Effects of amiprophosmethyl on massive and limited calcium loading of maize mitochondria

Cornelia HERTEL,* Hubert AFFOLTER,† Dieter MARME‡ and Christa DIERKS-VENTLING*

*Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland, †University Clinics, Department of Research, Kantonsspital Basel, CH-4031 Basel, Switzerland, and ‡Universität Freiburg, Institut für Biologie III, D-78 Freiburg, Federal Republic of Germany

(Received 20 December 1982/Accepted 15 February 1983)

In maize mitochondria the effect of the herbicide amiprophosmethyl was studied on massive and limited Ca^{2+} -loading and on mitochondrial energy transduction. Under massive Ca^{2+} -loading conditions amiprophosmethyl inhibited the Ca^{2+} transport system directly without a significant effect on the respiratory chain, the membrane potential or Ca^{2+} efflux. Under limited Ca^{2+} -loading conditions an increased Ca^{2+} efflux was noted, which could be partially responsible for the inhibition of the net Ca^{2+} accumulation.

Ca^{2+} is known to regulate a variety of cellular processes, including certain enzyme activities and microtubule assembly–disassembly in eukaryotic cells (Carafoli & Crompton, 1978). Whereas for animals, the function of mitochondria as Ca^{2+} -buffering organelles is well established (Nicholls, 1978), for plants, such a function is still under discussion (Hodges & Hanson, 1965; Earnshaw *et al.*, 1973; Chen & Lehninger, 1973; Moore & Bonner, 1977; Day *et al.*, 1978; Dieter & Marmé, 1980).

Recently it has been shown that mitochondria from maize are able to accumulate large amounts of Ca^{2+} in the presence of oxidizable substrates (Dieter & Marmé, 1980). The accumulation is inhibited by compounds that interfere with mitochondrial energy transduction and by Ruthenium Red, a specific inhibitor of the Ca^{2+} carrier in animal mitochondria (Moore, 1971). In the above experiments with maize mitochondria, conditions were used that had previously been established as massive Ca^{2+} -loading in animal mitochondria (Drahota *et al.*, 1965). Massive Ca^{2+} -loading is defined as Ca^{2+} accumulation under conditions of extramitochondrial Ca^{2+} and phosphate concentrations in the millimolar range, which stimulates irreversibly mitochondrial O_2 consumption. Limited Ca^{2+} -loading, on the other hand, is defined as Ca^{2+} accumulation under conditions of extramitochondrial Ca^{2+} concentration in the micromolar range without added phosphate; here, O_2 consumption will be stimulated transiently.

Abbreviations used: APM, amiprophosmethyl; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; TPP^+ , tetraphenylphosphonium ion.

Fig. 1. Structure of amiprophosmethyl

Recently it was shown that the herbicide APM (Fig. 1) inhibited Ca^{2+} accumulation under conditions of massive Ca^{2+} -loading (Hertel *et al.*, 1980). In the present paper we study the mechanism of inhibition by APM of Ca^{2+} accumulation in plant mitochondria under both massive and limited Ca^{2+} -loading conditions. We show that plant mitochondria are able to accumulate Ca^{2+} even under limited loading conditions and that this process is also inhibited by APM. Furthermore we demonstrate that the action of APM is due neither to inhibition of mitochondrial energy transduction, i.e. reduced membrane potential, nor to an increased Ca^{2+} efflux. Our data suggest that APM inhibits directly the Ca^{2+} transport system. Since APM, in contrast with the inorganic highly positively charged Ruthenium Red, is an uncharged organic compound inhibiting Ca^{2+} uptake, it may be a useful tool for further investigations of the hypothetical Ca^{2+} carrier in plant mitochondria.

