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ISOLATION AND CHARACTERIZATION OF A Ca^{2+} CARRIER CANDIDATE FROM CALF HEART INNER MITOCHONDRIAL MEMBRANE

by

Arco Yingcheu Jeng

Submitted in Partial Fulfillment

of the

Requirements for the Degree

DOCTOR OF PHILOSOPHY

Supervised by: Dr. Adil E. Shamoo

Department of Radiation Biology and Biophysics

School of Medicine and Dentistry

The University of Rochester

Rochester, New York

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DISCLAIMER

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Dedicated to

my father, Sek Fai Jeng

my mother, Su Yar Young Jeng

and

my wife, Er An Hsu Jeng

獻給

爸媽 鄭錫輝先生

鄭楊淑雅夫人

及

愛妻 鄭許爾安女士

VITAE

The author was born in Canton, China on May 5, 1947. He received all his education through college in Taiwan. He graduated with a Bachelor of Science degree in Physics from the National Taiwan Normal University, Taipei, Taiwan in 1970. He had taught mathematics and physics in a high school for two years as an obligatory duty of his college education before he came to the U.S.A.

The author married Er An Hsu in August 1971 and their first son, Bennie H., was born in January 1973.

In September 1972, the author enrolled as a graduate student in the Department of Physics at Brooklyn College of the City University of New York, and he was supported by a teaching assistantship there. He obtained a Master of Science degree in Physics in 1974. In June 1974 he began his graduate work in biophysics in the Department of Radiation Biology and Biophysics at the University of Rochester. He was supported by the Department in his tenure there. His research activities during this period were supervised by Dr. Adil E. Shamoo. He received his Master of Science degree in Biophysics from the University of Rochester in 1977.

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ABSTRACT

A protein was isolated from calf heart inner mitochondrial membrane with the aid of an electron paramagnetic resonance assay based on the relative binding properties of Ca^{2+}, Mn^{2+}, and Mg^{2+} to the protein. The molecular weight of this protein was estimated to be about 3000 by urea/sodium dodecyl sulfate gel electrophoresis and amino acid analysis. The protein had two classes of binding sites for Ca^{2+} by flow dialysis studies. The dissociation constants of the high- and low- affinity binding sites for Ca^{2+} were 9.5 and 33 μM, respectively. This protein could extract Ca^{2+} into an organic phase. The selectivity sequence of this protein determined from the organic solvent extraction experiments showed that it favored divalent cations over monovalent cations. Also, the relative selectivity sequence for divalent cations was Ca^{2+}, $Sr^{2+} > Mn^{2+} > Mg^{2+}$. Ruthenium red and La^{3+} were shown to inhibit the protein-mediated extraction of Ca^{2+} into the organic solvent. The calcium translocation in a Pressman cell mediated by this protein was selectively driven by a hydrogen ion gradient, with a higher pH on the donor aqueous phase. When the pH gradient was reversed, no Ca^{2+} translocation occurred. Also, no Ca^{2+} translocation was observed when a gradient of Na^{+}, K^{+}, or Ca^{2+} was substituted for the H^{+} gradient.

The isolated protein was found to be contaminated with a large amount of phospholipids, which was consistent with the fact that no extra lipophilic anions were required to extract Ca^{2+} into the organic phase. It was found that there were 150 moles of phospholipids associated with each mole of the protein. Partial delipidation of the protein was performed by using either the organic solvent extraction procedure or the silicic acid column chromatography. Control experiments indicated that the Ca^{2+} transport properties of the isolated protein were not due to the contaminating phospholipids.

A complete delipidation procedure was developed by using Sephadex LH-20 column chromatography. The mole ratio of phospholipids to the delipidated protein could be reduced to 0.1 mole of phospholipids per mole of protein. There were no free fatty acids, hexosamines, or sialic acids associated with the delipidated protein. The extraction of Ca^{2+} into an organic phase mediated by the delipidated protein required the presence of a lipophilic anion, picrate. Picrate also enhanced the rate of the delipidated protein-mediated Ca^{2+} translocation through a bulk organic phase.

Further characterization of the physical and chemical properties of the delipidated protein were investigated. It was found that the delipidated protein becomes more hydrophobic in the presence of Ca^{2+} and alkaline pH in the organic solvent extraction experiments. The pH

profile of the mole ratio of Ca^{2+} to protein exhibited a typical titration curve, showing a pK_a of 8.0-8.1. The relative cation selectivity of the delipidated protein determined from the organic solvent extraction experiments was $Zn^{2+} > Ca^{2+}, Sr^{2+} > Rb^{2+}, Na^{+} > Mn^{2+}$. Ruthenium red and La^{3+} were again shown to inhibit the protein-mediated Ca^{2+} extraction into the organic phase. Respiratory inhibitors, oligomycin, and an uncoupling agent had no effect on the Ca^{2+} extraction. Phosphate did not stimulate the protein-mediated Ca^{2+} extraction.

The Ca^{2+}-protein complex appears to have two positive charges. The delipidated protein only had one class of Ca^{2+}-binding sites as revealed from the flow dialysis studies. These Ca^{2+}-binding sites had a dissociation constant of 5.2 μM and bound 1 mole of Ca^{2+} per mole of calciphorin. Evidence suggests that this calcium ionophore protein (named "calciphorin") may be a strong candidate for the Ca^{2+} carrier responsible for the influx mechanism in mitochondrial Ca^{2+} transport system. Two possible models of calciphorin-mediated Ca^{2+} transport in mitochondria are proposed.

TABLE OF CONTENTS

CHAPTER 1

ISOLATION OF A CALCIUM IONOPHORE FROM CALF HEART INNER MITOCHONDRIAL MEMBRANE

CHAPTER 2

THE ELECTROGENICITY OF THE DELIPIDATED CALCIUM IONOPHORE

CHAPTER 3

FURTHER CHARACTERIZATION OF THE DELIPIDATED CALCIUM IONOPHORE

LIST OF TABLES

CHAPTER 3

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

LIST OF ABBREVIATIONS

ATP:	adenosine triphosphate
ATPase:	adenosine triphosphatase
BHT:	butylated hydroxytoluene
BSA:	bovine serum albumin
CCCP:	carbonyl cyanide m-chlorophenylhydrazone
C/M:	chloroform/methanol
DOC:	deoxycholic acid
EDTA:	ethylenediamine tetraacetic acid
EPR:	electron paramagnetic resonance
K DOC:	potassium deoxycholate
MES:	2 (N-morpholino) ethane sulfonic acid
Oe:	oersted
PC:	phosphatidylcholine
PE:	phosphatidylethanolamine
RR:	ruthenium red
SDS:	sodium dodecyl sulfate
TEMED:	N,N, N',N',-tetramethyl ethylene diamine
TLC:	thin-layer chromatography
Tris:	tris(hydroxymethyl) aminomethane

BACKGROUND

General features of Ca^{2+} transport in mitochondria.

