Lambeth, D. O., and Lardy, H. A. (1971), *Eur. J. Biochem. 22,* 355.

Landry, Y., and Goffeau, A. (1973), *Arch. Int. Physiol. Biochim. 81,* 590.

Lardy, H. A., Johnson, D., and McMurray, W. C. (1958), *Arch. Biochem. Biophys. 78,* 587.

Lardy, H. A., Reed, P., and Chiu-Lin, C. (1975), *Fed Proc., Fed. Am. Soc. Exp. Biol. 34,* 1707.

Linnett, P. E., Mitchell, A. D., and Beechey, R. B. (1975), *FEBS Lett. 53,* 180.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Mitchell, P., and Moyle, J. (1970), *FEBS Lett. 9,* 305.

Pedersen, P. L. (1976), *J. Biol. Chem. 251,* 934.

Pedersen, P. L., Levine, H., and Cintron, N. (1974), in Membrane Proteins in Transport and Phosphorylation, Azzone, G. F., Klingenberg, M. E., Quagliariello, E., and Siliprandi, N., Ed., Amsterdam, North-Holland Publishing Co., p 43.

Philippot, J., and Authier, M. H. (1973), *Biochim. Biophys. Acta 298,* 887.

Pullman, M. E., and Monroy, G. C. (1963), *J. Biol. Chem. 238,* 3762.

Robinson, N. C., Nozaki, Y., and Tanford, C. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1370.

Ryrie, I. J. (1975), *Arch. Biochem. Biophys. 168,* 712.

Sadler, M. H., Hunter, D. R., and Haworth, R. A. (1974), *Biochem. Biophys. Res. Commun. 59,* 804.

Schnaitman, C. A., and Greenawalt, J. W. (1968), *J. Cell Biol. 38,* 158.

Somlo, M., and Krupa, M. (1974), *Biochem. Biophys. Res. Commun. 59,* 1165.

Somlo, M., Avner, P. R., Cosson, J., Dujon, B., and Krupa, M. (1974), *Eur. J. Biochem. 42,* 439.

Sone, N., Yoshida, M., Hirata, H., and Kagawa, Y. (1975), *J. Biol. Chem. 250,* 7917.

Stekhoven, F. S., Waitkus, R. F., and van Moerkerk, H. (1972), *Biochemistry 11,* 1144.

Swanljung, P., Frigeri, L., Ohlson, K., and Ernster, L. (1973), *Biochim. Biophys. Acta 305,* 519.

Tzagoloff, A. (1969), *J. Biol. Chem. 244,* 5020.

Tzagoloff, A., Byington, K. H., and MacLennan, D. H. (1968), *J. Biol. Chem. 243,* 2405.

Tzagoloff, A., and Meagher, P. (1971), *J. Biol. Chem. 246,* 7328.

Vallejos, R. H., van Den Bergh, S. G., and Slater, E. C. (1968), *Biochim. Biophys. Acta 153,* 509.

Walter, P., Lardy, H. A., and Johnson, D. (1967), *J. Biol. Chem. 242,* 5014.

Yamamoto, G. (1970), *Acta Med. Okayama 24,* 143.

Some Effects of Ionophore A23187 on Energy Utilization and the Distribution of Cations and Anions in Mitochondria[†]

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ABSTRACT: The effects of ionophore A23187 on the movements of Ca^{2+}, Mg^{2+}, H^+, phosphate, and succinate and its effects on energy utilization by mitochondria have been studied as a function of ionophore concentrations. At a low ratio of the compound to mitochondrial protein, below that required for maximal uncoupling, an apparent steady state is established between intra- and extramitochondrial Ca^{2+} and H^+ which is maintained until anaerobiosis or inhibition of the mitochondrial Ca^{2+} pump. Mg^{2+} is continuously lost from the mitochondria during the steady state. At higher levels of the compound, both Ca^{2+} and Mg^{2+} are released very rapidly. In the medium used, the rate of respiration produced by cyclic Ca^{2+} uptake and release is a function of the ionophore level between 0 and 0.2 nmol per mg of protein and of the extramitochondrial Ca^{2+} concentration between 1 and 15 μM. The depletion of mitochondrial Ca^{2+} by A23187 appears to result in the complete reversal of the total ion movements occurring during energy-dependent Ca^{2+} accumulation by mitochondria. The ratio of H^+ uptake to Ca^{2+} released is nearly 1. During ionophore-induced Mg^{2+} depletion, no net H^+ uptake is observed, but the mitochondria are depleted of equal molar amounts of phosphate plus succinate. It is proposed that the initial H^+ uptake produced by the ionophore is reversed through the combined actions of the mitochondrial phosphate-hydroxide and phosphate-dicarboxylate exchangers. The data are discussed in terms of factors affecting the "turnover number" of A23187, the mechanism of Ca^{2+} uptake by mitochondria, and some considerations affecting interpretation of data obtained by use of A23187 in cellular systems.

The divalent cation selective ionophorous activity of antibiotic A23187 was discovered during studies of its effects on isolated mitochondria (Reed and Lardy, 1972a). Since that time, it has been used in a number of additional studies with mitochondria, both in studies on the properties of the ionophore (Reed and Lardy, 1972b; Wong et al., 1973; Case et al., 1974; Reed et al., 1975; Pfeiffer and Lardy, 1976) and to determine effects of altered divalent cation distribution on functional parameters, including the mechanism of divalent cation accumulation by these organelles (Rottenberg and Scarpa, 1974; Wohlrad, 1974; Sordahl, 1974; Schuster and Olson, 1974;

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Case, 1975). Further progress in these studies will be aided by a more detailed understanding of the effects of A23187 on isolated mitochondria which is the subject of this investigation. Aspects of this work should be of further interest to investigators using A23187 in studies on the involvement of Ca^{2+} in mechanisms controlling excitable cells and tissues, both because mitochondria are believed to be an important component controlling cytosolic Ca^{2+} levels in many systems (see Simkiss, 1974; Borle, 1973; and references therein) and because they are expected to be an important site of action of the ionophore on cells and tissues.