Experimental

Chemicals

Antimycin A and rotenone were obtained from Sigma (St. Louis, MO, U.S.A.), FCCP from Boehringer (Mannheim, Germany), the ionophores

A23187 and valinomycin from Calbiochem (Lucerne, Switzerland) and BSA (grade V) from Serva (Heidelberg, Germany). The radiochemicals [*carboxy*- ^{14}C]carboxylinulin (4.5×10^{13} Bq/mol), [*phenyl*- ^3H]tetraphenylphosphonium bromide (1.6×10^{14} Bq/mol), $^3\text{H}_2\text{O}$ (9.3×10^{14} Bq/ml), $^{45}\text{CaCl}_2$ (4.4×10^{13} Bq/mol) and Aquasol-2 scintillator were obtained from New England Nuclear (Dreieich, Germany). APM was a gift from Bayer (Leverkusen, Germany).

Preparation of maize mitochondria

For each experiment 500–1000 hybrid maize kernels (*Zea mays* L. cv. Inrakorn, from Nuncesser KG, Darmstadt, Germany) were germinated for 5.5 days on moist, absorbent cellulose sheets at 25°C in the dark. For the preparation of mitochondria, coleoptiles, including primary leaves, were placed in ice-cold extraction buffer (300 mM-sucrose/25 mM-4-morpholinepropanesulphonic acid, titrated with 25 mM-Tris to pH 7.5) at a concentration of 0.5 g/ml and coarsely cut with a razor blade. The chopped tissue was then ground with a mortar and pestle, filtered through a single layer of nylon cloth and the resulting homogenate was centrifuged at 1000 *g* for 15 min at 4°C to remove cell debris. The supernatant was centrifuged again at 6000 *g* for 15 min at 4°C. The resulting pellet containing the mitochondria was resuspended in extraction buffer at a final protein concentration of 3 mg/ml. An extensive characterization of the properties of this preparation procedure was published by Dieter & Marmé (1980).

Ca²⁺ transport assay

For this assay $^{45}\text{Ca}^{2+}$ (250 Bq/ml) was added to the incubation medium. The latter consisted of the extraction buffer with the following additions: (A) 5 mM-phosphate and 2 μmol of CaCl_2 /mg of mitochondrial protein or (B) 40 nmol of CaCl_2 /mg of mitochondrial protein, with or without 4 mM-succinate as energy source. The mitochondrial Ca^{2+} uptake assay was performed at 25°C and started by adding the mitochondria (50–100 μg /ml and 0.3 mg/ml for media A and B respectively) and stopped by filtration. A stock solution of APM dissolved in DMSO was diluted to give a range of 2–2000 nmol of APM per mg of mitochondrial protein as required by the experimental procedure. The final concentration of DMSO was 1%. Controls contained an equivalent volume of DMSO.

O₂ consumption

O₂ consumption was measured at 25°C with a Clark-type O₂-electrode (Bachofar, Reutlingen, Germany). The assay medium consisted of 2 ml of extraction buffer supplemented with 4 mM-phosphate, 10 mM-succinate, 2 mg of BSA and 0.5 mg of mitochondrial protein.

ADP (200 nmol) was added to determine the respiratory control index and the ADP/O ratio. The respiratory control index is the ratio of O₂ consumed in the presence of ADP (State-3 respiration) to that obtained after ADP is exhausted (State-4 respiration). The ADP/O ratio was calculated assuming that during ADP phosphorylation all other energy-consuming processes are negligible (Chance & Williams, 1956). Only mitochondria with respiratory control indices of at least 1.8 and ADP/O ratios of at least 1.4, i.e. well coupled according to the criteria of Chen & Lehninger (1973), were used. The effect of APM on State-4 respiration was determined before addition of ADP and compared with the stimulation of O₂ consumption obtained after addition of ADP, the latter set arbitrarily at 100%.