The interaction between Ca^{2+} and mitochondria has long been recognized since the early 1940's (DuBois and Potter, 1943). It was not until 1962 that mitochondria were found to be able to accumulate large amounts of Ca^{2+} (Vasington and Murphy, 1962). Since then, Ca^{2+} transport in mitochondria has been the subject of extensive study. Several recent reviews give a detailed analysis and critical evaluation of the large body of information accumulated to date (Mela, 1977; Bygrave, 1977, 1978; Carafoli and Crompton, 1978).

In general, the interaction of Ca^{2+} with mitochondria can be divided into two main categories: energy-linked accumulation and energy-independent binding. The energy-linked accumulation of Ca^{2+} in mitochondria can be supported by either respiration (Lehninger et al., 1967), or ATP hydrolysis (Lehninger et al., 1967; Spencer and Bygrave, 1973). In both cases, Ca^{2+} is known to be accumulated with high affinity (Carafoli and Azzi, 1972; Spencer and Bygrave, 1973), high rate (Vinogradov and Scarpa, 1973; Spencer and Bygrave, 1973), and high capacity in the presence of phosphate (Lehninger et al., 1967; Spencer and Bygrave, 1973). Respiration-supported Ca^{2+} accumulation is found to be inhibited by lanthanides (Mela, 1968, 1969; Vainio et al., 1970; Reed and Bygrave, 1974c), ruthenium red (Moore, 1971; Reed and Bygrave, 1974c), uncouplers of oxidative phosphorylation and inhibitors of respiration

(Carafoli, 1975), but it is not inhibited by oligomycin (Carafoli, 1975). On the other hand, Ca^{2+} accumulation supported by ATP hydrolysis is known to be inhibited by the phosphorylation inhibitor, oligomycin, but not by respiratory inhibitors (Spencer and Bygrave, 1973).

The energy-independent binding has been studied by many authors (Rossi et al., 1967; Scarpa and Azzone, 1968, 1969; Reynafarje and Lehninger, 1969). Scarpa and Azzone (1968) showed that the Ca^{2+} bound at the mitochondrial surface under anaerobic conditions was then translocated into the mitochondria when respiration was initiated. Furthermore, they showed that the rate of aerobic Ca^{2+} translocation was related to the amount of Ca^{2+} bound to the mitochondrial surface. Later, Reynafarje and Lehninger (1969) found high and low-affinity Ca^{2+}-binding sites in respiration-inhibited mitochondria. They suggested that the high-affinity Ca^{2+}-binding sites may be the Ca^{2+} transport sites in mitochondria. Also, it was suggested by Reed and Bygrave (1974c) that the low affinity binding sites are probably the Ca^{2+}-binding sites at the mitochondrial surface described by Scarpa and Azzone (1968). These results imply that Ca^{2+} transport in mitochondria is a sequential process: Ca^{2+} binds to low-affinity Ca^{2+} binding sites first, and then translocates via Ca^{2+} transport sites under aerobic conditions.

Most authors now agree that the energy - supported penetration of Ca^{2+} into mitochondria is a carrier-mediated passive process driven by a membrane potential (for review, see Bygrave, 1977; Cara-

foli and Crompton, 1978). By applying the Nernst equation in different experimental conditions, many investigators have reported the estimated membrane potentials (Mitchell and Moyle, 1969; Rottenberg and Scarpa, 1974; Nicholls, 1974; Puskin et al., 1976; Azzone et al., 1976; Massari and Pozzan, 1976). However, the application of the Nernst equation requires a knowledge of free ion concentrations both inside and outside of the membranes. Because part of the accumulated cations can be bound to fixed anionic sites in the mitochondrial membrane, or complexed with endogenous anions, e.g. phosphates (Lehninger, 1970), the extent that Ca^{2+} remains free in the intramitochondrial matrix is still a point of argument. The magnitude of the gradient against which Ca^{2+} can be transported is thus not yet certain. Rottenberg and Scarpa (1974) have suggested that a large fraction of the Ca^{2+} accumulated in the absence of permeant anions is free in mitochondrial matrix, while Gunter and Puskin (1972) observed from the electron paramagnetic resonance (EPR) spectra that Mn^{2+}, a Ca^{2+} analog, is mostly bound in regions of high local concentrations within the mitochondria. The latter data may represent a more realistic picture since free and bound Mn^{2+} can be distinguished by using EPR spectroscopy (Gunter and Puskin, 1972).

The specificity of divalent cations accumulated in mitochondria has been determined by several groups (for review, see Bygrave,1977). Vainio et al. (1970) used dual-wavelength spectroscopy and murexide to measure the rates of oxidation of cytochrome b induced by

divalent cations in rat liver mitochondria. The specificity sequence arrived at is: $Ca^{2+} > Sr^{2+} > Mn^{2+} > Ba^{2+} . Mg^{2+}$ is not accumulated in rat liver mitochondria under the conditions favorable for the uptake of Ca^{2+} and Sr^{2+} (Lehninger et al.,1967). Although Mg^{2+} accumulation in beef heart mitochondria has been observed (Brierly et al., 1962), there is no evidence that the Mg^{2+} uptake is a carrier-mediated process (Brierly, 1976). Small amounts of La^{3+} have also been found to be accumulated by rat liver mitochondria (Reed and Bygrave, 1974b). It has been suggested that the transport of Ca^{2+}, Sr^{2+}, and Mn^{2+} may operate by a similar mechanism (Carafoli, 1965; Vinogradov and Scarpa, 1973). Kinetic studies have shown a sigmoidal curve of Ca^{2+} uptake versus Ca^{2+} concentration plot (Vinogradov and Scarpa, 1973; Spencer and Bygrave, 1973). In fact, it is almost certain that two molecules of Ca^{2+} must be bound to the transport system to have Ca^{2+} transported at significant rates (Vinogradov and Scarpa, 1973; Reed and Bygrave, 1975).