The present work is also preliminary to studying ion transport by A23187 in mitochondria at a detailed level. This ionophore contains two aromatic chromophores (Chaney et al., 1974) and may be readily studied by spectroscopic techniques. At present several studies are available on aspects of the ionophore's solution chemistry (Caswell and Pressman, 1972; Case et al., 1974; Pfeiffer et al., 1974; Pfeiffer and Lardy, 1976) as well as on the structure of some metal ion complexes (Deber and Pfeiffer, 1976; Puskin and Gunter, 1975) and on the effects of this ionophore on the permeability of artificial membranes to divalent cations (Case et al., 1974; Hyono et al., 1975; Kafka and Holz, 1976). Although it is probable that this ionophore forms as many as five complexes with differing stoichiometries (Pfeiffer and Lardy, 1976), the available data indicate that the 2:1, A23187-M^{2+} charge neutral complex is responsible for transport of divalent cations across membranes. This concept is basic to the interpretation of the present data.

Materials and Methods

Rat liver mitochondria were prepared according to the procedure of Johnson and Lardy (1967). The homogenizing medium contained in addition, 0.1 mM EGTA[1] and 0.5 mg/ml bovine serum albumin (low fatty acid type) which were absent from the washing medium. All mannitol-sucrose solutions were deionized with a mixed bed resin (Amberlite MB-3) prior to use. Protein was determined by the biuret reaction in the presence of 1% deoxycholate. Subsequent incubations of mitochondria were at 25 °C in 0.23 M mannitol, 0.07 M sucrose, 10 mM sodium succinate plus rotenone (0.4 nmol per mg of protein), 3 mM triethanolamine, pH 7.4 (normal medium), or with modifications as indicated in the figure and table legends.

A variety of techniques was used to monitor the movements of cations, anions, and energy utilization in mitochondria under conditions of interest. Ca^{2+} and Mg^{2+} movements were monitored spectrophotometrically using the indicating dyes murexide (Mela and Chance, 1968) and eriochrome blue SE (Scarpa, 1974), respectively. The metal-induced difference absorptions were recorded with an American Instrument Co. DW-2 spectrophotometer with simultaneous recording of oxygen consumption by the vibrating platinum electrode accessory. Alternatively, intramitochondrial cation movements as observed by the fluorescent probe chlorotetracycline (Caswell, 1972) were determined with an Aminco Bowman spectrophotofluorometer or by direct measurements of atomic absorption. Samples for atomic absorption (Perkin-Elmer 403) were prepared by rapidly sedimenting mitochondria in a microcentrifuge and analyzing aliquots of the supernatant or by subsequently dispersing the pellet with 1% deoxycholate plus 1 mM EDTA in H_2O and analyzing the extract.

The redox state of cytochrome *b* was observed by dual wavelength spectroscopy at 430–410 nm together with oxygen consumption as described above. Alternatively, oxygen consumption was determined with a Clark electrode using a Gilson oxygraph equipped with a hydrogen electrode for simultaneous recording of H^+ (OH^-) movements across the mitochondrial inner membrane. The electrode was standardized by additions of known amounts of HCl. $CaCl_2$ and EGTA solutions were standardized by titration against a primary standard EDTA solution.

Extra- and intramitochondrial inorganic phosphate was determined chemically or in some cases by scintillation counting of $^{32}P_i$. For the chemical determinations, mitochondria were first sedimented by centrifugation. P_i was then determined, essentially by the method of Parvin and Smith (1969) on the supernatant or 5% Cl_3CCOOH extracts from the pellet. Cl_3CCOOH was removed by extraction with water-saturated ether before analysis. Where indicated, cation concentrations on these same fractions were determined by atomic absorption.

For the determination of mitochondrial $^{32}P_i$ and succinate-^{14}C by scintillation counting, aliquots of mitochondria were filtered through Millipore filters (0.45-μm pore size) at 0–5 °C, which were subsequently washed twice with 5-ml amounts of cold 0.23 M mannitol–0.07 M sucrose which contained either 1 mM P_i (for ^{32}P) or 3 mM triethanolamine (for succinate-^{14}C), pH 7.4. The radioactivity retained by the filters was determined by counting cerenkov radiation in H_2O for ^{32}P or by drying the filters and counting succinate-^{14}C in toluene based scintillation medium (Amersham Searle Isocap 300).

[1,4-^{14}C]Succinate was obtained from Amersham Searle. $^{32}P_i$ was obtained from New England Nuclear. Ruthenium red was obtained from K & K Laboratories and used without further purification. Murexide was obtained from Eastman. Eriochrome blue SE was a gift from Dr. Antonio Scarpa of the Johnson Foundation. A23187 was a gift from Dr. Robert Hamill of Eli Lilly Co. All other reagents were obtained from commercial sources and were reagent grade or better.

Results

Effects of Varying Levels of A23187 and Ca^{2+} on the Divalent Cation Contents of and Energy Utilization by Mitochondria. The consequences of treating mitochondria, or more complicated systems as well, with A23187 may be varied by altering the amount of the ionophore added. Figure 1 shows the effects of a relatively low and high dose of the compound on the depletion of mitochondrial Ca^{2+} and Mg^{2+} as indicated by the specific indicating dyes murexide (series A) or eriochrome blue SE (series B), respectively. A decrease in the difference absorbance of these dyes results from an increase in the medium concentration of the ion. In series C, the intramitochondrial levels of "membrane bound" divalent cations as indicated by the fluorescent probe, chlorotetracycline, are shown under similar conditions, with an increase in fluorescence indicating an increased level of intramitochondrial divalent cation. After addition of A23187 at the relatively low level of 0.15 nmol per mg of protein, the release of intramitochondrial Mg^{2+} began immediately (B,1) whereas following release of a small amount of Ca^{2+} (about 5 μM under these conditions), an apparent steady-state distribution of Ca^{2+} across the mitochondrial inner membrane persisted and was not further altered until the system went anaerobic. Following exhaustion of oxygen and subsequent deenergization, the remaining Ca^{2+} was released (A,1). The same level of A23187

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[1] Abbreviations used: EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