Determination of membrane potential

Membrane potential was calculated by using Nernst's equation by measuring the distribution of the membrane-permeable cation [^3H]TPP⁺ in the intra- and extra-mitochondrial space (Chappell & Crompton, 1979). Mitochondria (0.3 mg of protein) were incubated at 25°C for 15 min with 10³ Bq of [^3H]TPP⁺/0.2 ml and sedimented by centrifugation for 2 min in an Eppendorf centrifuge. Portions of the supernatant fluid were removed for determining the [^3H]TPP⁺ concentration. The pellet was resuspended in 0.1 ml of extraction buffer and quantitatively transferred to a scintillation vial containing 10 ml of Aquasol-2 for determination of the radioactivity. The amount of [^3H]TPP⁺ bound to mitochondria that had been inactivated at 95°C for 5 min was assumed to be non-specific binding. The same amount of [^3H]TPP⁺ was obtained when 10 μM each of FCCP, antimycin A, rotenone and valinomycin was present. To determine the mitochondrial volume, mitochondria were incubated with 10⁵ Bq of $^3\text{H}_2\text{O}$ and 3×10^4 Bq of [^{14}C]inulin per 0.2 ml at 25°C for 10 min. Radioactivity in the mitochondria and in the supernatant fluid was determined as described above. The mitochondrial volume was calculated by the method of Chappell & Crompton (1979).

Protein determination

Protein was determined by the dye-binding method of Spector (1978) with BSA as standard.

Results

Ca²⁺ accumulation under massive loading conditions

The kinetics of massive Ca^{2+} loading are shown in Fig. 2. Ca^{2+} accumulation was linear for 13 min and reached a maximum of approx. 400 nmol of Ca^{2+} /mg of mitochondrial protein after 20 min. Ca^{2+} accumulation was markedly inhibited by the ad-

Fig. 2. Ca^{2+} accumulation in maize mitochondria under massive loading conditions

The reaction was started by adding the mitochondria (0.1 mg of mitochondrial protein/ml) to the medium. After 1 min 4 mM-succinate was added (arrow 1). After 5 min the sample was divided into two equal portions (arrow 2) and DMSO (1% final concentration; open symbols) or APM (500 nmol/mg of mitochondrial protein; filled symbols) was added. At $t = 13$ min (arrow 3) each sample was further divided into three portions: $\circ$ and $\bullet$, no further addition; $\triangle$ and $\blacktriangle$, 10 μM -antimycin A and 10 μM -FCCP; $\square$ and $\blacksquare$, 10 μM -A23187. A typical experiment is shown ($n = 6$).

dition of 500 nmol of APM/mg of mitochondrial protein. When the calcium ionophore A23187 was added to either non-treated (control) or APM-treated mitochondria, a fast efflux of Ca^{2+} was observed. However, addition of antimycin A, an inhibitor of the electron-transport chain, together with FCCP, an uncoupler of energy transduction, promoted a slow Ca^{2+} release.

The inhibitory effect of APM was dependent on its concentration: half-maximal inhibition of Ca^{2+} accumulation was reached at 140 ± 8 (mean $\pm$ s.e.m., $n = 8$) nmol of APM/mg of mitochondrial protein (Fig. 3).

To measure Ca^{2+} uptake and not Ca^{2+} accumulation, i.e. the sum of Ca^{2+} efflux and Ca^{2+} uptake (see Fig. 2), mitochondria were partially loaded with unlabelled Ca^{2+} . The effect of APM was determined on additional loading with labelled Ca^{2+} (Fig. 4). By this procedure, $^{45}\text{Ca}^{2+}$ taken up is diluted and unidirectional Ca^{2+} influx rather than net Ca^{2+} accumulation can be measured.

Mitochondria were incubated with unlabelled Ca^{2+} for 2 min, at which time $^{45}\text{Ca}^{2+}$ alone, with APM or with Ruthenium Red was added to the

Fig. 3. Dose-response relationship of APM to Ca^{2+} accumulation ($\blacktriangle$) and to State-4 O_2 consumption ($\bullet$) of maize mitochondria

Ca^{2+} accumulation is given as a percentage of the control value set arbitrarily at 100%. The effect on State-4 O_2 consumption is given as a percentage of the stimulation caused by ADP set arbitrarily at 100%. The effect of DMSO was negligible. Each value is the mean of at least four independent experiments. The absolute value for Ca^{2+} accumulation in control mitochondria was 632 ± 55 nmol of Ca^{2+} /mg of mitochondrial protein per 15 min and that for ADP-stimulated O_2 consumption was 120 ± 13 nmol of O_2 consumed/mg of mitochondrial protein per min.

