Biochimica et Biophysica Acta, 345 (1974) 243–251
© Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

BBA 76617

PROPERTIES OF Ca^{2+}-ATPase FROM THE GILL OF RAINBOW TROUT (*SALMO GAIRDNERI*)

S. W. Y. MA, Y. SHAMI, H. H. MESSER and D. H. COPP

Department of Physiology, University of British Columbia, Vancouver, B.C., V6T 1W5, (Canada)

(Received December 7th, 1973)

SUMMARY

1. An ATPase, independent of Na^+ and K^+ and insensitive to ouabain, is present in plasma membranes of gill tissues from the rainbow trout, *Salmo gairdneri*.

2. This enzyme is preferentially activated by Ca^{2+}, with an apparent K_m of 0.33 mM. Mg^{2+} can substitute for Ca^{2+}, but with a V only 70% of that for Ca^{2+} and an apparent K_m of 0.50 mM. Ca^{2+} and Mg^{2+} were found to act at the same site.

3. ATP is the preferential substrate for the enzyme, though GTP, ITP and ADP can also be hydrolyzed to a small extent. AMP is not hydrolyzed.

4. The pH optimum for enzyme activation is 7.9–8.1.

5. A possible role for the enzyme in calcium transport across the gill is suggested.

INTRODUCTION

Although mechanisms of calcium regulation in teleost fish are still poorly understood, there is indirect evidence that the gill may be involved [1, 2]. The fish gill is known to play an important role in sodium transport between the animal's internal and external environments through an active transport mechanism involving Mg^{2+}-dependent, (Na^+–K^+)-ATPase [3]. We are investigating the possibility that the gill is similarly involved in calcium transport. Ca^{2+}-ATPases have been described in several vertebrate tissues where active transport of calcium occurs [4–8]. In this paper, we report the existence of a Ca^{2+}-activated ATPase located in the plasma membranes isolated from gill tissue of the rainbow trout, *Salmo gairdneri*.

MATERIALS AND METHODS

Rainbow trout (*S. gairdneri*) weighing 200–300 g were acclimated at 12.5± 1.0 °C for 4 weeks before sacrifice by a blow on the head. Gill filaments were freed from the gill arches and rinsed in ice-cold 0.9% saline. The isolated gill tissue was then homogenised in 30 ml of a cold (4 °C) solution containing 87 g sucrose, 1 g NaCl, 1.86 g EDTA (disodium salt), 0.2 g $MgCl_2 \cdot 6H_2O$ and 0.68 g imidazole per l,

using a Tri-R tissue grinder with Teflon pestle. Plasma membranes were isolated by a method similar to that described by Post and Sen [9] for the isolation of kidney cortical plasma membranes, except that the urea stage was omitted. The final membrane preparation was suspended in 0.5 mM imidazole, 5 mM Tris buffer (pH 7.6) and stored at −20 °C until use.

To assay for ATPase activity, the reaction mixture contained, in a final volume of 1.2 ml, 0.1 ml of enzyme suspension (20–50 μg protein), 20 mM Tris–HCl buffer (pH 8.0), 70 mM Na^+ (as NaCl), and divalent cations (Ca^{2+}, Mg^{2+}) and ATP (disodium salt) at different concentrations as indicated. Divalent cations were omitted in blank determinations. Incubations were carried out in a water-bath at 12.5±1.0 °C for 1 h during which time the reaction was linear. The reaction was then stopped by quickly placing the tubes in an ice-bath and adding 1 ml 10% (w/v) trichloroacetic acid. ATP hydrolysis was measured by the release of inorganic phosphate as determined by the automated Gomori method [10]. Total protein was determined by the method of Lowry et al. [11]. ATPase activity was expressed as μmoles P_i released per mg protein per h.

Purity of the membrane preparations was checked by electron microscopy. The final enzyme preparation was further tested for mitochondrial contamination by using succinate dehydrogenase as a marker for mitochondria [12] and alkaline phosphatase as a marker for plasma membranes [13]. As described by Post and Sen [9], the pellet following centrifugation at 35000 × g contains an upper layer consisting predominantly of plasma membranes and a lower layer containing mitochondria and heavy plasma membrane fragments. At the end of each high-speed centrifugation, the upper membrane layer was scraped free from the remainder of the pellet and resuspended in buffer for the next centrifugation. The lower layer from all purifying steps constituted the "pooled residue". Both the final membrane preparation and the pooled residue were assayed for enzyme activities.