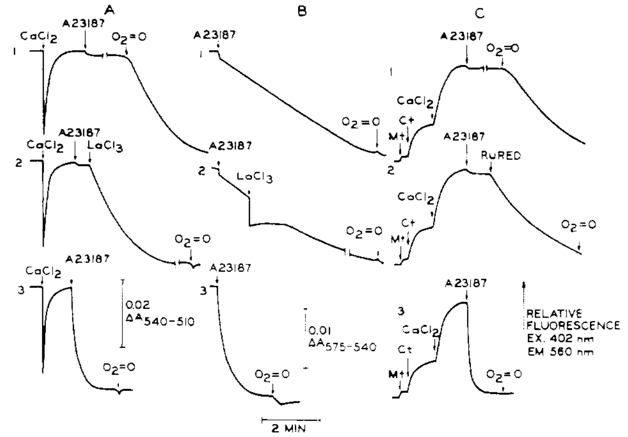

FIGURE 1: The effect of a low and high level of A23187 on the loss of Ca^{2+} and Mg^{2+} from mitochondria. Mitochondria were incubated in 3.0 ml of the normal medium with the further additions described. For the three sets (A, B, and C), A23187 was added where indicated at 0.15 nmol per mg of protein in traces 1 and 2 or 2.0 nmol per mg of protein in trace 3. (Series A) The media contained 100 μM murexide. The protein concentration was 2.7 mg per ml with $CaCl_2$ additions of 250 nmol. (Series B) The media contained 100 μM eriochrome blue SE. The protein concentration was 2.5 mg per ml. (Series C) Where indicated, chlorotetracycline was added at 2.0 nmol per mg of protein. The protein concentration was 3.2 mg per ml with $CaCl_2$ additions of 60 nmol. Where indicated, the inhibitors of Ca^{2+} transport, $LaCl_3$ and ruthenium red, were utilized at 2 nmol per mg of protein. Oxygen consumption was monitored simultaneously in series A and B or in parallel experiments for series C.

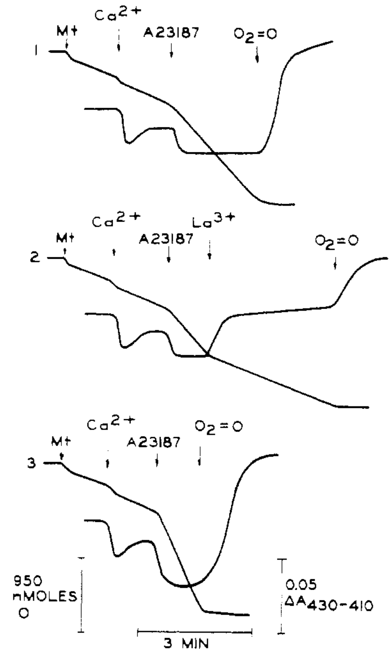

FIGURE 2: The effect of a low and high level of A23187 on the rate of succinate oxidation and the oxidation-reduction state of cytochrome *b* in mitochondria. Mitochondria were incubated at 2.6 mg per ml of the normal reaction medium (total volume 3.0 ml). The rate of succinate oxidation (initially the upper tracings) and the redox state of cytochrome *b* (initially the lower tracings) were monitored simultaneously. The further additions, indicated in 1, 2, and 3, were the same as described in 1, 2, and 3 of series A in Figure 1.

will release intramitochondrial Ca^{2+} extensively prior to deenergization if the mitochondrial Ca^{2+} uptake system is inhibited by the addition of La^{3+} (A,2) without appreciably altering the course of Mg^{2+} depletion induced by the compound (B,2). The transient decrease in the difference absorbance of the Mg^{2+}-indicating dye that occurred on addition of La^{3+} was due to interference by La^{3+} and to a shift in the medium pH under these conditions (see below), rather than a rapid release of mitochondrial Mg^{2+} (verified by atomic absorption, data not shown). We find that the successful use of eriochrome blue SE as a Mg^{2+}-specific indicator is critically dependent on maintaining a constant pH. Traces A,3 and B,3 show that A23187 at 2.0 nmol per mg of protein, and without interference with the Ca^{2+} pump, promptly released both Ca^{2+} and Mg^{2+} with no further release indicated at attainment of anaerobiosis.

The fluorescence signal of chlorotetracycline, indicating intramitochondrial divalent cations (Caswell, 1972), was altered by the accumulation, and ionophore induced depletion, of mitochondrial Ca^{2+} in a manner which indicated that the dye was responding only to mitochondrial Ca^{2+} (compare series A and series C). Similar data have been reported by Binet and Volfin (1975). In other experiments not shown, we find that the magnitude of chlorotetracycline fluorescence in rat liver mitochondria is a linear function of the mitochondrial Ca^{2+} content up to approximately 25 nmol per mg of protein and thus this technique may be useful as a relatively specific indicator of intramitochondrial Ca^{2+}.

In Figure 2, the effects of the varying conditions employed in Figure 1 on energy utilization by the mitochondria have been examined. The separate experiments 2-1, 2-2, and 2-3 were performed in parallel to 1, 2, and 3 of series A in Figure 1; the initially upper traces are recordings of oxygen consumption and the initially lower traces show the redox state of cytochrome *b*. The addition of Ca^{2+} under these "limited loading" conditions produced the normal transient increases of oxygen uptake and cytochrome *b* oxidation accompanying the energy-dependent accumulation of the added Ca^{2+}. The subsequent addition of A23187 at 0.15 nmol per mg of protein in Figure 2-1 accelerated respiration to a submaximal value under these conditions of 60 ng-atoms of oxygen per min per mg of protein and shifted cytochrome *b* to a more oxidized state. This condition, like the Ca^{2+} depletion shown in A-1 and C-1 of Figure 1, was maintained as an apparent steady state until oxygen was exhausted. The addition of La^{3+} to block subsequent Ca^{2+} uptake (Figure 2-2) returned both the respiration rate and the redox state of cytochrome *b* to state 4 values. Figure 2-3 shows that the higher level of A23187, which gave immediate depletion of both Ca^{2+} and Mg^{2+} in Figure 1, A-3, B-3, and C-3, results in a maximal rate of respiration and highly oxidized state of cytochrome *b* which are both maintained until anaerobiosis.

In Figure 3, experiments of the type presented in Figure 1, A-1, and Figure 2-1 have been performed over a wide range of ionophore concentrations. Levels of compound below 0.06 nmol per mg of protein produced little detectable effect on either the distribution of Ca^{2+} across the mitochondrial inner membrane or on the rate of respiration. Further increasing the level of A23187 steadily increased respiration until a maximal rate was attained at 0.22 nmol per mg of protein; higher levels slightly inhibited respiration. The parallel determination of the intra- and extramitochondrial Ca^{2+} distribution showed that little net Ca^{2+} is released by A23187 until a high enough level is added to maximally release respiration. Further increase beyond that level progressively shifts the distribution to increasing extramitochondrial levels until, at 2 nmol per mg of protein, effectively all of the Ca^{2+} is released by the compound and remains extramitochondrial during the period of enhanced respiration.