Fig. 4. Effect of APM on Ca^{2+} uptake under massive Ca^{2+} loading conditions

Mitochondria were preloaded with unlabelled Ca^{2+} for 2 min, after which time 250 Bq/ml of $^{45}\text{Ca}^{2+}$ was added (indicated by the arrowhead). The sample was immediately divided into three equal portions, to which one of the following was added: 1% DMSO ($\bullet$); 10 nmol of Ruthenium Red ($\blacktriangle$); 40 nmol of APM ($\blacksquare$). To a further (control) sample ($\circ$) $^{45}\text{Ca}^{2+}$ was added at $t = 0$. A typical experiment is shown ($n = 3$).

suspension. In the absence of Ruthenium Red or APM, $^{45}\text{Ca}^{2+}$ uptake occurred, whereas in their presence Ca^{2+} uptake was prevented as expected.

To determine the effect of APM on Ca^{2+} efflux (Fig. 5), mitochondria were preloaded for 2 min with $^{45}\text{Ca}^{2+}$, after which time the specific radioactivity of

Fig. 5. Effect of APM on Ca^{2+} efflux and massive Ca^{2+} -loading conditions

Mitochondria were preloaded with $^{45}\text{Ca}^{2+}$ for 2 min. At the point marked by the arrowhead the sample was divided into four equal portions to which the following substances were immediately added: control, $\circ$; 4 mM-CaEGTA, $\bullet$; 4 mM-CaEGTA + 10 nmol of Ruthenium Red, $\blacktriangle$; 4 mM-CaEGTA + 50 nmol of APM, $\blacksquare$. All samples contained, in addition, 1% DMSO. Since CaEGTA reduced the specific radioactivity in the media by a factor of 20, the accumulated radioactivity in mitochondria is given as Bq/mg of mitochondrial protein. A typical experiment is shown ($n = 3$).

Fig. 6. Ca^{2+} accumulation and limited Ca^{2+} -loading conditions

The incubation medium was supplemented with $10\mu\text{M}$ - CaCl_2 and $10\mu\text{M}$ -rotenone. Ca^{2+} accumulation was started by adding 0.3 mg of mitochondrial protein/ml. The mitochondria were incubated with ($\bullet$) or without ($\blacksquare$) succinate. After 4 min incubation the succinate-containing sample was divided (arrowhead 1) into two equal portions, to which 1% DMSO ($\bullet$) or 200 nmol of APM/mg of mitochondrial protein ($\blacktriangle$) were added. After 15 min of incubation $10\mu\text{M}$ -A23187 was added (arrowhead 2) to each of the samples. A typical experiment is shown ($n = 2$).

Ca^{2+} was reduced to less than 5% of the original value by addition of 4 mM-CaEGTA, which increased the Ca^{2+} activity by less than 0.5%. Since the specific radioactivity was altered during the experiment, unidirectional Ca^{2+} efflux could thus be measured. Fig. 5 shows that the mitochondria released only a very small amount of the already accumulated $^{45}\text{Ca}^{2+}$, even when APM or Ruthenium Red had been added.

Ca^{2+} accumulation under limited loading conditions

In a medium containing $10\mu\text{M}$ - Ca^{2+} (corresponding to approx. 40 nmol of Ca^{2+} /mg of mitochondrial protein), mitochondria accumulated up to 10 nmol of Ca^{2+} /mg of mitochondrial protein in the presence of $10\mu\text{M}$ -rotenone and in the absence of succinate (Fig. 6). Most of this Ca^{2+} could be released by the addition of $10\mu\text{M}$ -A23187. In the presence of 4 mM-succinate, an additional 6 nmol of Ca^{2+} /mg of mitochondrial protein accumulated. The addition of 200 nmol of APM/mg of mitochondrial protein released Ca^{2+} very slowly.