The mechanism of Ca^{2+} transport in mitochondria is not certain. The uniport mechanism has been proposed by Selwyn et al. (1970), Lehninger (1974), and Rottenberg and Scarpa (1974). The antiport mechanism has been suggested by Moyle and Mitchell (1977a, 1977b, 1977c). Recent studies favor that an influx and an efflux mechanisms are responsible for regulating the Ca^{2+} transport in mitochondria (Puskin et al., 1976; Crompton et al., 1977, 1978; Gunter et al., 1978; Akerman, 1978).

Evidence of the Ca^{2+} carrier in mitochondria

The first evidence for the Ca^{2+} carrier in mitochondria is that mitochondria show high specificity and high affinity in taking up divalent cations. Only three divalent cations (Ca^{2+}, Sr^{2+}, and Mn^{2+}) are accumulated significantly by intact rat liver mitochondria in an energy-linked process stoichiometric with electron transport (Lehninger et al., 1967). The specificity among these three divalent ions is $Ca^{2+} > Sr^{2+} > Mn^{2+}$ (Vainio et al.,1970). The initial rate versus Ca^{2+} concentration plot shows a saturation phenomenon (Vinogradov and Scarpa, 1973; Spencer and Bygrave, 1973) which is thought to be a general characteristic of carrier-mediated transport processes (Wyssbrod et al., 1971).

Substrate competition has been reported by Carafoli (1965). Carafoli showed that when mitochondria were incubated simultaneously with Sr^{2+} and Ca^{2+}, uptake of each ion was found not to be influenced by the presence of the other ion when their concentrations were 0.5 mM or less. At higher concentrations, Ca^{2+} uptake was still almost maximal, whereas the Sr^{2+} uptake was inhibited by Ca^{2+}.

Another piece of evidence for the existence of a Ca^{2+} carrier in the mitochondrial membranes is obtained from the dose response relationship between lanthanide concentration and the rate of Ca^{2+} accumulation (Mela, 1968, 1969). By measuring the oxidation-reduction change of the respiratory chain carriers during Ca^{2+} accumulation, Mela (1969) found that lanthanides at an extrapolated concentration of about 0.06 nmoles per mg of

mitochondrial protein specifically inhibited Ca^{2+} uptake. This number, 0.06 nmoles per mg of mitochondrial protein, has been used by Mela (1969) as the concentration of Ca^{2+} carrier sites in mitochondria.

The genetic relationship between the high-affinity Ca^{2+} binding in respiration-inhibited mitochondria and their ability to accumulate Ca^{2+} provides another hint for the existence of the carrier. Using Scatchard plots, Reynafarje and Lehninger (1969) have reported that there are high-affinity Ca^{2+}-binding sites in respiration-inhibited rat liver mitochondria. Carafoli and Lehninger (1971) found that mitochondria from all vertebrate tissues they examined showed high-affinity Ca^{2+} binding. However,mitochondria from yeast and blowfly flight muscle, which are unable to accumulate Ca^{2+} in a respiration-dependent process, were reported to possess no high-affinity Ca^{2+}-binding sites (Carafoli and Lehninger, 1971).

Estimated number of Ca^{2+} carrier sites in mitochondria.

The first attempt in estimating the number of Ca^{2+} carrier sites was made by Reynafarje and Lehninger (1969). Using Scatchard plots, Reynafarje and Lehninger (1969) have reported that respiration-inhibited rat liver mitochondria possess two major classes of Ca^{2+}-binding sites with widely different affinities. They further found important similarities between the respiration-inhibited high-affinity Ca^{2+} binding and the energy-linked Ca^{2+} uptake. These observations have led Reynafarje and Lehninger (1969) to conclude that the high-affinity Ca^{2+} binding sites are identical to the Ca^{2+} carrier sites.

The number of high-affinity Ca^{2+}-binding sites, 1.2 nmoles per mg of mitochondrial protein, is therefore taken by Reynafarje and Lehninger (1969) as the number of Ca^{2+} carrier sites.

By measuring the oxidation-reduction change of the respiratory chain carriers during Ca^{2+} accumulation, Mela (1969) found that lanthanides at an extrapolated concentration of about 0.05-0.07 nmoles per mg of mitochondrial protein specifically inhibited Ca^{2+} uptake. Thus, this number, 0.05-0.07 nmoles per mg of protein, has been used by Mela (1969) as the concentration of Ca^{2+} carriers that exists in mitochondria.

Ruthenium red has recently been found to be another potent inhibitor of mitochondrial Ca^{2+} transport (Moore, 1971; Reed and Bygrave, 1974a, 1974 c). By applying Henderson's kinetic analysis of tightly bound inhibitors (Henderson, 1972), Reed and Bygrave (1974c) were able to estimate the concentration of ruthenium red-sensitive Ca^{2+}-binding sites in mitochondria. The number they estimated was 0.08 nmoles per mg of protein, which is approximately the same as the number of carrier sites estimated by Mela (1969) from the lanthanide experiments. In the same paper, Reed and Bygrave (1974c) used the same method to estimate La^{3+}-sensitive Ca^{2+}-carrier sites in mitochondria. The number they published was less than 0.001 nmole per mg of protein. However, there was a miscalculation in this number. The corrected value should be less than 0.0001 nmole per mg of protein , an extremely small number compared to 0.4 nmoles per mg of protein for adenine nucleotide translocase

estimated by Henderson (1972) and 0.28 nmoles per mg of protein for both cytochrome b and cytochrome oxidase estimated by Williams (1968). The main source of error in the estimation of the number of La^{3+}-sensitive Ca^{2+} carrier sites by Reed and Bygrave came from the applicability of Henderson's derivation under their experimental conditions. There are two main assumptions in Henderson's simplified deriviation. These are the enzyme-inhibitor complex is a 'dead-end' complex and that only one molecule of the inhibitor combines with a single form of the enzyme. While the ruthenium red experiments did not seem to be too far away from these criteria, the La^{3+} experiments did. La^{3+} has been shown to be accumulated by intact mitochondria (Reed and Bygrave, 1974b). Thus, the La^{3+}-carrier complex can not be 'dead end'. Also, Ca^{2+} and Mn^{2+}, which are known to be accumulated by intact mitochondria, show positive cooperativity (Vinogradov and Scarpa, 1973). Two molecules of Ca^{2+} or Mn^{2+} are found to bind to the transport system in the process of ion translocation (Vinogradov and Scarpa, 1973). Although there is no direct evidence regarding how many La^{3+} ions are needed to bind to the transport system when translocation of La^{3+} occurs, it seems reasonable to assume that more than one is needed. Thus the use of Henderson's derivation to estimate La^{3+}-sensitive Ca^{2+} carrier concentration is questionable.