The insert in Figure 3 shows a double-reciprocal plot of that

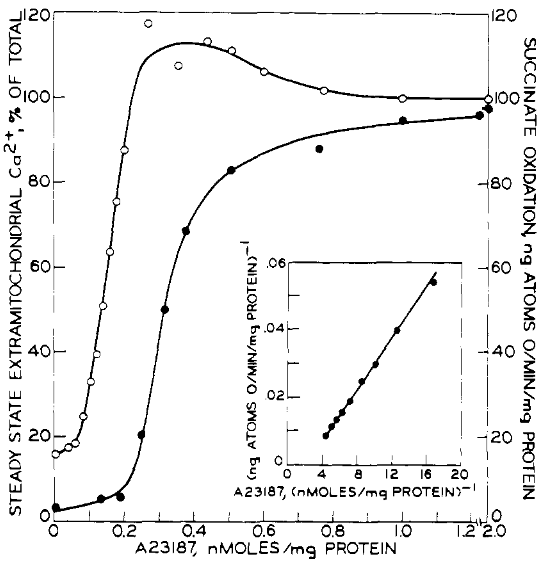

FIGURE 3: The effect of increasing levels of A23187 on the rate of succinate oxidation and the steady-state distribution of Ca^{2+} across the inner mitochondrial membrane. (○) Succinate oxidation. Mitochondria (2.5 mg of protein per ml) were incubated in the normal medium. After being allowed to accumulate Ca^{2+} (30 nmol per mg of protein), A23187 was added at the indicated levels and the rate of oxygen consumption was observed. (●) Ca^{2+} distribution data. The data were obtained in parallel experiments where the media contained in addition 100 μM murexide (analogous to Figure 1, series A). The percent of total Ca^{2+} which was extramitochondrial during the period of accelerated respiration was estimated from the value of $\Delta A_{540-510}$ after addition of A23187 compared with the final value obtained at anaerobiosis with subsequent release of the remaining Ca^{2+}. The insert in this figure is a double-reciprocal plot of that portion of the respiration data between 0.08 and 0.22 nmol of A23187 per mg of protein.

portion of the respiration data where increasing amounts of the compound give a steadily increasing rate of oxygen consumption. Significant aspects of these data are the linearity of the plot and its slope from which a nominal rate constant for the overall transport sequence may be calculated. These data are further discussed below.

In Figure 3, it is difficult to assess whether or not the submaximal acceleration of respiration produced by the lower levels of A23187 is due to an increase in the medium Ca^{2+} concentration or some other property of the ionophore since the concentrations of free Ca^{2+} are near to the detection limit of murexide under these conditions. In Figure 4, however, the rate of energy utilization by mitochondria treated with A23187 is shown to be a function of the level of free Ca^{2+} present in the incubation medium. Mitochondria, after being allowed to accumulate Ca^{2+}, were treated with varying levels of EGTA and A23187 at 2.0 nmol per mg of protein. As shown in Figures 1 and 2, under these conditions essentially all intramitochondrial Ca^{2+} is released promptly and establishes a free Ca^{2+} concentration in the media which is determined by the equilibrium between Ca^{2+} and EGTA (as well as other Ca^{2+} binding components of the medium). In this experiment, the added plus endogenous Ca^{2+}, if free in solution, would produce a concentration of 91.5 μM. Lower levels of free Ca^{2+} would be present with increasing concentrations of EGTA. The data indicate that decreasing the concentration of free Ca^{2+} had no effect on respiration until the mole ratio of EGTA–Ca^{2+} was increased to about 0.86 (corresponding to a free Ca^{2+} concentration of 13 μM assuming quantitative Ca^{2+} binding by EGTA and no other significant Ca^{2+} binding by other components of the media). Further increases in this ratio progressively decrease the respiration rate until, at a mole ratio of between 1.10 and 1.15, the respiration rate is returned to a near state 4 value. For the present considerations, these data demonstrate further that it is an increase in the medium Ca^{2+} concentration which is responsible for the enhanced respiration produced by the compound and that only low levels of free Ca^{2+} (in the range of 1–15 μM) together with A23187 are required to produce a marked effect on rate of energy utilization by mitochondria. This procedure, however, is also useful for studying the kinetics of the mitochondrial Ca^{2+} pump under steady conditions as will be presented in a subsequent communication (Hutson et al., submitted for publication).

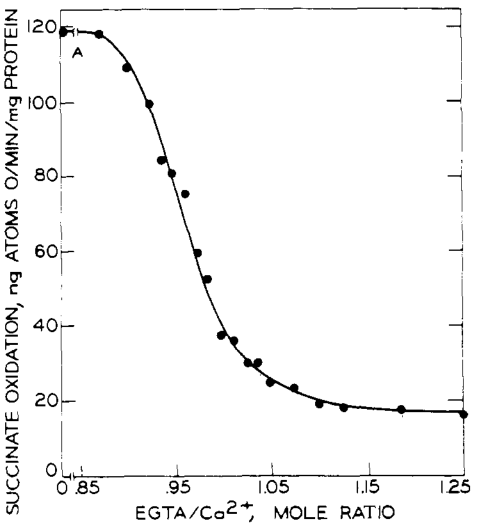

FIGURE 4: The effect of decreasing Ca^{2+} concentrations on the rate of succinate oxidation stimulated by A23187. Mitochondria (10.2 mg of protein) were incubated in the normal reaction medium (4.0 ml) and were allowed to accumulate 29 nmol of Ca^{2+} per mg of protein. After addition of varying amounts of EGTA, A23187 was added at 2.0 nmol per mg of protein and the rate of respiration observed. Including the endogenous mitochondrial Ca^{2+} content (6.9 nmol per mg of protein), the potential Ca^{2+} concentration present was 91.5 μM.