Fig. 7. Stimulation of O_2 consumption by Ca^{2+} and ADP. To 2 ml of incubation medium the following additions were made: M, 0.5 mg of mitochondrial protein; S, $20\mu\text{mol}$ of succinate; Ca, $2\mu\text{mol}$ of CaCl_2 ; APM, 500 nmol; DMSO, $20\mu\text{l}$; ADP, 200 nmol. The numbers beside the traces indicate the rate of O_2 consumption as nmol of O_2 /mg of mitochondrial protein per min. Each trace is from a typical experiment ($n = 4$).

Table 1. *Effect of APM on membrane potential*
Each value is the mean of two independent experiments performed in triplicate.

Condition	Other additions	Membrane potential (mV)
Massive loading (2 μmol of Ca^{2+} /mg of mitochondrial protein plus 5 mM-phosphate)	Control (+1% DMSO)	-160
	1 μmol of APM/mg of mitochondrial protein	-178
Limited loading (40 nmol of Ca^{2+} /mg of mitochondrial protein)	Control (+1% DMSO)	-177
	1 μmol of APM/mg of mitochondrial protein	-151

Effects of APM on respiratory chain and on mitochondrial membrane potential

APM had no significant effect on basal O_2 consumption up to concentrations of 2 μmol /mg of mitochondrial protein, as was demonstrated in Fig. 3. Fig. 7 shows O_2 consumption induced by Ca^{2+} (traces A, B and C) or by ADP (traces D and E). Addition of Ca^{2+} stimulated O_2 consumption markedly under these conditions (trace A). This stimulation was comparable with that induced by ADP (trace D). A respiratory control index of 1.9 ± 0.1 and an ADP/O ratio of 1.6 ± 0.2 were obtained (means $\pm$ S.E.M., $n=8$); 1 μmol of APM/mg of mitochondrial protein inhibited Ca^{2+} -induced stimulation of O_2 consumption (trace B) when added after Ca^{2+} , and prevented this stimulation (trace C) when added before Ca^{2+} . The stimulation of O_2 consumption by ADP was only slightly inhibited by APM (trace E), decreasing the respiratory control index from 1.9 to 1.6 ± 0.1 (mean $\pm$ S.E.M., $n=8$), while maintaining the ADP/O ratio. The effect of DMSO alone was always negligible. Furthermore the effect of APM on the mitochondrial energy transduction was determined by measuring membrane potentials. For this determination the $[\text{H}]\text{TPP}^+$ distribution between extra- and intra-mitochondrial space was measured as described previously for mitochondria by Kamo *et al.* (1979), for sarcoplasmic-reticulum vesicles by Caroni *et al.* (1980) or by using an anionic TPP^+ analogue for chloroplasts (Casadio *et al.*, 1980). The mitochondrial volume was measured under massive and limited Ca^{2+} loading conditions. It was calculated to be 2 μl /mg of mitochondrial protein ($n=6$), regardless of the experimental conditions employed. Table 1 shows that APM did not significantly change the membrane potential under conditions of massive and limited Ca^{2+} loading.

Discussion

The question of whether plant mitochondria possess an active Ca^{2+} transport system has been a topic of considerable interest for the past 20 years. However, the results obtained have often been contradictory, partly because different techniques were used to prepare mitochondria (Hodges & Hanson, 1965; Chen & Lehninger, 1973; Earnshaw

et al., 1973; Moore & Bonner, 1977; Day *et al.*, 1978; Dieter & Marmé, 1980). In the present investigation we used, as a source of our mitochondria, maize coleoptiles including primary leaves.

