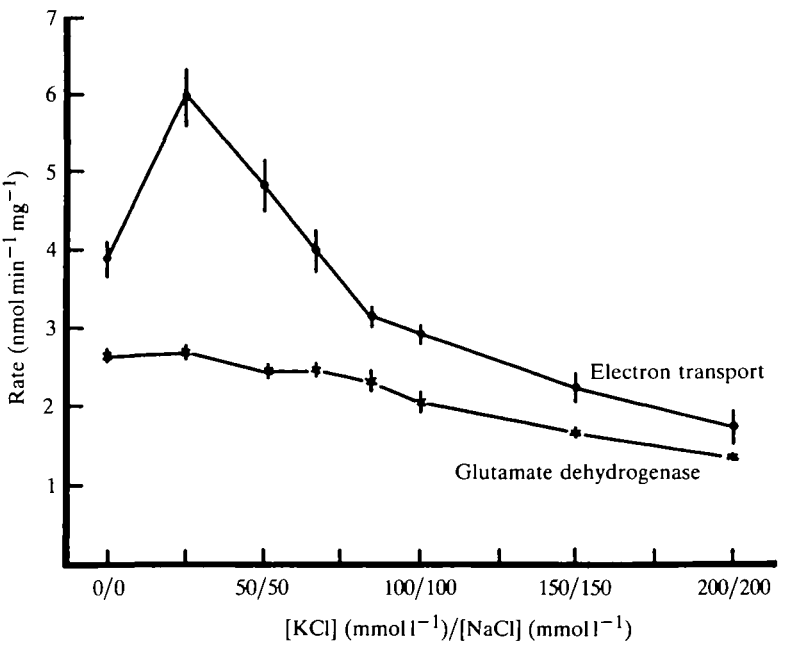

Fig. 5. The effects of variable ionic strength on the rate of electron transport (●) and glutamate dehydrogenase (×) activity. The rate of electron transport was determined by measuring oxygen consumption in response to the addition of NADH (0·2 mmol l^{-1}) using broken mitochondria. Mitochondria were osmotically swollen then sonicated as described in Materials and Methods. Glutamate dehydrogenase activity was determined in the deaminating direction as described in Materials and Methods, using mitochondria broken with the Brinkman Polytron. Rates (nmol substrate converted min^{-1} mg mitochondrial $protein^{-1}$) are expressed as mean ± S.E. for five determinations.

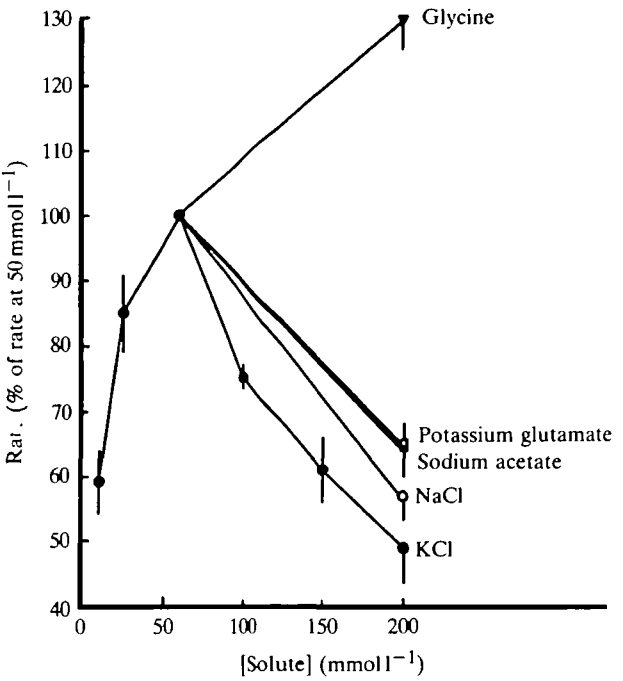

Fig. 6. The effects of several monovalent salts and glycine on the rate of electron transport. Mitochondria were prepared as described in Materials and Methods. Assay conditions are as described in the legend for Fig. 5. Rates are expressed as mean (± S.E.) rate of oxygen consumption relative to the rate using 50 mmol l^{-1} salt, for three determinations.

NaCl + 85 mmol l^{-1} KCl and 200 mmol l^{-1} NaCl + 200 mmol l^{-1} KCl, less of an effect of ionic strength is observed. As KCl and NaCl had similar effects on the rate of electron transport (Fig. 6), it is clear that neither Na^+ nor K^+ alone are responsible for the observed effects. Salts of organic anions (glutamate, acetate) had inhibitory effects similar to those of chloride salts (Fig. 6), supporting the possibility that changes in matrix ionic strength are responsible for the changes in the rate of electron transport. Similar changes in concentration of glycine had no inhibitory effect, and in fact markedly stimulated the rate of electron transport.