The Movement of Protons and Anions in Mitochondria Treated with A23187. The above data are consistent with the hypothesis by Reed and Lardy (1972a) that A23187 catalyzes an electroneutral exchange of divalent cation for two H^+ through the formation of a $[A23187^-]_2Me^{2+}$ complex. Thus, besides perturbing the transmembrane distribution of divalent cations, the ionophore produces a primary alteration in transmembrane ΔpH which in turn would be expected to result in a secondary alteration in the distribution of some anions of physiological importance. The net movements of H^+ accompanying the A23187-induced depletion of mitochondrial divalent cations are shown in Figure 5. In the normal medium, mitochondria showed a net extrusion of between 25 and 30 nmol of H^+ per mg of protein upon energization by succinate plus rotenone. In agreement with reports from many laboratories, Ca^{2+} uptake resulted in further H^+ extrusion which attained a final value, H^+/Ca^{2+}, of 1.0 (see Lehninger, 1967, for review). Figure 5-1, -2, and -3 are experiments parallel to Figure 1A, -1, -2, and -3. After Ca^{2+} uptake, the subsequent addition of A23187 at 0.15 nmol per mg protein produced only a small net H^+ uptake by mitochondria unless the oxygen was depleted (Figure 5-1) or the reaccumulation of Ca^{2+} by the mitochondria was prevented (Figure 5-2). The higher level of the ionophore, however, produced a prompt net H^+ uptake (Figure 5-3). A comparison of these data with Figure 1 demonstrates that net H^+ uptake under these conditions parallels closely the loss of Ca^{2+}. Relatively extensive depletion of Mg^{2+}

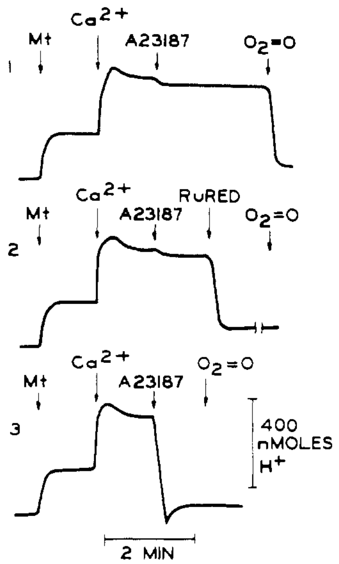

FIGURE 5: The effect of a low and high level of A23187 on net H^+ movement in mitochondria. Mitochondria were incubated at 2.5 mg of protein per ml in the normal reaction medium. The pH of the medium was recorded simultaneously with the O_2 concentration as described in Materials and Methods. Where indicated, $CaCl_2$ was added at 25 nmol per mg of protein, ruthenium red (RuRed) at 2 nmol per mg of protein, and A23187 at 0.15 nmol per mg of protein in 1 and 2 or 2.0 nmol per mg of protein in 3.

TABLE I: The Effect of a Low Level of A23187 on Mitochondrial Content of Ca^{2+}, Mg^{2+}, and Inorganic Phosphate.[a]

Conditions[b]	Ca^{2+} (nmol mg^{-1} protein)	Mg^{2+} (nmol mg^{-1} protein)	P_i (nmol mg^{-1} protein)
Nonenergized			
A. Intramitochondrial	6.7	30.3	2.6
B. Extramitochondrial	0.5	4.9	6.8
Energized; no A23187			
A. Intramitochondrial			
5 min	7.1	30.5	2.5
10 min	7.2	31.1	2.6
B. Extramitochondrial			
5 min	0.2	5.9	1.4
10 min	0.1	5.8	0.9
Energized; plus A23187			
A. Intramitochondrial			
2 min	6.8	17.4	1.1
4 min	6.4	13.9	0.7
6 min	5.7	12.0	0.6
8 min	5.3	10.0	0.6
10 min	5.4	10.7	0.6
B. Extramitochondrial			
2 min	0.6	18.6	3.6
4 min	0.7	22.2	4.4
6 min	1.9	25.4	3.7
8 min	1.8	24.6	4.4
10 min	1.8	24.5	4.2

[a] Mitochondria were prepared in the normal manner, except 0.30 M sucrose was used in the final wash medium to prevent the interference by mannitol with the phosphate determinations. Mannitol was also excluded from the normal medium which was otherwise as described in the Materials and Methods section. [b] Mitochondria were incubated at 3.2 mg of protein per ml and subsequently separated by centrifugation. For "nonenergized", succinate was excluded from the media. Ca^{2+}, Mg^{2+}, and inorganic phosphate were determined on aliquots of the supernatants or of pellet extracts as described in the Materials and Methods section. When present, A23187 at 0.15 nmol per mg of protein was added after a 2-min preincubation. Subsequent time points are times after the addition of A23187.

occurs due to transport by A23187 without net H^+ movement. In other experiments of the type in Figure 5, increasing levels of A23187 between 0.15 and 2.0 nmol per mg of protein resulted in increasing net H^+ uptake which closely paralleled the steady-state Ca^{2+} distribution data in Figure 3. During the steady state before exhaustion of O_2, the ratio of net H^+ taken up to Ca^{2+} released was near 1, indicating reversal of the net movements which had occurred during Ca^{2+} uptake.

In the present medium, a number of components could potentially be altered in their distribution across the mitochondrial inner membrane by an electroneutral mechanism which could reverse the ionophore-induced H^+ movements as indicated in Figure 5. Since we have shown that A23187 can catalyze the exchange of monovalent cation for H^+ under some conditions (Pfeiffer and Lardy, 1976), we considered the possibility that net Na^+ uptake occurred during A23187-induced Mg^{2+} depletion. Experiments of the type in Figure 1, A1 and B1, and Figure 5-1 were repeated in different media where alternatively Li^+, Na^+, K^+, Rb^+, or Cs^+ was the only monovalent metal cation present significantly in the bulk medium. No variations from the results presented for the all-Na^+ medium were observed. Since the stability of monovalent cation complexes with A23187 decreases markedly with increasing ionic radius (Pfeiffer and Lardy, 1976), the lack of variation in the rate of Mg^{2+} depletion or in the accompanying net movements of H^+ indicated that A23187 was not exchanging Mg^{2+} for extramitochondrial monovalent cation. In addition, no A23187-induced Na^+ uptake was seen by atomic absorbance determinations. The reversal of ionophore-induced H^+ uptake during Mg^{2+} depletion indicated in Figure 5 must, therefore, result from subsequent loss of intramitochondrial anions.

The only transportable or permeant anions in the medium which are present in significant quantities are succinate and inorganic phosphate. The effects of A23187 on the mitochondrial content of inorganic phosphate are shown in Table I. The intramitochondrial levels of Ca^{2+}, Mg^{2+}, and P_i were not much altered by energization and subsequent 10-min incubations. The reduction in extramitochondrial P_i following energization without increasing the intramitochondrial content indicates that the P_i is incorporated into ATP. The subsequent addition of A23187 at 0.15 nmol per mg of protein resulted in the normal Mg^{2+} depletion without extensive Ca^{2+} depletion. These direct measurements by atomic absorption agree well with the indicating dyes in Figure 1. The ionophore reduced the content of mitochondrial Mg^{2+} by about 18 nmol per mg of protein. During this time, 3-4 nmol of P_i per mg of protein was also lost, apparently derived in part from the intramitochondrial P_i pool together with some hydrolysis of the endogenous nucleotides (Walajtys et al., 1974).