RESULTS

Purity of membrane preparation

Electron microscopy showed that the final preparation consisted mostly of plasma membranes. Contamination of the final membrane preparation by mitochondria, as indicated by succinate dehydrogenase activity, was very low (Table I). Ca^{2+}-ATPase activity followed a distribution similar to that of alkaline phosphatase. The Ca^{2+}-ATPase activity in the pooled residue probably resulted from incomplete recovery of the heavier plasma membrane fragments.

Activation of the enzyme by divalent cations

Ca^{2+} or Mg^{2+} alone activated the enzyme. Maximum enzyme activity occurred at a divalent cation concentration of 5 mM (Fig. 1). The apparent K_m for Ca^{2+} was 0.33±0.01 mM (mean ±S.E., n = 10) and for Mg^{2+} was 0.50±0.02 mM (mean±S.E., n = 7) (Fig. 2). Maximum activity with Mg^{2+} alone was only 70% that with Ca^{2+} alone. Equimolar concentrations of both Mg^{2+} and Ca^{2+} in the incubation medium produced a V intermediate between those for Ca^{2+} and Mg^{2+} alone (Fig. 1). With Ca^{2+} and ATP in equimolar concentrations, the apparent K_m was found to be 0.18 mM (Fig. 2).

TABLE I

PURITY OF MEMBRANE PREPARATIONS

Succinate dehydrogenase was used as a marker for mitochondria and alkaline phosphatase as a marker for plasma membranes. The pooled residue consisted of the remaining pellet after the upper membrane layer had been scraped off. It includes mitochondria and heavy plasma membrane fragments (see Materials and Methods for details).

Fraction	Ca^{2+}-ATPase (μmoles P_i per mg protein per h)	Alkaline phosphatase*	Succinate dehydrogenase**
Final membrane fraction	13.9	0.93	0.086
Pooled residue	8.4	0.50	0.728
Final membrane fraction / Pooled residue	1.66	1.86	0.12

* Activity in μmole p-nitrophenol per mg protein per h.
** Activity in $A_{490\ nm}$ units per mg protein per h.

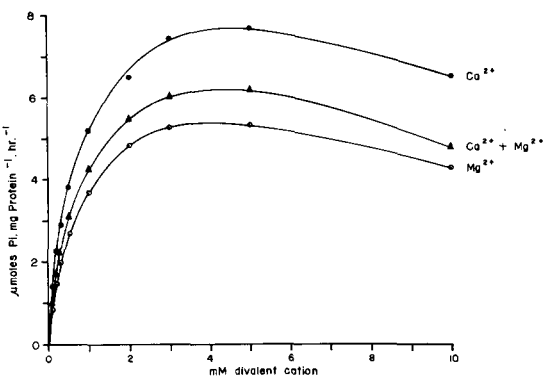

Fig. 1. Activation of ATP hydrolysis by increasing concentrations of divalent cations. ●–●, activation by Ca^{2+}; ○–○, activation by Mg^{2+}; ▲–▲, activation by Ca^{2+} plus Mg^{2+} (in equimolar concentrations). Incubation mixtures contained 20 mM Tris–HCl (pH 8.0), 70 mM Na^+ and 5 mM ATP (disodium salt).

Maximum activity at 5 mM Ca^{2+} for 10 different fresh gill membrane preparations ranged from 8 to 14 μmoles P_i released per mg protein per h.

Activation of the enzyme by Ca^{2+} or Mg^{2+} did not require the presence of Na^+ or K^+. Incubation without Na^+ and using 5 mM Tris–ATP resulted in a slightly higher activation than that with 70 mM Na^+ and 5 mM ATP (disodium salt).

Effect of ATP concentration and substrate specificity

The effect of ATP concentration on enzyme activity was examined by incubating the enzyme preparation with 5 mM Ca^{2+}, 20 mM Tris–HCl (pH 8.0), 70 mM Na^+ and various concentrations of ATP (disodium salt) (0.1–10 mM). Maximal ATP hydrolysis occurred at 5 mM ATP (Fig. 3) and the apparent K_m was 0.13 ± 0.01 mM (Fig. 2).