DISCUSSION

The role of divalent cations

Osmotically induced changes in matrix divalent cation concentration have been implicated as the effector for changes in enzyme activity and the rate of transport of metabolites and ions in mitochondria (Garlid, 1980; Kovacevic *et al.* 1980; Joseph *et al.* 1981). If a similar mechanism operates in oyster gill mitochondria, then a direct reduction of matrix divalent cation concentration should mimic the effects of hypo-osmotic incubation. Joseph *et al.* (1981) found that addition of A23187 stimulated mitochondrial glutaminase activity and addition of extramitochondrial magnesium

reversed the stimulation by A23187. In the present study, A23187 markedly reduced glutamate oxidation, but did not affect the dependence on assay medium tonicity (Fig. 2), suggesting that neither Ca^{2+} nor Mg^{2+} are involved in controlling the rate of glutamate oxidation at different tonicities. Addition of magnesium to magnesium-depleted mitochondria stimulated glutamate oxidation, but at each $MgCl_2$ concentration the dependence on osmolarity remained. Maximal stimulation occurred with 3–5 mmol l^{-1} $MgCl_2$, which may reflect mitochondrial magnesium levels in this species. This concentration is slightly higher than concentrations found in rat liver mitochondria (Williamson, Corkey & Murphy, 1979). The apparent magnesium requirement is not related to the Mg^{2+} requirement for the Mg-ADP chelate, the substrate for the F_1ATPase (Mitchell, 1974), as similar effects of A23187 and magnesium were produced in the presence of CCCP (Table 2), an uncoupler of oxidative phosphorylation. As both CCCP and A23187 dissipate the mitochondrial pH gradient (Skulachev, 1971; Pressman, 1976), it is clear that osmotic effects on the pH gradient are not responsible for the effects on glutamate oxidation, *via* glutamate/OH^- exchange. The magnesium-dependent step in glutamate oxidation by oyster gill mitochondria is not known.

Given the inhibition of glutamate oxidation in the presence of A23187 (Fig. 2), it is surprising that EDTA markedly stimulated oxidation at both osmolarities tested and removed the osmotic sensitivity (Fig. 3A). As EDTA affects glutamate oxidation in the same manner as hypo-osmotic incubation, this could be taken as evidence that osmotically induced changes in mitochondrial magnesium concentration are responsible for the osmotic sensitivity of glutamate oxidation. In their study on the role of magnesium in the osmotic sensitivity of glutamine oxidation, Joseph *et al.* (1981) found that EDTA and A23187 had similar stimulatory, but not markedly additive, effects. The marked differences in the effects of EDTA and A23187 in the present study appear to be due to indirect effects of EDTA on mitochondrial volume (Fig. 4). That is, EDTA directly causes swelling in the present study whereas in the study by Joseph *et al.* (1981) EDTA produced changes which mimicked the effects of swelling.

As the swelling response to EDTA is much reduced if potassium and sodium are replaced with sucrose (Fig. 3A,B), it is possible that EDTA causes swelling by activation of cation transport into the mitochondria. Hormonally induced mitochondrial volume changes appear to be mediated by changes in matrix Ca^{2+} concentration (Halestrap *et al.* 1985). Both Mg^{2+} and Ca^{2+} appear to control mitochondrial volume by altering the steady-state levels of matrix K^+. An increase in matrix Ca^{2+} concentration stimulates swelling by increasing the rate of influx of K^+ on its electrogenic uniport (Halestrap, Quinlan, Whipps & Armston, 1986). A decrease in Mg^{2+} concentration causes shrinking by stimulation of K^+ efflux on the K^+/H^+ exchanger (Garlid, 1980; Brierley, Jurkowitz, Farooqui & Jung, 1984). In mammalian mitochondria, the K^+/H^+ exchanger is capable of transporting both K^+ and Na^+, but K^+ movements are generally considered to be the most important because of the high intracellular levels relative to Na^+ (Brierley, 1976). In oysters, the intracellular levels of K^+ and Na^+ are comparable (Bricteux-Grégoire, Duchâteau-