The number of Ca^{2+} carrier sites appears to be between 0.05 to 1.0 nmole per mg of mitochondrial protein, and is probably closer to the former value.

Mechanism of Ca^{2+} transport in mitochondria.

The most convincing data showing that Ca^{2+} transport in mitochondria is a passive process, driven by a membrane potential across the inner mitochondrial membrane, has been demonstrated by Scarpa and Azzone (1970). They found that Ca^{2+} translocation into mitochondria could be initiated by adding valinomycin in the suspension medium to induce the efflux of K^+. Since K^+ is thought to be translocated independently via valinomycin, the only possible coupling between K^+ efflux and Ca^{2+} uptake under this condition is believed to be the membrane potential across the inner mitochondrial membrane generated by the diffusion of K^+. Recently, the driving force for Ca^{2+} transport in inner mitochondrial membrane vesicles has also been demonstrated to be the negative-inside membrane potential (Niggli et al., 1978).

Selwyn et al. (1970) was the first group to investigate whether the passive Ca^{2+} movement into non-respiring mitochondria down an electrochemical gradient occurred via a neutral antiport ($Ca^{2+}/2H^+$ or $Ca^{2+}/2K^+$), an electrogenic antiport (Ca^{2+}/H^+ or Ca^{2+}/K^+), or an electrogenic uniport mechanism. They used light scattering to monitor respiration-inhibited mitochondrial swelling induced by high external concentrations of various Ca^{2+} salts. Swelling should have occured only when both pH balance and charge balance are satisfied. In the presence of acetate, Selwyn et al. (1970) found that swelling did not occur. However, swelling did occur in the acetate system when an uncoupler which could act to

transport H^+ was added. Acetate is known to penetrate the membrane only in its undissociated free acid form (Chappell, 1968). This finding showed that the carrier could not be a $Ca^{2+}/2H^+$ antiporter, since this mechanism would balance both charge and pH; and the mitochondria should have swollen before the addition of uncoupler. They also found that thiocyanate induced swelling to the same degree both in the presence and the absence of an uncoupler. These results showed that a neutral antiport $Ca^{2+}/2K^+$ was not likely, since the replacement of K^+ by divalent Ca^{2+} would have lowered the osmolarity of the intramitochondrial fluid and caused the shrinkage of the mitochondria. According to Selwyn et al. (1970), the thiocyanate experiment also ruled out Ca^{2+}/H^+ antiport mechanism, because this mechanism would have resulted in an imbalance of pH and hence inhibited the swelling due to lowering the availability of protons for exchange with the Ca^{2+} ions. However, Puskin et al. (1976) suggested that this Ca^{2+}/H^+ antiport could occur in conjunction with a substantially larger Ca^{2+} uniport activity. The mechanisms which were not ruled out by Selwyn et al. (1970) were uniport and Ca^{2+}/K^+ electrogenic antiport.

Rottenberg and Scarpa (1974) measured the distributions of Ca^{2+} and Rb^+ (in the presence of valinomycin) across the mitochondrial membrane. They found that the distribution ratio for Ca^{2+} was about the same as the square of the distribution ratio for Rb^+. Assuming that the Rb^+ distribution in the presence of valinomycin was governed by membrane potential obeying the Nernst equation, Rotten-

berg and Scarpa (1974) interpreted their results as evidence that Ca^{2+} uptake in mitochondria is an electrogenic process driven by membrane potential with a net charge transfer of 2.

An entirely different mechanism was proposed by Moyle and Mitchell (1977a). They estimated the electric charge stoichiometry of Ca^{2+} translocation from the number of acid equivalents that were exported through the respiratory chain system per Ca^{2+} imported through the La^{3+}-sensitive Ca^{2+} carrier. It was concluded that the specific Ca^{2+} carrier is not a Ca^{2+} uniporter, but is a Ca^{2+}-anion symporter or a Ca^{2+}/cation antiporter with a net charge transfer of one. Later, Moyle and Mitchell suggested that the Ca^{2+}-anion symporter is a Ca^{2+}-phosphate (Moyle and Mitchell, 1977b) or a Ca^{2+}-monocarboxylate (Moyle and Mitchell, 1977c) symporter. However, there are serious doubts against a Ca^{2+}-anion symporter being the mechanism for Ca^{2+} transport in mitochondria. If this mechanism were true, Azzone et al. (1977) calculated that the concentration of the anion in the matrix would be either 10 times lower or equal to that of the outer space assuming that the steady state divalent cation distribution of either 10^4 or 10^3 (Azzone et al.,1976) in the presence of a membrane potential of 180 mV. However, the distribution of phosphate was found to be opposite (Klingenberg, 1970). Another criticism of Ca^{2+}-phosphate symport mechanism comes from Reynafarje and Lehninger (1977). They showed that during Ca^{2+} uptake, a net 2 positive charges were transferred even under the experimental conditions described by Moyle and Mitchell (1977a).

There have been numerous attempts to measure the membrane potential across the inner mitochondrial membrane. The measured high values vary from 130 to 200 mV (Mitchell and Moyle, 1969; Nicholls, 1974; Azzone et al., 1976). If the uniport mechanism were the only functional mechanism, a membrane potential of 180 mV would yield an equilibrium gradient of 10^6 for Ca^{2+}. Since the divalent cation concentration in the matrix compartment is not likely to exceed a few mM (Azzone et al., 1975; Puskin et al., 1976), an accumulation ratio of 10^6 would reduce the cytoplasmic Ca^{2+} concentration to 10^{-9}M. At such a low level of Ca^{2+}, many cytoplasmic enzyme systems can not function (Carafoli and Crompton, 1978). Thus, the Ca^{2+} uniporter alone is not able to regulate the Ca^{2+} transport in mitochondria.