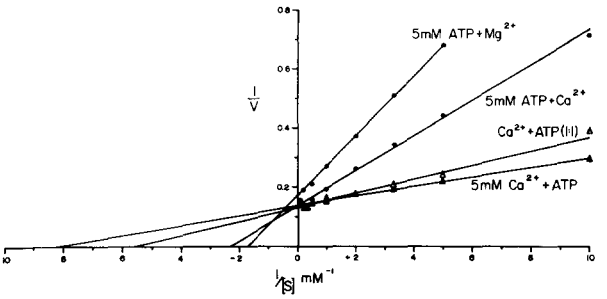

Fig. 2. Lineweaver–Burk plots of ATPase activity at various concentrations of Ca^{2+}, Mg^{2+} and ATP. V is expressed in μmoles P_i per mg protein per h, [S] in mM. ●–●, 5 mM ATP+increasing concentration of Ca^{2+}; ○–○, 5 mM ATP+increasing concentration of Mg^{2+}; △–△, increasing concentration of Ca^{2+} plus ATP (in equimolar concentrations); ▲–▲, 5 mM Ca^{2+}+increasing concentration of ATP. Incubation mixtures contained 20 mM Tris–HCl (pH 8.0) and 70 mM Na^+.

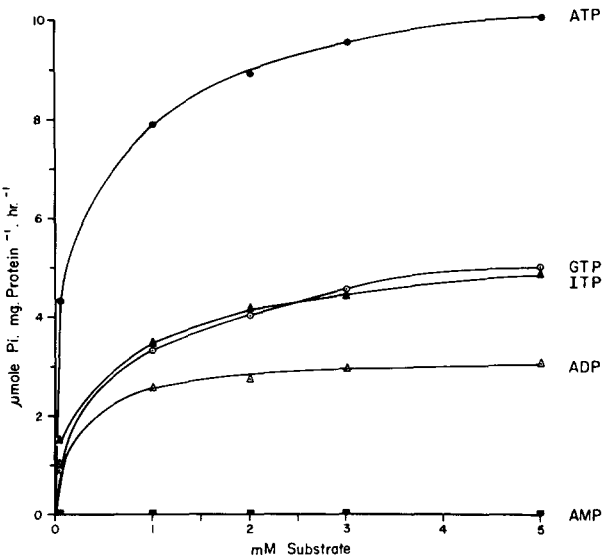

Fig. 3. Enzyme activity with different nucleotides as substrate. ●–●, ATP; ○–○, GTP, ▲–▲, ITP; △–△, ADP; ■–■, AMP. Incubation mixtures contained 5 mM Ca^{2+}, 20 mM Tris–HCl (pH 8.0) and 70 mM Na^+.

The substrate specificity of the Ca^{2+}-activated enzyme was tested, using sodium salts of GTP, ITP, ADP and AMP (Fig. 3). The V with GTP and ITP as substrates was approximately 50% and with ADP was only 30% that obtained with ATP. P_i production with AMP as substrate was undetectable.

Effect of pH on Ca^{2+}-ATPase activity

The effect of pH on enzyme activity was determined in the range pH 4.1–10.9. The pH optimum was between 7.8 and 8.1 (Fig. 4).

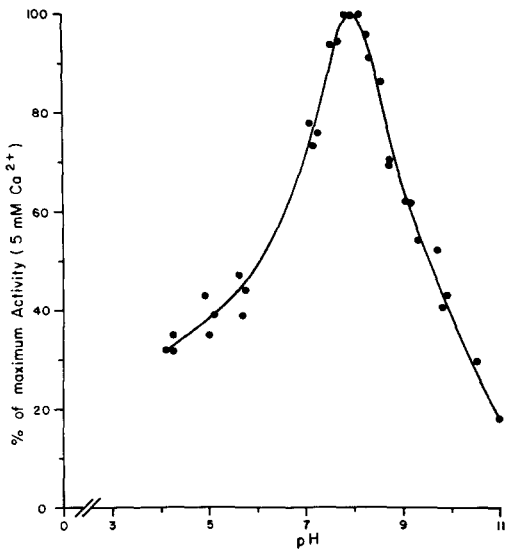

Fig. 4. Effect of pH on enzyme activity. Incubation mixtures contained 5 mM Ca^{2+}, 20 mM Tris–HCl, 70 mM Na^{+} and 5 mM ATP (disodium salt).