Bosson, Jeuniaux & Florkin, 1964): thus movements of Na^+ are potentially as important as movements of K^+. Garlid's 'carrier brake' hypothesis (Garlid, 1980) states that a reduction in the concentration of matrix Mg^{2+} occurring during mitochondrial swelling unmasks the K^+/H^+ exchanger. As this stimulates K^+ efflux and reverses osmotic swelling, it may be an important mechanism for control of mitochondrial volume in mammals. Jung & Brierley (1986) have questioned the physiological relevance of Garlids 'carrier brake' hypothesis on the basis that too large a depletion of matrix Mg^{2+} is required to produce effects on mitochondrial volume. The observations in the present study regarding the effects of EDTA differ from those of Garlid in the location of the critical Mg^{2+} as well as in the direction of cation movement. In the present study, the mitochondrial location of the critical Mg^{2+} does not appear to be in the matrix. EDTA can reduce the concentration of extramitochondrial Mg^{2+} and consequently also reduce the amount of Mg^{2+} bound to the outer side of the inner membrane. Although EDTA cannot enter mitochondria at near neutral pH (Wehrle, Jurkowitz, Scott & Brierley, 1976), it could reduce matrix Mg^{2+} concentrations if a mechanism for Mg^{2+} efflux exists in these mitochondria. However, as A23187, which would directly reduce matrix Mg^{2+} concentrations, did not stimulate oxidation as did EDTA, it is apparent that the critical Mg^{2+} involved in the EDTA effect is not in the matrix. A second difference is that removal of the critical Mg^{2+} in the present study causes swelling, presumably by stimulation of cation influx, but in mammals mitochondrial shrinkage is produced as a result of stimulation of cation efflux (Garlid, 1980). While the effect of EDTA on mitochondrial glutamate oxidation does not explain the *in vitro* osmotic sensitivity of glutamate oxidation, it does present a potentially important mechanism for Mg^{2+}-mediated regulation of osmolyte oxidation in the absence of osmotic stress. Reductions in cytosolic Mg^{2+} levels, induced indirectly by hypotonic conditions or actively by cell membrane Mg^{2+}-ATPase, could enhance mitochondrial swelling as a mechanism for further stimulating osmolyte oxidation *in vivo*.

The role of ionic strength

The activities of various enzymes involved in amino acid metabolism, including citrate synthase (Sarkissian & Boatwright, 1974) and glutamate dehydrogenase (Gilles, 1974; Male & Storey, 1983; Batrel & Le Gal, 1984), have been reported to be sensitive to the ionic strength of the assay medium. The role of GDH in amino acid metabolism of bivalve molluscs has been suggested to be minimal, because the enzyme activities are lower than in mammals (Reiss, Pierce & Bishop, 1977). Recently, we have shown that GDH activities *in vitro* are sufficient to account for all of the glutamate oxidation occurring in intact mitochondria (Moyes *et al.* 1985). It appears, however, that GDH from this species is not very sensitive to the ionic strength of the assay medium *in vitro* (Fig. 5) and thus could not be responsible for the osmotic effects on mitochondrial glutamate oxidation.

Osmotic effects on glutamate oxidation by this oyster gill mitochondrial preparation may be due to ionic strength effects on the rate of electron transport. The rate of glutamate oxidation in clam mantle mitochondria is apparently limited by the rate

of electron transport over a wide pH range (Moyes *et al.* 1985). A similar situation probably exists in oyster gill mitochondria, although the low *in vitro* rates make a direct comparison between the electron transport rate (Fig. 5) and the rate of glutamate oxidation (Fig. 3A) impossible. Typically, no metabolite is oxidized at greater rates than glutamate in these mitochondria (J. S. Ballantyne & C. D. Moyes, unpublished observations), supporting the possibility that the electron transport system is rate-limiting for glutamate oxidation in this tissue.

The release of matrix K^+ (and presumably Na^+) is slow under most conditions (Brierley, 1976) such that volume changes induced by anisotonic media would also produce changes in matrix ionic strength. Isolated mitochondria are also only slowly permeable to chloride ions (Weiner, 1975). The exact concentrations of these ions in oyster gill mitochondria are not known and would be expected to be markedly different from concentrations in rat mitochondria, because of differences in the intracellular milieu (particularly, higher Na^+ and Cl^- levels). However, it is clear from Figs 5 and 6 that, except at high matrix ion concentrations, the rate of electron transport would be highly dependent on matrix ionic strength. While this observation accounts for the *in vitro* osmotic sensitivity of glutamate oxidation, other osmotically active solutes are typically oxidized by intact mitochondria at lower rates than is glutamate. The effect of ionic strength on the electron transport system observed in the present study could not be responsible for the osmotic sensitivity of oxidation of other osmolytes observed *in vitro*. The osmotically sensitive component of the oxidative pathway probably differs for each metabolite or group of metabolites *in vitro*. However, *in vivo* the rate of mitochondrial oxidation may be limited by the maximum rate of electron transport, as mitochondria are constantly presented with high concentrations of oxidizable substrates, i.e. the intracellular amino acids. The role of the adenylate pool in regulation of mitochondrial oxidation in these tissues is not known. An overall increase in the oxidative capacity of mitochondria of osmoconformers may have important significance *in vivo*. During hypo-osmotic stress, an increase in the rate of oxidation of most of the organic osmolytes employed by osmoconforming bivalve molluscs would be required as part of the cell volume regulatory response (Strange & Crowe, 1979). The metabolic energy derived from their oxidation may be important for fuelling other components of the cell volume regulatory response such as ion pumps.