Puskin et al.(1976) used EPR to measure the Mn^{2+} distribution in the presence of K^+ and valinomycin. Since free Mn^{2+} in mitochondria can be measured by EPR (Gunter and Puskin, 1972), this method of calculating membrane potential is thought to be more accurate. However, the membrane potential calculated by using Mn^{2+} was usually 40-50 mV lower than that calculated from the K^+ distribution in the presence of valinomycin. This finding, together with the fact that ruthenium red addition to mitochondria after Ca^{2+} accumulation causes a slow efflux of Ca^{2+} from mitochondria (Sordahl, 1974) and alters the steady-state distribution of divalent cations across mitochondrial membranes (Puskin et al. 1976) has led Puskin et al. (1976) to suggest that there may be more than

one Ca^{2+} transport mechanism in mitochondria. One of the Ca^{2+} transport mechanisms is ruthenium red sensitive (presumably Ca^{2+} uniport) and the other is less ruthenium red sensitive.

Ca^{2+} efflux has attracted much attention recently. The efflux of Ca^{2+} from mitochondria can be induced by prostaglandins (Malmstrom and Carafoli, 1975), fatty acids (Harris, 1977), phosphoenolpyruvate (Roos et al., 1978), and Na^{+} (Crompton et al., 1976, 1977, 1978). Whether the influx and efflux mechanisms are separate entities is still debatable. While many authors favor separate mechanisms for influx and efflux (Puskin et al., 1976; Gunter et al., 1977, 1978; Crompton et al., 1977, 1978; Akerman, 1978), Pozzan et al. (1977) suggest that Ca^{2+} efflux occurs always through the native carrier responsible for the Ca^{2+} influx mechanism.

Attempts at the isolation of the Ca^{2+} carrier.

Using Scatchard plots, Lehninger and his co-workers (Reynafarje and Lehninger , (1969) Lehninger and Carafoli, 1971) have reported that respiration-inhibited rat liver mitochondria possess two major classes of Ca^{2+}-binding sites with widely different affinities. At pH 7.4, the low-affinity sites can bind about 40 nmoles of Ca^{2+} per mg of mitochondrial protein with a K_d larger than 30 μM, whereas the high-affinity sites can bind 0.1-1.0 nmoles of Ca^{2+} per mg of protein with a K_d of about 1.0 μM (Lehninger and Carafoli, 1971). Reynafarje and Lehninger (1969) also found important similarities between the respiration-inhibited high-affinity Ca^{2+} binding and the energy-linked Ca^{2+} uptake. These similarities include the number of

of binding sites for Ca^{2+}, the affinity of the binding sites, and the competitive effects of various divalent cations. These observations led Reynafarje and Lehninger to conclude that the high-affinity Ca^{2+}-binding sites are the sites of the Ca^{2+} carrier molecule.

Since the suggestion that high - affinity Ca^{2+}-binding sites are the sites of the Ca^{2+} carrier, the isolation of several high-affinity Ca^{2+}-binding proteins from mitochondria has been reported. Lehninger (1971) first extracted a soluble, heat-labile, high-affinity Ca^{2+}-binding fraction from mitochondria by using osmotic shock. From this soluble fraction, Gomez-Puyou et al. (1972) extracted an insoluble fraction, by ammonium sulfate precipitation, which contained a high-affinity Ca^{2+}-binding glycoprotein. Upon application to analytical gel electrophoresis, this insoluble fraction showed a major protein band of molecular weight around 67,000 daltons and four other minor bands . No further characteristics of this glycoprotein have been reported.

Later, Utsumi and Oda (1974) extracted from liver mitochondria a water-soluble fraction containing a glycoprotein. This fraction can bind Ca^{2+} and ruthenium red tightly and is able to reverse the inhibition of Ca^{2+} transport when added to ruthenium red-treated mitochondria. But this water-soluble fraction contains more than six bands according to SDS gel electrophoresis. It is not clear whether this fraction is identical to the water soluble Ca^{2+}-binding fraction isolated by Lehninger (1971).

Sottocasa et al.(1972) have also extracted a soluble Ca^{2+}-

binding glycoprotein from ox liver mitochondria. The protein moiety of this glycoprotein has a molecular weight of about 42,000 daltons. This glycoprotein has both high- and low-affinity Ca^{2+} binding sites as revealed from Scatchard plots. It has been strongly suggested that this glycoprotein is localized between the two membranes of mitochondria as can be realized from the method of isolation (Sottocasa et al., 1971; Sottocasa et al., 1972). They have also shown that the glycoprotein is associated with the mitochondrial membrane only when Ca^{2+} is present and is released into intermembrane space when Ca^{2+} is removed by EDTA. These findings led Sandri et al.(1976) to classify the glycoprotein as a "mobile carrier" which associates with the membrane and translocates Ca^{2+} when Ca^{2+} is present in the medium, but is localized in the intermembrane space when Ca^{2+} is not present.

The fact that ruthenium red is a potent inhibitor of Ca^{2+} transport in mitochondria (Moore, 1971) combined with the belief that ruthenium red is specific for mucopolysaccharides and glycoproteins have led Carafoli and Sottocase (Carafoli et al., 1971; Carafoli and Sottocasa, 1974) to concentrate on the isolation of glycoproteins from mitochondria. They used a chaotropic agent specific for membrane glycoproteins, lithium diiodosalicylate (Marchesi and Andrews, 1971), to extract glycoproteins. Two classes of glycoproteins with slightly different amino acid compositions have been isolated. The monomer molecular weight of the protein moiety of both glycoproteins is about 30,000 daltons. These glycoproteins have been tested on artificial

lipid bilayers for the possibility of translocating Ca^{2+} across the apolar region (Carafoli et al., 1974; Prestipino et al, 1974). An increase of membrane conductance induced by these glycoproteins in the presence of Ca^{2+} has been shown. However, no Nernst potential due to a Ca^{2+} gradient across the lipid bilayers can be measured. Also, La^{3+} inhibition of Ca^{2+} transport is not demonstrated. These observations, together with the finding that these glycoproteins are very hydrophilic, led Carafoli (1975) to postulate that these isolated glycoproteins may be superficial Ca^{2+}-binding sites and are not involved in the transmembrane translocation of Ca^{2+}. The observed increase of membrane conductance due to these glycoproteins in the presence of Ca^{2+} may indicate that Ca^{2+} is not the charge carrier species but is required for the absorption of these glycoproteins to the membrane. This may be due to the acidic nature of these glycoproteins, as well as to the presence of sialic acid in these glycoproteins.

Recently, Carafoli et al. (1977) used ^{106}Ru-ruthenium red to label mitochondria and then removed the soluble proteins by extensive sonication. A hydrophobic fraction containing a major 20,000 dalton protein and 7 other minor protein bands as identified by SDS gel electrophoresis can bind ruthenium red with high affinity. Whether this fraction is specific for Ca^{2+} has not been reported.