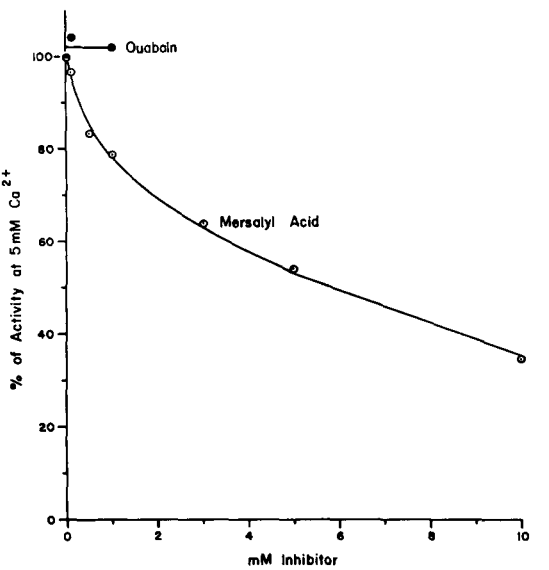

Fig. 5. Effect of inhibitors on enzyme activity. ●–●, effect of ouabain; ○–○, effect of mersalyl acid. Incubation media contained 5 mM Ca^{2+}, 20 mM Tris–HCl (pH 8.0), 70 mM Na^{+} and 5 mM ATP (disodium salt).

Effect of inhibitors

Fig. 5 shows the effect of two inhibitors on Ca^{2+}-ATPase activity, when the protein concentration in the final incubation mixture was 0.07 mg/ml. Ouabain at concentrations of 0.1 and 1.0 mM did not inhibit the enzyme. Mersalyl acid, when

added to the incubation mixture at concentrations between 0.1 and 10 mM, exhibited a progressive inhibitory effect on enzyme activity; 50% inhibition occurred at a concentration of 5.8 mM mersalyl acid.

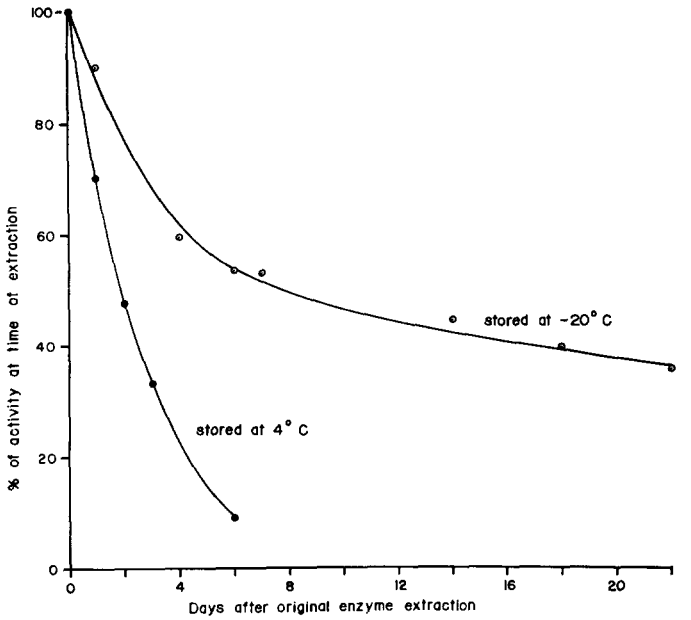

Fig. 6. Effect of storage on Ca^{2+}-ATPase activity. ●–●, enzyme preparation stored at +4 °C; ○–○, enzyme preparation stored at −20 °C. Incubation mixtures contained 5 mM Ca^{2+}, 20 mM Tris–HCl (pH 8.0), 70 mM Na^+ and 5 mM ATP (disodium salt).