The movements of succinate and its metabolites under these conditions are shown in Figure 6. The mitochondria accumulated 50 nmol of succinate per mg of protein subsequent to energization under state 4 conditions. With further incubation, this content decreased only slowly unless depletion of divalent cations was induced by addition of A23187. The ionophore at 0.15 nmol per mg of protein reduced the content of succinate by about 15 nmol per mg of protein which was maintained during the period of accelerated respiration until anaerobiosis after which further release occurred. Thus, these data together with Table I demonstrate that, under conditions where A23187 depletes the mitochondria of 18-20 nmol of Mg^{2+} per mg of protein, the mitochondria also lose 3-4 nmol of P_i and 15 nmol of succinate (or its metabolites). Together the loss of P_i and succinate is sufficient to balance the Mg^{2+} depleted by A23187 without net H^+ uptake. The loss of succinate is not dependent on the "uncoupling" produced by the ionophore plus Ca^{2+} since it occurs as well (and is enhanced by about 5 nmol per mg of protein) in the presence of EGTA where state 4 respiration is maintained.

Discussion

It is apparent from Figures 1 to 4 that the differential effects of low levels of A23187 on the depletion of intramitochondrial Ca^{2+} and Mg^{2+} can be adequately accounted for by ability of these mitochondria actively to accumulate Ca^{2+} and not Mg^{2+}. The addition of A23187 to mitochondria initially results in an increase in the medium concentration of both Ca^{2+} and Mg^{2+}. As the extramitochondrial Ca^{2+} concentration increases to the region of 1-15 μM, the energy-dependent Ca^{2+} pump progressively increases its rate of reaccumulation as indicated by enhanced rates of energy utilization demonstrated in Figures 2-4. If the outward flux of Ca^{2+} produced by a given level of the compound is less than the inward flux that can be generated by the mitochondrial Ca^{2+} pump under these conditions, a steady-state extramitochondrial Ca^{2+} concentration in the range of a few micromolar is established and not further altered until re-uptake ceases due to anerobiosis or by otherwise interfering with the subsequent activity of the Ca^{2+} pump. Mg^{2+} depletion continues during the steady-state period and can become maximal prior to exhaustion of O_2. At higher levels of the ionophore, the rate of Ca^{2+} loss exceeds that which can be reversed by the Ca^{2+} pump and so both Ca^{2+} and Mg^{2+} can be depleted promptly. From these considerations as well as others (Pfeiffer and Lardy, 1976), we see no support for an alternative interpretation that Ca^{2+} depletion in A23187-treated mitochondria occurs due to a general increase in membrane permeability which has been proposed to be secondary to Mg^{2+} depletion (Binet and Volfin, 1975).

A number of aspects of the data in Figure 3 requires additional discussion. In the region of A23187 concentration between 0.05 and 0.2 nmol per mg of protein, the observed rates of respiration are presumably limited by the rate of energy-dependent Ca^{2+} uptake which is in turn limited by the rate of efflux produced by the ionophore. If it is assumed that the ionophore is "saturated" under these conditions (further increasing the mitochondrial Ca^{2+} content gives no further respiration increase) and that the Ca^{2+} to O ratio equals 4, then an apparent rate constant or turnover number for A23187-mediated Ca^{2+} transport can be calculated. From the slope of the double-reciprocal plot, the value is 44 per s per pair of A23187 molecules. It is then necessary to explain why the level of the ionophore which completely releases respiration (0.22 nmol per mg) must be increased by about eightfold to effect the complete release of 25 nmol per mg of protein of Ca^{2+} during the period of accelerated respiration. Since a sustainable increase in the Ca^{2+}/O ratio produced by elevating the Ca^{2+} concentration beyond that required maximally to release respiration seems improbable, a reduction in the turnover number of the ionophore is indicated. Such a reduction in turn could result from the collapse of ΔpH across the inner membrane brought about by the uncoupling due to Ca^{2+} cycling. That condition would be expected to diminish the ability of the ionophore to complex Ca^{2+} on the inner surface of the inner membrane by favoring the protonated over the carboxylate anion form of the compound. As seen in Figure 3, this condition can be overcome by a further increase in ionophore concentration. More generally, the turnover number of this ionophore could be limited by the rate constants of the ion complexing or decomplexing reactions, or by the diffusion constants for the passage of the free acid or metal ion complex through the membrane, together with the equilibrium constants which establish the level of the species involved in the rate-limiting step. Clearly further work is required before the overall transport process can be treated quantitatively. In the meantime, the above considerations indicate that investigators interested in determining the actions of A23187 on a cellular level could expect the ionophore to produce more rapid fluxes of cations across subcellular membranes which possess a ΔpH. The overall transport process produced by A23187 is also sufficiently complex that the linearity of the double-reciprocal plot in Figure 3 need not indicate that it is a monomeric cation complex of A23187 which is transporting Ca^{2+}. This linearity could occur with the proposed dimeric complex being responsible for transport if, for instance, the second stepwise binding constant for formation of the dimeric complex were greater than the first or from kinetic considerations such as the decomplexing reactions being rate limiting. Thus, we continue to find no objection to the hypothesis that A23187 catalyzes an electroneutral exchange of Me^{2+} for $2H^+$ via formation of a charge neutral complex of the type Me^{2+} $(A23187^-)_2$ (Reed and Lardy, 1972a).

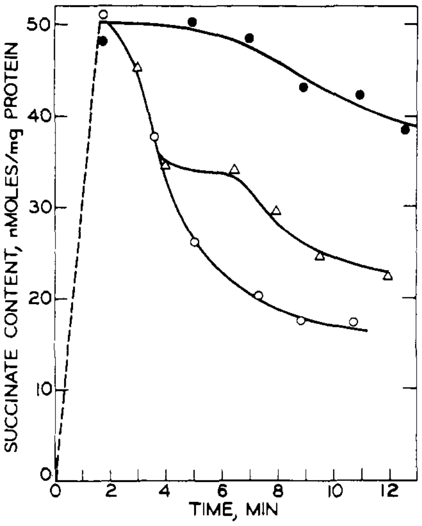

FIGURE 6: The effect of a low level of A23187 on the mitochondrial content of C-4 dicarboxylic acids. Mitochondria were incubated at 2.5 mg of protein per ml in the standard reaction medium which contained in addition 1,4-^{14}C-succinate at 42 mCi per mol. The mitochondrial content of succinate (plus metabolites) was determined on aliquots as described in Materials and Methods. (●) No further additions; (△) 0.15 nmol of A23187 per mg of protein added at 2 min; (○) the same as for △, except the medium contained, in addition, EGTA at 20 nmol per mg of protein. Without further additions or in the presence of A23187 plus EGTA, the mitochondria remained aerobic throughout the experiment. In the presence of A23187 alone, anaerobiosis occurs at about 7 min.