Another approach to the isolation of a Ca^{2+} ionophore from mitochondria is to release the ionophore by proteolytic digestion. Blondin (1974, 1975) isolated some oxyoctadecadienoic acid compounds

from a partial tryptic digestion of lipid deleted mitochondria. These compounds are able to interact with Ca^{2+} and Mg^{2+} and facilitate the transfer of Ca^{2+} and Mg^{2+} across the mitochondrial inner membrane or across a carbon tetrachloride phase separating two discontinuous aqueous compartments. Makhmudova et al. (1975) investigated the effect of these compounds on phospholipid bilayers. An increase of electrical conductance with slight cation-selectivity was reported. But the sensitivity of Ca^{2+} transport to ruthenium red was not mentioned. By using prolonged tryptic digestion, Blondin et al. (1977) could also release a K^{+}/Ca^{2+} ionophore from a 10,000 dalton protein. However, the specificity of this ionophore among divalent cations has not been investigated.

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CHAPTER 1

ISOLATION OF A CALCIUM IONOPHORE FROM CALF HEART INNER MITOCHONDRIAL MEMBRANE

SUMMARY

A protein was isolated from calf heart inner mitochondrial membrane with the aid of an electron paramagnetic resonance assay based on the relative binding properties of Ca^{2+}, Mn^{2+}, and Mg^{2+} to the protein. The molecular weight of this protein has been estimated to be about 3000 by urea/sodium dodecyl sulfate gel electrophoresis and amino acid analysis. The protein is shown to have two classes of binding sites for Ca^{2+} by flow dialysis studies and can extract Ca^{2+} into an organic phase. The selectivity sequence of this protein determined from the organic solvent extraction experiments shows that it favors divalent cations over monovalent cations. Also, the relative selectivity sequence for divalent cations is $Ca^{2+}, Sr^{2+} > Mn^{2+} > Mg^{2+}$. Ruthenium red and La^{3+} are shown to inhibit the protein-mediated extraction of Ca^{2+} into the organic solvent. The calcium translocation in a Pressman cell by this protein is selectively driven by a hydrogen ion gradient. Control experiments indicate that the Ca^{2+} transport properties of the protein are not due to the contaminating phospholipids. Evidence suggests that the isolated protein may be a Ca^{2+} carrier in the mitochondrial Ca^{2+} transport system.

INTRODUCTION

Many reactions in cells are known to be Ca^{2+} dependent. These reactions include activation of enzyme systems, activation of contractile and motile systems, hormonal regulations and several membrane-linked functions (for examples of these reactions, see Carafoli, 1976). Because of the important roles played by calcium in these reactions, the intracellular level of Ca^{2+} is thought to be very well regulated.

Based on certain assumptions, Carafoli and Crompton (1978) have calculated that in the heart cells, the total membrane area available for Ca^{2+} transport is about 12.2 m^2/gm of tissue. Among which, 87% of the total area belongs to the inner mitochondrial membrane, whereas the sarcoplasmic reticulum and the sarcolemma occupy 12% and 1%, respectively, of the total area. Thus, in tissue like heart where sarcoplasmic reticulum is scarce and is less active in the transport of Ca^{2+} than in its skeletal muscle counterpart (Tada et al., 1978), mitochondria may play an important role in sequestering Ca^{2+} (Affolter et al., 1976; Carafoli, 1974).

Ca^{2+} transport in mitochondria appears to be a carrier-mediated process (Lehninger, 1970; Selwyn et al., 1970; Carafoli and Sottocasa, 1974; Reed and Bygrave, 1974) that responds to a negative-inside membrane potential across the inner mitochondrial membrane (Scarpa and Azzone, 1970). There have been numerous attempts (Lehninger, 1971; Sottocasa et al., 1972; Gomez-Puyou et al., 1972; Carafoli and Sottocasa, 1974; Utsumi and Oda, 1974; Carafoli, 1975) to isolate a protein

with characteristics consistent with those expected of a carrier, i.e., high-affinity Ca^{2+} binding (Carafoli and Azzi, 1972; Spencer and Bygrave, 1973), high specificity for Ca^{2+} (Lehninger et al.,1967) and high sensitivity to Ca^{2+} transport inhibitors (Moore, 1971; Mela, 1968, 1969; Reed and Bygrave, 1974). Among these proteins, a 67,000 dalton protein (Gomez-Puyou et al., 1972) and a 33,000 dalton glycoprotein (Carafoli, 1975) have been implicated in Ca^{2+} transport. However, it appears that these proteins may be extrinsic membrane binding proteins rather than the Ca^{2+} carrier (Carafoli, 1976; Carafoli et al., 1974;Prestipino et al., 1974).

It has been suggested that Ca^{2+} and Mn^{2+} are transported into mitochondria via the same carrier (Lehninger and Carafoli, 1971; Vinogradov and Scarpa, 1973). Free Mn^{2+} shows sextet hyperfine spectrum in electron paramagnetic resonance (EPR) spectroscopy (Gunter and Puskin, 1972; Puskin and Gunter, 1972). When Mn^{2+} is bound to proteins (Reed and Cohn, 1970) or phospholipids (Allen et al., 1966), the EPR spectra do not show hyperfine structure. Thus, the bound Mn^{2+} can be distinguished from the free Mn^{2+} in a biological sample. Using Mn^{2+} as a Ca^{2+} analog, a novel approach of isolating a low molecular weight Ca^{2+} ionophore from calf heart inner mitochondrial membrane is reported in this chapter. The isolation method is based on the relative binding properties of Ca^{2+},Mn^{2+}, and Mg^{2+} to the protein and uses EPR spectroscopy to follow the high affinity Mn^{2+}-binding site(s) through a series of isolation steps. The possible role of this isolated protein in the mitochondrial Ca^{2+} transport system is discussed.

Some of the results described here have been reported previously (Jeng and Shamoo, 1978; Shamoo and Jeng, 1978; Jeng et al, 1978).