We would like to thank Ted White for his efficient operation of the Marine Biology Laboratory at the University of Guelph. We also wish to thank the anonymous referees for their helpful suggestions. This study was funded by an operating grant and University Research Fellowship to JSB from the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

ARMSTON, A. E., HALESTRAP, A. P. & SCOTT, R. D. (1982). The nature of the changes in liver mitochondrial function induced by glucagon treatment of rats. The effects of intramitochondrial volume, aging and benzyl alcohol. *Biochim. biophys. Acta* **681**, 429–439.

ATSMON, A. & DAVIS, R. P. (1967). Mitochondrial respiration under conditions of varying osmolarity. *Biochim. biophys. Acta* **131**, 221–233.

BALLANTYNE, J. S. & MOON, T. W. (1985). Hepatopancreas mitochondria from *Mytilus edulis*: substrate preferences and effects of pH and osmolarity. *Mar. Biol.* **87**, 239–244.

BALLANTYNE, J. S. & MOON, T. W. (1986). Solute effects on mitochondria from an elasmobranch (*Raja erinacea*) and a teleost (*Pseudopleuronectes americanus*). *J. exp. Zool.* **239**, 319–328.

BALLANTYNE, J. S., MOYES, C. D. & MOON, T. W. (1986). Osmolarity affects oxidation of sarcosine by isolated hepatocytes and mitochondria from a euryhaline elasmobranch. *J. exp. Zool.* **238**, 267–271.

BALLANTYNE, J. S. & STOREY, K. B. (1983). Mitochondria from the ventricle of the marine clam, *Mercenaria mercenaria*: substrate preferences and effects of pH and salt on proline oxidation. *Comp. Biochem. Physiol.* **76**B, 133–138.

BALLANTYNE, J. S. & STOREY, K. B. (1984). Mitochondria from the hepatopancreas of the marine clam, *Mercenaria mercenaria*: substrate preferences and salt and pH effects on the oxidation of palmitoyl-L-carnitine and succinate. *J. exp. Zool.* **230**, 165–174.

BALLANTYNE, J. S. & STOREY, K. B. (1985). Solute effects on mitochondrial respiration: the kinetics of proline oxidation by mitochondria from the ventricle of the marine clam, *Mercenaria mercenaria*. *Comp. Biochem. Physiol.* **81**B, 777–780.

BATREL, Y. & LE GAL, Y. (1984). Nitrogen metabolism in *Arenicola marina*: Characterization of a NAD-dependent glutamate dehydrogenase. *Comp. Biochem. Physiol.* **78**B, 119–124.

BRICTEUX-GRÉGOIRE, S., DUCHÂTEAU-BOSSON, G., JEUNIAUX, C. & FLORKIN, M. (1964). Constituents osmotiquement actifs des muscles adducteurs d'*Ostrea edulis* adaptée à l'eau de mer ou à l'eau saumâtre. *Archs int. Physiol. Biochim.* **72**, 267–275.

BRIERLEY, G. P. (1976). The uptake and extrusion of monovalent cations by isolated heart mitochondria. *Molec. cell. Biochem.* **10**, 41–61.

BRIERLEY, G. P., JURKOWITZ, M. S., FAROOQUI, T. & JUNG, D. W. (1984). K^+/H^+ antiport in heart mitochondria. *J. biol. Chem.* **259**, 14 672–14 678.

CAMPBELL, L. C., RAISON, J. K. & BRADY, C. J. (1975). Factors limiting mitochondrial respiration in media of high solute content. *J. Bioenerg.* **7**, 189–200.

ELLIS, L. L., BURCHAM, J. M., PAYNTER, K. T. & BISHOP, S. H. (1985). Amino acid metabolism in euryhaline bivalves; regulation of glycine accumulation in ribbed mussel gills. *J. exp. Zool.* **233**, 347–358.

GARLID, K. D. (1980). On the mechanism of regulation of the mitochondrial K^+/H^+ exchanger. *J. biol. Chem.* **255**, 11 273–11 279.