A final consideration in Figure 3 is the apparent lag between 0 and 0.06 nmol of A23187 per mg of protein before the ionophore elevates respiration above a state 4 level. Other investigators have reported that state 4 respiration can support a low rate of Ca^{2+} uptake and that a portion of state 4 respiration is referable to recycling of endogenous Ca^{2+} (Stucki and Ineichen, 1974; Drahota et al., 1965; Carafoli et al., 1965). Since there is no reason to assume that levels of A23187 below 0.06 nmol per mg of protein are not active for inducing Ca^{2+}

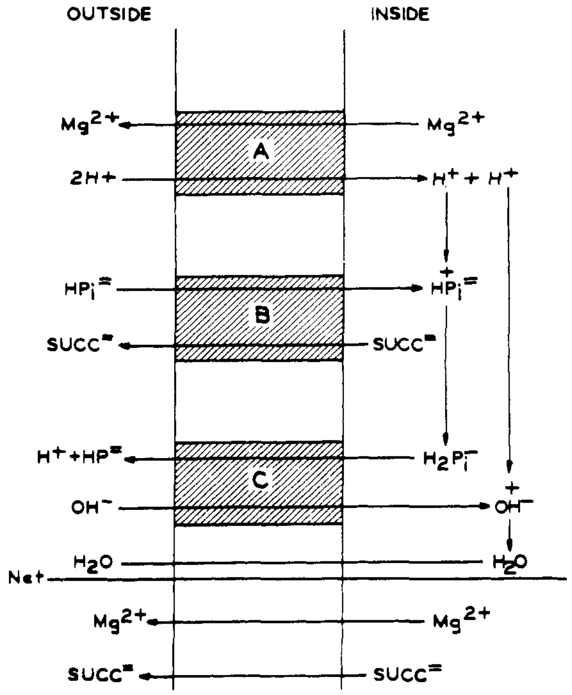

FIGURE 7: The relationship between the movements of Mg^{2+}, H^+, succinate, and phosphate in mitochondria treated with A23187.

efflux, this lag period represents further support for the concept of diversion of state 4 respiration to support Ca^{2+} uptake. Indeed, extrapolating the data at higher levels of the ionophore suggests that essentially all the energy generated by state 4 respiration can be diverted to Ca^{2+} uptake as the medium Ca^{2+} concentration increases.

The Interrelationship between A23187-Induced Divalent Metal Movements and the Secondary Movements of Anions. The depletion of mitochondrial Ca^{2+} and Mg^{2+} produces a substantial depletion of mitochondrial anions. In the case of Mg^{2+} depletion, the present data indicate that all of the ionophore mediated proton uptake is reversed by the secondary depletion of mitochondrial P_i and dicarboxylates. A proposed mechanism for coupling the ionophore-induced exchange of intramitochondrial Mg^{2+} for two extramitochondrial H^+ with subsequent reversal of the net H^+ uptake by anion depletion is shown in Figure 7. In this figure, A represents two molecules of A23187 producing an electroneutral exchange of Mg^{2+} for $2H^+$; B represents the phosphate–dicarboxylate exchanger catalyzing the neutral exchange of dicarboxylate for dibasic phosphate; and C represents the phosphate hydroxide exchanger catalyzing the neutral exchange of monobasic phosphate for OH^- (Chappell, 1968; Papa et al., 1970; Palmieri et al., 1970; McGivan and Klingenberg, 1971; Hock et al., 1971; Coty and Pedersen, 1974). Following the alterations in ΔpH produced by A23187-induced exchange of Mg^{2+} for $2H^+$, the activity of the two endogenous anion exchangers, cycling endogenous phosphate catalytically can produce a net loss of succinate with recovery of the initial value for ΔpH. As indicated in Figure 7, no coupled or net movement of H_2O is required by the scheme (other than to maintain osmotic equilibria) since H_2O is permeant to the inner mitochondrial membrane. Alternatively, the phosphate–hydroxide exchanger alone could reverse the initial reduction in ΔpH by producing a net loss of phosphate derived from the free matrix pool or from hydrolysis of endogenous nucleotides. The data in Figures 5 and 6 and in Table I indicate that Mg^{2+} depletion produced by ionophore alone is balanced largely by net succinate depletion in the normal medium with a smaller net depletion of P_i. In the presence of EGTA, A23187 does not produce hydrolysis of endogenous nucleotides (Walajtys et al., 1974) and so less matrix P_i is available for net depletion. This condition, together with immediate depletion of endogenous Ca^{2+}, is responsible for the enhanced depletion of succinate in the presence of EGTA seen in Figure 6.

The present data show a balance for charge and mass between the A23187-induced movements of Mg^{2+}, H^+, and anions which is complete within experimental error. Demonstrating a similar balance for A23187-induced depletion of Ca^{2+} is more difficult since the complete reaction for Ca^{2+} uptake is uncertain. During energy-dependent Ca^{2+} uptake in this medium, there is a net extrusion of one H^+ per Ca^{2+} accumulated. To what extent and by what means charge neutrality is maintained during Ca^{2+} uptake by mitochondria is not yet clear, but the present data has some bearing on this problem. According to many formulations of the chemiosmotic hypothesis for energy coupling in mitochondria, $2H^+$ are extruded electrogenically from mitochondria per electron pair per coupling site. In addition, it is widely accepted that overall mitochondria can accumulate two Ca^{2+} ions per electron pair per coupling site (see Lehninger, 1967). The present data demonstrate that, during the cyclic uptake and release of Ca^{2+} produced by low levels of A23187 and the Ca^{2+} pump, an apparent steady state is maintained between the movement of Ca^{2+}, H^+, P_i, and succinate (or its metabolites), providing the loss of P_i and succinate due to Mg^{2+} depletion is accounted for. In addition, no significant depletion of mitochondrial K^+ occurs under these conditions (Pfeiffer and Lardy, 1976). Thus, to avoid a large transmembrane charge imbalance accumulating during this steady state, we must assume that the total ion movements associated with Ca^{2+} uptake are electroneutral overall under these conditions. At a Ca^{2+} per site ratio of 2, overall electroneutrality for Ca^{2+} uptake would require a H^+ per site ratio of 4. Recent work by others has also indicated that the H^+ per site ratio is 4 rather than 2 (Brand et al., 1976a,b). In agreement with previous reports (Rossi et al., 1967), we find that Ca^{2+} uptake by mitochondria under limited loading conditions produces an accompanying accumulation of succinate, presumably by combined action of the phosphate–carboxylate and phosphate–hydroxide exchanges (Pfeiffer, unpublished results). This accumulation is reversed by A23187 in a manner similar to that indicated in Figure 7 when the net H^+ movement shown in Figure 5 is accounted for. The continuation of these studies should be useful for clarifying the overall reaction of Ca^{2+} uptake by these mitochondria.