EXPERIMENTAL PROCEDURE

MATERIALS

$^{86}RbCl$, $^{22}NaCl$, $^{54}MnCl_2$, $^{65}ZnCl_2$ and $^{85}SrCl_2$ were purchased from New England Nuclear. $^{45}CaCl_2$ was obtained from Amersham. Sephadex G-50 (fine) was the product of Pharmacia. Silicic acid (Bio-Sil A, 100-200 mesh) was obtained from Bio-Rad. Silica gel G plates (250 μm) were products of Fisher Sci. Sequanal grade pyridine and constant boiling HCl (6N) were obtained from Pierce. Digitonin, ruthenium red, deoxycholic acid, methylene chloride, bovine serum albumin, soybean trypsin inhibitor, horse heart cytochrome c and bacitracin were all purchased from Sigma. Insulin was obtained from Lilly. All other reagents used were of the highest commercial purity available.

Phospholipase C was a generous gift from Dr. Richard D. Mavis. Digitonin and deoxycholic acid were recrystallized in ethanol before use. Ruthenium red was used without further purification.

METHODS

Isolation of calf heart mitochondria.

Calf heart mitochondria were prepared as previously described (Crane et al., 1956) with slight modification. The hearts were obtained from a slaughter house and were packed in ice immediately on the killing floor. All fat and connective tissue were trimmed off, and the muscle was cut into small pieces and passed through a precooled electric meat grinder. The material was then blended in a high speed blender. To every 100 ml of mince, 300 ml of buffer (0.25 M sucrose, 0.5 mM EDTA, and 10 mM Tris.HCl, pH 7.6-7.7) and 1 ml of 6 N KOH were added. The blender was operated for 40 seconds at full speed. The pH of the homogenate was adjusted to 7.8 with 6 N KOH. This homogenate was immediately centrifuged for 5 minutes at 3,000 rpm (1000 x g) in a Beckman JA-10 rotor. The red supernatant was decanted through a 4-fold cheesecloth and kept, and the sediment and a partially sedimented buff layer were discarded. The same centrifugation procedure was repeated. The supernatant of the second centrifugation was spun for 35 minutes at 10,000 rpm (11,000 x g) in the same rotor. The resulting supernatant was discarded and the mitochondria which had been sedimented were resuspended in one liter of buffer containing 0.25 M sucrose and 10 mM Tris.HCl, pH 7.6-7.7. The homogenized suspension was again subjected to centrifugation for 35 minutes at 10,000 rpm. The last step was repeated and the mitochondria were resuspended in a small volume of the sucrose and Tris.HCl buffer as described in

the previous step. Protein concentration was determined by the biuret method (Jacobs et al., 1956). The concentration was then adjusted to 50 mg/ml. This mitochondrial suspension was divided into several plastic bottles (50 ml each) and kept in the freezer.

Further fractionation of mitochondria.

Submitochondrial particles were prepared according to the method of Schnaitman and Greenawalt (1968). Recrystallized digitonin (1.2 mg per 10 mg of mitochondrial protein) was dissolved in a small volume of buffer solution (0.25 M sucrose and 10 mM Tris, pH 7.8) prior to use. Mitochondria were thawed on the day of the experiment. While stirring gently, ice-cooled digitonin solution was added to the mitochondria suspension. The final digitonin concentration was 0.5%. This resulting suspension was gently stirred for 15 minutes over ice and then diluted with 3 volumes of buffer solution. The diluted suspension was centrifuged for 10 minutes at 8,000 x g in a Beckman JA-20 rotor. The supernatant was carefully decanted and the pellet was gently resuspended in the same volume of isolation medium. The suspension was centrifuged again at the same speed for 10 minutes. The pellet contained submitochondrial particles and was resuspended in the buffer solution at a concentration of about 35 mg/ml.

The submitochondrial particles obtained from the previous step contain both inner mitochondrial membranes and matrix compartments. This preparation was fractionated further by treatment with 1.2 mg of Lubrol per 10 mg of protein. The final concentration of Lubrol was 0.3%. This Lubrol-treated suspension was gently stirred for 15

minutes over ice and then centrifuged at 144,000 x g for one hour. The pellet obtained is enriched with inner mitochondrial membranes (Schnaitman and Greenawalt, 1968). This pellet was resuspended in 10 mM Tris, pH 7.8 and the protein concentration was adjusted to 35 mg/ml.

Inner mitochondrial membranes were fractionated by adding 0.3 mg of potassium deoxycholate (K DOC) per mg of protein in the presence of 1 M KCl. After stirring for 15 minutes over ice, the protein suspension was centrifuged at 105,000 x g for 30 minutes. The supernatant was divided into 5 ml fractions and stored in a freezer.

For further fractionation, 5 ml of the DOC-soluble inner mitochondrial membrane protein was thawed and dialyzed against 500 ml of column buffer (1% K DOC, 0.02% NaN_3, and 10 mM Tris.HCl, pH 7.8) at room temperature for 2 hours. The dialysate was centrifuged at 105,000 x g for 30 minutes at room temperature. The supernatant was then applied to a Sephadex G-50 column equilibrated with 1% K DOC, 0.02% NaN_3, and 10 mM Tris.HCl, pH 7.8. The column was 2 m long and 2.5 cm in inner diameter. The operational pressure was usually 30 cm and the flow rate was 10 ml per hour. The elution profile was monitored by 280 nm absorbance.

EPR assay of the high-affinity Mn^{2+}-binding protein.

EPR spectroscopy was carried out at 9.5 GHz in a Varian E-12 system. The central field was 3400 gauss. The magnetic field was swept to meet the resonance conditions. To test for high-affinity binding, a suitable concentration of $MnCl_2$ was added to a sample and

and the EPR spectrum was taken. $MgCl_2$ was then added to the same sample at a concentration 50 times the $MnCl_2$ concentration and the EPR spectrum was taken again. Free Mn^{2+} concentration in the solution could be determined from the formula:

$$C = \frac{K \times I}{R \times S \times \beta}$$

where

C = free Mn^{2+} concentration in mM

K = constant

I = height of the first line

R = receiver gain

S = average height of the g=2 standard before and after the scan

β = correction factor related to the width of the lines.

The constant in the formula could be determined from the slope of the known concentration of $MnCl_2$ in aqueous solutions vs. $I/(R \times S \times \beta)$ plot. The concentration of free Mn^{2+} in the sample could then be determined. The concentration of bound Mn^{2+} was calculated from the difference of the concentrations of Mn^{2+} added to the sample and the free Mn^{2+} calculated from EPR spectrum. The calculated concentration of bound Mn^{2+} after the Mg^{2+} flush is referred to as the concentration of Mg^{2+}-irreplaceable high-affinity Mn^{2+}binding sites. The protein concentration was determined and the binding per mg of protein was then calculated. High-affinity Mn^{2+} binding was measured starting with intact mitochondria. In the binding experiments using mitochondria

and submitochondrial particles, 30 μM rotenone was added to the medium to prevent the uptake of Mn^{2+}. Fractions containing a high concentration of Mg^{2+}-irreplaceable high-affinity Mn^{2+} binding sites were chosen for further purification.