It has been proposed that Ca^{2+} accumulation by mitochondria from higher plants is catalysed by an active energy-dependent Ca^{2+} transport mechanism (Dieter & Marmé, 1980). The accumulation is inhibited by compounds interfering with mitochondrial energy transduction, i.e. inhibitors of the respiratory chain (antimycin A), or by uncouplers (FCCP). In the present paper we show that disturbance of the mitochondrial energy supply not only prevented Ca^{2+} accumulation, but induced a marked release of the already accumulated Ca^{2+} (Fig. 2). Furthermore most of the accumulated Ca^{2+} was released faster when the Ca^{2+} ionophore A23187 was added (Figs. 2 and 7; see also Dieter & Marmé, 1980), indicating that the Ca^{2+} in the mitochondrial space was still in an exchangeable form. The A23187-induced Ca^{2+} release indicates that the mitochondria accumulate Ca^{2+} against a concentration gradient. Under conditions of massive Ca^{2+} -loading phosphate is needed for Ca^{2+} accumulation (Drahota *et al.*, 1965), precipitating an unpredictable amount of Ca^{2+} as phosphate complexes. Therefore, under these conditions, the free Ca^{2+} concentration in the mitochondrial matrix cannot be estimated. In the case of limited Ca^{2+} -loading, however, no exogenous phosphate was required in the suspension in order to obtain an accumulation of Ca^{2+} . Since the amount of Ca^{2+} accumulated was 16 nmol/mg of mitochondrial protein (Fig. 6) and the intramitochondrial volume was found to be 2 μl /mg of mitochondrial protein, we calculated that the internal Ca^{2+} concentration was approx. 8 mM. Even if 90% of this Ca^{2+} were complexed, e.g. by endogenous phosphate, the free Ca^{2+} concentration would still be 0.8 mM. As the Ca^{2+} concentration in the suspending medium was approx. 8 μM , a Ca^{2+} concentration gradient across the mitochondrial membrane of at least two orders of magnitude can be assumed. This value is in good agreement with the free Ca^{2+} concentration in mitochondria from rat liver as assumed by Nicholls (1978).

We determined that 1 mg of mitochondrial protein accumulated as much as 10 nmol of Ca^{2+} in a

saturable manner in the absence of succinate and in the presence of rotenone under conditions of limited Ca^{2+} -loading (Fig. 6). We suggest that this may be due to endogenous oxidizable substrates, which may support Ca^{2+} transport by the cyanide-insensitive pathway (Wilson, 1980), which itself is not inhibited by rotenone (Moore & Rich, 1980). Addition of succinate increased this Ca^{2+} accumulation to 16 nmol of Ca^{2+} /mg of mitochondrial protein (see above). In either case, A23187 released most of the accumulated Ca^{2+} . Furthermore Ca^{2+} uptake is inhibited by Ruthenium Red (Figs. 4 and 5), a well characterized and specific inhibitor of the Ca^{2+} carrier in animal mitochondria (Moore, 1971). The data provide evidence that Ca^{2+} accumulation by maize mitochondria is similar to that by rat liver mitochondria (Carafoli & Crompton, 1978; Nicholls, 1978). However, we cannot exclude that in maize mitochondria endogenous phosphate is necessary for Ca^{2+} uptake as suggested by Day *et al.* (1978).

It has been shown that the addition of the organophosphorus herbicide APM impedes Ca^{2+} accumulation in plant mitochondria under conditions of massive Ca^{2+} -loading (Hertel *et al.*, 1980). The mode of action of APM, however, has not yet been fully elucidated. Inhibition of Ca^{2+} accumulation can be explained by four mechanisms: (1) a disturbance of membrane integrity; (2) an impairment of mitochondrial energy transduction; (3) an increased Ca^{2+} efflux; (4) a direct inhibition of the Ca^{2+} transport system itself. As neither State-4 (Fig. 3) or State-3 O_2 consumption (Fig. 7) nor the membrane potential (Table 1) were significantly changed by APM at concentrations that completely inhibited Ca^{2+} accumulation, the first two possibilities are unlikely. The third possibility, i.e. an increased Ca^{2+} efflux, can only partially explain the inhibitory effect of APM, for the following reasons. (a) If APM increased Ca^{2+} efflux, the mitochondria would have to compensate the energy dissipation induced by Ca^{2+} cycling with an increased respiratory rate. APM, however, prevented the stimulation of State-4 O_2 consumption by Ca^{2+} and blocked the Ca^{2+} -induced increase in the respiratory rate (Fig. 7). (b) There was a substantial inhibition of further Ca^{2+} uptake by APM, thus eliminating the possibility of a potential stimulation of Ca^{2+} efflux (Fig. 4). (c) A very slow Ca^{2+} efflux (approx. 16 nmol/mg of mitochondrial protein per min) was measured under massive loading conditions (Fig. 5). This efflux was not increased by APM compared with the control or with that obtained in the presence of Ruthenium Red. (d) Under conditions of limited Ca^{2+} -loading, where a more sensitive estimation of Ca^{2+} efflux is possible, a Ca^{2+} efflux rate of approx. 0.7 nmol of Ca^{2+} /mg of mitochondrial protein per min was measured (Fig. 6). Here increased Ca^{2+}