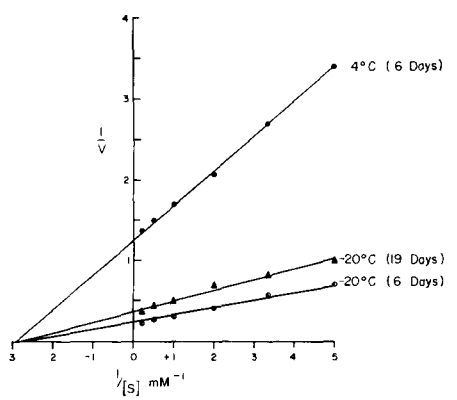

Fig. 7. Effect of storage on Lineweaver–Burk plots of enzyme activity at various Ca^{2+} concentrations. V is expressed in μmoles P_i per mg protein per h, [S] in mM. ●–●, enzyme preparation stored at +4 °C for 6 days; ○–○, stored at −20 °C for 6 days; ▲–▲, stored at −20 °C for 19 days. Incubation fluids contained 20 mM Tris–HCl (pH 8.0), 70 mM Na^+ and 5 mM ATP (disodium salt).

Effect of temperature on enzyme activity

Ca^{2+}-ATPase activity was determined in the range 0–80 °C. Enzymatic hydrolysis of ATP increased progressively up to 54 °C. Complete enzyme inactivation occurred at 70 °C. The Q_{10} (5–15 °C) calculated according to the method of Giese [14] was 2.3.

Stability of the enzyme

To determine the stability of Ca^{2+}-ATPase, aliquots of enzyme preparation were tested for activity after storage at 4 or −20 °C for up to 21 days. Enzyme activity declined progressively with increasing time of storage (Fig. 6). The loss of activity in samples stored at −20 °C was much less than that of samples stored at 4 °C. The apparent K_m for Ca^{2+} was not affected by storage conditions (Fig. 7). Therefore, frozen samples as well as fresh preparations were used in this study.

DISCUSSION

In the present investigation, a Ca^{2+}-activated ATPase has been demonstrated in the plasma membranes from the gills of rainbow trout. The enzyme can be stimulated by either Ca^{2+} or Mg^{2+} alone, but the V and affinity are higher for Ca^{2+} than for Mg^{2+}. This preferential activation of the enzyme by Ca^{2+} is similar to that described for placental Ca^{2+}-ATPase [8]. However, such Ca^{2+} preference is not observed in other Ca^{2+}-, Mg^{2+}-ATPases which vary considerably in their divalent cation requirements [4–7, 15].

Shami and Radde [16] have derived an equation for determining whether two ions activate the same site while the enzyme is saturated. The V and K_m for Ca^{2+} and Mg^{2+} obtained from this study were introduced into the equation. The maximum velocity observed for Ca^{2+} plus Mg^{2+} (1 : 1) was found to deviate from the calculated value by only 2%, indicating that Ca^{2+} and Mg^{2+} activate the gill ATPase at the same site.

ATP is the preferred substrate for the enzyme. Although other nucleotide triphosphates (GTP and ITP) and ADP may also be utilized, the degree of hydrolysis is much lower than that of ATP; AMP cannot serve as substrate. Unlike the Ca^{2+}-ATPase of the red blood cell membrane [5] which does not hydrolyse other triphosphates to any appreciable extent, this low degree of substrate specificity is common to many other Ca^{2+}-ATPases [8, 15, 17]. However, in all cases, ATP is the preferential substrate.

The apparent K_m for equimolar Ca^{2+}+ATP was greater than that for ATP when Ca^{2+} was held constant (at 5 mM) and smaller than that for Ca^{2+} when ATP was held constant (at 5 mM). Thus, at substrate concentrations below saturation, excess Ca^{2+} (i.e. greater than equimolar) enhanced enzyme activity, while excess ATP inhibited it. Ca^{2+}-ATP is believed to be the actual substrate for the enzyme [6, 18]. If this is so, then for low substrate concentrations, excess Ca^{2+} may be necessary for complete formation of the Ca^{2+}-ATPcomplex.

The optimum pH for the gill Ca^{2+}-ATPase (8.0) lies within the range reported for a majority of the Ca^{2+}-ATPases (pH 7.5–8.2) [6–8, 17]. The narrow bell-shaped curve with a sharp pH optimum suggests that, under the assay conditions employed, it is unlikely that more than one enzyme is contributing significantly to ATP hydrolysis.

Gill Ca^{2+}-ATPase activity is inhibited by mersalyl acid, indicating that free-SH groups are essential for enzyme activity [19]. Hasselbach and Seraydarian [19] have demonstrated that both calcium transport and Ca^{2+}-activated ATPase activity are abolished when sarcoplasmic reticulum membranes are incubated in the presence of mersalyl acid. Similar results are reported in human red cells where both the Ca^{2+}-ATPase and the calcium pump are inhibited by mersalyl acid [4].