A23187 is proving to be widely useful in studies on Ca^{2+} involvement in cellular excitation phenomena. The effects of this compound on mitochondria most certainly are involved at the cellular level and an increase in cytosolic Ca^{2+} concentration at the expense of intramitochondrial Ca^{2+} is among these effects. However, other effects of the ionophore (e.g., changes in energy utilization, ATP/ADP ratios or levels of free phosphate and tricarboxylic acid cycle intermediates altered secondarily to movements of Ca^{2+} and Mg^{2+} as well as the possibility of monovalent cation transport by the compound (Pfeiffer and Lardy, 1976)) may be rather important in its action at a cellular level and should be considered in interpreting effects of the ionophore on cellular systems.

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References

Binet, A., and Volfin, P. (1975), *FEBS Lett. 49,* 400.

Borle, A. D. (1973), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 1944.

Brand, M. D., Chen, C.-H., and Lehninger, A. L. (1976a), *J. Biol. Chem. 251,* 968.

Brand, M. D., Reynafarje, B., and Lehninger, A. L. (1976b), *Proc. Natl. Acad. Sci. U.S.A. 73,* 437.

Carafoli, E., Rossi, C. S., and Lehninger, A. L. (1965), *Biochem. Biophys. Res. Commun. 19,* 609.

Case, G. D. (1975), *Biochim. Biophys. Acta 375,* 69.

Case, G. D., Vanderkooi, J. M., and Scarpa, A. (1974), *Arch. Biochem. Biophys. 161,* 174.

Caswell, A. H. (1972), *J. Membr. Biol. 7,* 345.

Caswell, A. H., and Pressman, B. C. (1972), *Biochem. Biophys. Res. Commun. 49,* 292.

Chaney, M. O., Demarco, P. V., Jones, N. D., and Occolowitz, J. L. (1974), *J. Am. Chem. Soc. 96,* 1932.

Chappell, J. B. (1968), *Br. Med. Bull. 24,* 150.

Coty, W. A., and Pedersen, P. L. (1974), *J. Biol. Chem. 249,* 2593.

Deber, C. M., and Pfeiffer, D. R. (1976), *Biochemistry 15,* 132.

Drahota, Z., Carafoli, E., Rossi, C. S., Gamble, R. L., and Lehninger, A. L. (1965), *J. Biol. Chem. 240,* 2712.

Hock, J. B., Lofrumento, N. E., Meyer, A. J., and Tager, J. M. (1971), *Biochim. Biophys. Acta 226,* 297.

Hyono, A., Hendriks, Th., Daemen, F. J. M., and Bonting, S. L. (1975), *Biochim. Biophys. Acta 389,* 34.

Johnson, D., and Lardy, H. A. (1967), *Methods Enzymol. 10,* 94.

Kafka, M. S., and Holz, R. W. (1976), *Biochim. Biophys. Acta 426,* 31.

Lehninger, A. L. (1967), *Adv. Enzymol. 29,* 259.

McGivan, J. D., and Klingenberg, M. (1971), *Eur. J. Biochem. 20,* 392.

Mela, L., and Chance, B. (1968), *Biochemistry 7,* 4059.

Palmieri, F., Quagliariello, E., and Klingenberg, M. (1970), *Eur. J. Biochem. 17,* 230.

Papa, S., Lofrumento, N. E., Quagliariello, E., Meijer, A. J., and Tager, J. M. (1970), *Bioenergetics 1,* 287.

Parvin, R., and Smith, R. A. (1969), *Anal. Biochem. 27,* 65.

Pfeiffer, D. R., and Lardy, H. A. (1976), *Biochemistry 15,* 935.

Pfeiffer, D. R., Reed, P. W., and Lardy, H. A. (1974), *Biochemistry 13,* 4007.

Puskin, J. S., and Gunter, T. E. (1975), *Biochemistry 14,* 187.

Reed, P. W., and Lardy, H. A. (1972a), *J. Biol. Chem. 247,* 6970.

Reed, P. W., and Lardy, H. A. (1972b), in The Role of Membranes in Metabolic Regulation, Mehlman, M. E., and Hanson, R. W., Ed., New York, N.Y., Academic Press, p 111.

Reed, P. W., Pfeiffer, D. R., and Lardy, H. A. (1975), in Proceedings of the Second Annual New England Bioengineering Conference, Peura, R. E., et al., Ed., Burlington, Vt., Vermont University Press, p 73.

Rossi, C., Azzone, G. F., and Azzi, A. (1967), *Eur. J. Biochem. 1,* 141.

Rottenberg, H., and Scarpa, A. (1974), *Biochemistry 13,* 4811.

Scarpa, A. (1974), *Biochemistry 13,* 2789.

Schuster, S. M., and Olson, M. S. (1974), *J. Biol. Chem. 249,* 7151.

Simkiss, K. (1974), *Endeavor 33,* 119.

Sordahl, L. A. (1974), *Arch. Biochem. Biophys. 167,* 104.

Stucki, J. W., and Ineichen, E. A. (1974), *Eur. J. Biochem. 48,* 365.

Walajtys, E. I., Gottesman, D. P., and Williamson, J. R. (1974), *J. Biol. Chem. 249,* 1857.

Wohlrad, H. (1974), *Biochemistry 13,* 4014.

Wong, D. T., Wilkinson, J. R., Hamill, R. L., and Horng, J. (1973), *Arch. Biochem. Biophys. 156,* 578.