efflux may partially be responsible for the APM inhibition but under massive loading conditions it is unlikely to have a significant influence.

Therefore the fourth possibility, i.e. a direct inhibition of the Ca^{2+} transport, best explains the mechanism of action of APM. This explanation is further supported by the Ca^{2+} uptake experiments with preloaded mitochondria (Fig. 4). However, the results obtained so far cannot exclude the possibility that the inhibition of Ca^{2+} uptake by APM is mediated by inhibition of Ca^{2+} /phosphate co-transport. The inhibition of Ca^{2+} uptake in the absence of extramitochondrial phosphate, on the other hand, strongly favours the hypothesis that Ca^{2+} uptake is directly inhibited by APM.

Part of this work was supported by the Deutsche Forschungsgemeinschaft (SFB 46, Teilprojekt C4), by the Stiftung Volkswagenwerk and by the Bundesminister für Forschung und Technologie (01 QV 318-ZA/WF/WRK 0275/5).

References

- Carafoli, E. & Crompton, M. (1978) *Curr. Top. Membr. Transp.* **10**, 151–216.
- Caroni, P., Reinlib, L. & Carafoli, E. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6354–6358.
- Casadio, R., Ventuoli, G. & Melandri, B. A. (1980) in *Short Reports of the First European Bioenergetics Conference*, pp. 231–232, Patron, Bologna.
- Chance, B. & Williams, G. R. (1956) *Adv. Enzymol. Relat. Areas Mol. Biol.* **17**, 65–134.
- Chappell, J. B. & Crompton, M. (1979) in *Membrane Biochemistry: A Laboratory Manual on Transport and Bioenergetics* (Carafoli, E. & Semenza, G., eds.), pp. 92–97, Springer Verlag, Berlin, Heidelberg and New York.
- Chen, C. H. & Lehninger, A. L. (1973) *Arch. Biochem. Biophys.* **137**, 183–196.
- Day, D. A., Bertagnolli, B. L. & Hanson, J. B. (1978) *Biochim. Biophys. Acta* **502**, 289–297.
- Dieter, P. & Marmé, D. (1980) *Planta* **150**, 1–8.
- Drahota, Z., Carafoli, E., Rossi, C. S., Gamble, R. L. & Lehninger, A. L. (1965) *J. Biol. Chem.* **240**, 2712–2720.
- Earnshaw, M. J., Madden, D. M. & Hanson, J. B. (1973) *J. Exp. Bot.* **24**, 828–840.
- Hertel, C., Quader, H., Robinson, D. G. & Marmé, D. (1980) *Planta* **149**, 336–340.
- Hodges, T. K. & Hanson, J. B. (1965) *Plant Physiol.* **40**, 101–108.
- Kamo, N., Muratsugu, M., Hongoh, R. & Kobatake, Y. (1979) *J. Membr. Biol.* **49**, 105–121.
- Moore, A. L. & Bonner, W. D., Jr. (1977) *Biochim. Biophys. Acta* **460**, 455–466.
- Moore, A. L. & Rich, P. R. (1980) *Trends Biochem. Sci.* **3**, 284–288.
- Moore, C. L. (1971) *Biochem. Biophys. Res. Commun.* **42**, 298–305.
- Nicholls, D. G. (1978) *Biochem. J.* **176**, 463–474.
- Spector, T. (1978) *Anal. Biochem.* **86**, 142–146.
- Wilson, S. B. (1980) *Biochem. J.* **190**, 349–360.