GILLES, R. (1974). Studies on the effect of NaCl on the activity of *Eriocheir sinensis* glutamate dehydrogenase. *Int. J. Biochem.* **5**, 623–628.

GORNALL, A. G., BARDAWILL, C. J. & DAVID, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *J. biol. Chem.* **177**, 751–766.

HALESTRAP, A. P., QUINLAN, P. T., ARMSTON, A. E. & WHIPPS, D. E. (1985). Mechanisms involved in hormone signal transduction across the mitochondrial membrane. *Biochem. Soc. Trans.* **13**, 659–663.

HALESTRAP, A. P., QUINLAN, P. T., WHIPPS, D. E. & ARMSTON, A. E. (1986). Regulation of the mitochondrial matrix volume *in vivo* and *in vitro*: the role of calcium. *Biochem. J.* **236**, 779–787.

JOSEPH, S. K., MCGIVAN, J. D. & MEIJER, A. J. (1981). The stimulation of glutamine hydrolysis in isolated liver mitochondria by Mg^{2+} depletion and hypo-osmotic incubation conditions. *Biochem. J.* **194**, 35–41.

JUNG, D. W. & BRIERLEY, G. P. (1986). Matrix magnesium and the permeability of heart mitochondria to potassium ion. *J. biol. Chem.* **261**, 6408–6415.

KOVACEVIC, Z., BAJIN, K. & PAVLOVIC, M. (1980). Volume changes of rat kidney mitochondria, transport of glutamine and its inhibition by mersalyl. *Int. J. Biochem.* **12**, 139–143.

MALE, K. B. & STOREY, K. B. (1983). Kinetic characterization of NADP-specific glutamate dehydrogenase from the sea anenome, *Anthopleura xanthogrammica*: control of amino acid biosynthesis during osmotic stress. *Comp. Biochem. Physiol.* **76**B, 823–829.

MITCHELL, P. (1974). A chemiosmotic molecular mechanism for proton-translocating adenosine triphosphatases. *FEBS Letts* **43**, 189–194.

MOYES, C. D., MOON, T. W. & BALLANTYNE, J. S. (1985). Glutamate catabolism in mitochondria from *Mya arenaria* mantle: effects of pH on the role of glutamate dehydrogenase. *J. exp. Zool.* **236**, 293–301.

MOYES, C. D., MOON, T. W. & BALLANTYNE, J. S. (1986). Osmotic effects on amino acid oxidation in skate liver mitochondria. *J. exp. Biol.* **125**, 181–195.

POLLOCK, A. S. & ARIEFF, A. I. (1980). Abnormalities of cell volume regulation and their functional consequences. *Am. J. Physiol.* **239**, F195–F205.

PRESSMAN, B. C. (1976). Biological applications of ionophores. *A. Rev. Biochem.* **45**, 501–530.

QUINLAN, P. T., THOMAS, A. P., ARMSTON, A. E. & HALESTRAP, A. P. (1983). Measurement of intramitochondrial volume in hepatocytes without cell disruption and its elevation by hormones and valinomycin. *Biochem. J.* **214**, 395–404.

REISS, P. M., PIERCE, S. K. & BISHOP, S. H. (1977). Glutamate dehydrogenase from tissues of the ribbed mussel *Modiolus demissus*: ADP activation and possible physiological significance. *J. exp. Zool.* **202**, 253–258.

SARKISSIAN, I. V. & BOATWRIGHT, D. T. (1974). Regulation by salt and Krebs cycle metabolites of citrate synthase from an osmoregulator, white shrimp, *Penaeus setiferus*, and from a non-osmoregulator, sea anenome, *Bunedosoma cavernata*. *Comp. Biochem. Physiol.* **49**B, 325–333.

SKULACHEV, V. P. (1971). Energy transformations in the respiratory chain. *Curr. Top. Bioenerg.* **4**, 127–190.

STRANGE, K. B. & CROWE, J. H. (1979). Acclimation to successive short term salinity changes by the bivalve *Modiolus demissus*. II. Nitrogen metabolism. *J. exp. Zool.* **210**, 227–235.

WEHRLE, J. P., JURKOWITZ, M., SCOTT, K. M. & BRIERLEY, G. P. (1976). Mg^{2+} and the permeability of heart mitochondria to monovalent cations. *Archs Biochem. Biophys.* **174**, 312–323.

WEINER, M. W. (1975). Mitochondrial permeability to chloride ion. *Am. J. Physiol.* **228**, 122–126.

WILLIAMSON, J. R., CORKEY, B. E. & MURPHY, E. (1979). Measurement of free Ca^{2+} and Mg^{2+} concentration in hepatocytes and mitochondria. *Biophys. J.* **25**, 46a.