The inability of ouabain to inhibit enzyme activity and the lack of requirement for Na^+ and K^+ distinguish this ATPase from the reported (Na^+–K^+)-ATPase in trout gill membranes [20].

Mg^{2+}-dependent, (Na^+–K^+)-ATPase has been studied in gill tissue preparations of a large variety of teleosts [20–24]. In all cases, a high "baseline" Mg^{2+}-ATPase activity, insensitive to ouabain, was observed. On the basis of the present findings, we suggest that this Mg^{2+}-stimulated ATP hydrolysis may in fact be due to activation of the gill Ca^{2+}-ATPase which is also stimulated by Mg^{2+}. Since (Na^{2+}–K^+)-ATPase in those studies [20–24] was assayed in the presence of 5 mM Mg^{2+}, it is very likely that the Ca^{2+}-ATPase system was also activated.

In teleost fish, three sites are considered capable of ion exchange between the animal's internal and external environments, namely the gill, the skin and the gut. The gill, being the respiratory organ where blood comes into intimate contact with the external environment, is likely to be a major site for ion transport. One of the proposed mechanisms for active transport of calcium across cell membranes is via the action of Ca^{2+}(Mg^{2+})-ATPase [6]. The presence of a Ca^{2+}-ATPase in the gill plasma membrane, with properties similar to those of other Ca^{2+}-ATPases, is presumptive evidence for a role for the gill in Ca^{2+} transport in the teleost fish.

ACKNOWLEDGEMENTS

The authors thank Miss E. McGowan for her expert technical assistance and Dr W. K. Ovalle, Jr., Department of Anatomy, University of British Columbia, for the electron microscopic work.

REFERENCES

1 Chan, D. K. O., Chester Jones, I., Henderson, I. W. and Rankin, J. C. (1967) J. Endocrinol. 37, 297–317
2 Lopez, E. (1970) Z. Zellforsch. Mikrosk. Anat. 109, 566–572
3 Motais, R. and Garcia-Romeu, F. (1972) Annu. Rev. Physiol. 34, 141–176
4 Schatzmann, H. J. and Vincenzi, F. F. (1969) J. Physiol. 201, 369–395
5 Cha, Y. N., Shiu, B. C. and Lee, K. S. (1971) J. Gen. Physiol. 57, 202–215
6 Melancon, M. J. and DeLuca, H. F. (1970) Biochemistry 9, 1658–1664
7 Parkinson, D. K. and Radde, I. C. (1971) Biochim. Biophys. Acta 242, 238–246
8 Shami, Y. and Radde, I. C. (1971) Biochim. Biophys. Acta 249, 345–352
9 Post, R. L. and Sen, A. K. (1967) Methods Enzymol. 10, 762–768
10 Gomori, G. (1942) J. Lab. Clin. Med. 27, 955–960
11 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
12 Pennington, R. J. (1961) Biochem. J. 80, 649–654
13 Linhardt, K. and Walter, K. (1965) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 783–785, Academic Press, New York

14 Giese, A. C. (1968) Cell Physiology, 3rd edn, p. 248, B. Saunders Co., Philadelphia
15 Nakamaru, Y., Kosakai, M. and Konish, K. (1967) Arch. Biochem. Biophys. 120, 15–21
16 Shami, Y. and Radde, I. C. (1972) Biochim. Biophys. Acta 255, 665–679
17 MacLennan, D. H. (1970) J. Biol. Chem. 245, 4508–4518
18 Hyde, M. E. and Rimai, L. (1971) Biochemistry 10, 1121–1128
19 Hasselbach, W. and Seraydarian, K. (1966) Biochem. Z. 345, 159–172
20 Pfeiler, E. and Kirschner, L. B. (1972) Biochim. Biophys. Acta 282, 301–310
21 Kamiya, M. and Utida, S. (1968) Comp. Biochem. Physiol. 26, 675–685
22 Kamiya, M. and Utida, S. (1969) Comp. Biochem. Physiol. 31, 671–674
23 Kirschner, L. B. (1969) Comp. Biochem. Physiol. 29, 871–874
24 Zaugg, W. S. and McLain, L. R. (1971) Comp. Biochem. Physiol. 38B, 501–506