Determination of protein concentration.

When the protein concentration to be determined was in the range of 10-20 mg/ml, the biuret method (Gornall et al., 1949) was applied and crystalline bovine serum albumin (BSA) was used as protein standard. One hundred microliters of protein solution were solubilized in 3 ml of 0.2% K DOC as modified by Jacobs et al. (1956). Three milliliters of biuret solution (3% NaOH, 0.15% cupric sulfate, and 0.6% sodium potassium tartrate) was then added. Corrections were made for non-biuret color and turbidity by using appropriate blanks. The absorbance at 560 nm was measured after 20 minutes of incubation period.

Lower protein concentration (0.1-10 mg/ml) was determined according to the method of Lowry et al. (1951) The protein concentration was calculated from the absorbance at 750 nm measurements with BSA as the standard.

Gel electrophoresis and glycoprotein identification

Urea/SDS polyacrylamide gel electrophoresis was carried out according to the method of Swank and Munkres (1971). Cylindrical gels (5.5 x 120 mm) containing 10% acrylamide, 1% bis-acrylamide, 0.1% SDS, 8 M urea and 100 mM H_3PO_4 , at a final pH of 6.8 adjusted by Tris, were made on the day of electrophoresis. Gels were polymerized by the addition of 0.03% (vol/vol) TEMED and 0.02% ammonium

persulfate. The samples to be analyzed were dissolved at 2 mg/ml in 1% SDS, 8 M urea, 1% mercaptoethanol, and 0.01 M H_3PO_4 adjusted to pH 6.8 with Tris, and heated for 10 minutes at 60° prior to electrophoresis . Bromophenol blue (0.005%) was used as the tracking dye. The reservoir solution contained 0.1% SDS and 0.1 M H_3PO_4 adjusted to pH 6.8 with Tris. Gels were run at constant current of 2 mA per gel overnight and stained for two hours in a solution containing 0.25% Coomassie blue, 45% methanol, and 9% acetic acid. These gels were then destained in 10% acetic acid. Apparent molecular weights were estimated from log (molecular weight) versus relative mobility plots. Molecular weight standards used were soybean trypsin inhibitor (21,300), cytochrome c (11,700), insulin (5,700), insulin B (3,400), insulin A (2,300), and bacitracin (1,400).

Gels were also stained with periodic acid/Schiff base (Fairbanks et al., 1971) to identify glycoproteins. After electrophoresis, gels were immersed in 25% (vol/vol) isopropanol and 10% acetic acid overnight, in 10% isopropanol and 10% acetic acid for 6 hours, and then in 10% acetic acid alone for several hours until the background was clear. Afterwards, gels were dipped in the following solutions in sequence: 0.5% periodic acid for 2 hours, 0.5% sodium arsenite and 5% acetic acid for 1 hour, 0.1% sodium arsenite and 5% acetic acid for 20 minutes for 3 times, 5% acetic acid for 15 minutes, and finally, Schiff reagent (0.45% basic fuchsin, 0.9% sodium metabisulfite, and 0.09 N HCl) overnight. These gels were rinsed with 0.1% sodium metabisulfite and 0.01 N HCl until the rinse solution failed to turn pink

upon addition of formaldehyde.

Amino acid analysis.

Amino acid analysis was performed according to the method of Spackman et al. (1958). The purified protein sample (0.1 mg) was lyophilized in a vacuum reaction flask. One milliliter of Pierce constant boiling HCl (6N) was added and the liquid was deaerated under vacuum. The stopper of the reaction flask was then closed and the flask was placed in a heating block at 110°C for 24 hours. After cooling, the sample was dried in a vacuum dessicator over NaOH.The dry sample was then mixed with 100 µl of citrate starting buffer and run on a Durrum D500 analyzer.

Ca^{2+}-binding studies.

To remove the bulk of DOC, purified protein was dialyzed against 10 mM Tris.HCl, pH 7.8, for 72 hours. Ca^{2+} binding was measured by flow dialysis at 23° in a Teflon chamber set according to the method of Colowick and Womack (1969). The Teflon chamber set consisted of a lower and an upper cylindrical chamber separated by a piece of Spectrapor 2000-dalton molecular weight cutoff dialysis membrane. Both the lower and upper chambers were 9.5 mm in diameter. The depths of the lower and the upper chambers were 5 and 25 mm,respectively. Each chamber had a 7 x 2 mm Teflon flea stirrer. Protein was added to the upper chamber. The experiment was initiated by adding $^{45}CaCl_2$ to the upper chamber. Increasing amounts of non-radioactive $CaCl_2$ were added at later times to displace the ^{45}Ca bound to the protein. The flow rate of the dialysis buffer was about 1 ml per minute.

One milliliter fractions were collected in a fraction collector and 500 µl was pipetted from each tube to count the radioactivity.

Organic solvent extraction experiments.

In order to prepare the protein for these experiments, column fractions containing the pure protein were pooled and dialyzed against 50% (vol/vol) ethanol at 4° for 5 days. The resulting cloudy dialysate was centrifuged at 228,000 x g for 30 minutes. The yellowish pellet was dissolved in a small volume of sequanal grade pyridine at a concentration of 1.0 mg/ml. The experiments were carried out at room temperature. Depending on the experiments, different amounts of purified protein in pyridine were added to 2 ml of methylene chloride. One milliliter of aqueous phase containing the described concentration of radioactive cation was then added to the organic phase. The two phases were then mixed by vortexing for one minute and separated by centrifugation in a Beckman TJ-6 table-top centrifuge at 900 x g for 15 minutes at room temperature. Aliquots (0.5 ml) of the methylene chloride phase were then assayed for radioactivity by liquid scintillation or γ counting.

Bulk phase transport experiments.

Protein-mediated Ca^{2+} transport through an organic phase was measured by using a Pressman cell (Pressman, 1973) at 22°C. This cell consists of two aqueous phases separated by a stirred organic phase. Methylene chloride was used as the organic phase. The volume of the aqueous and organic phases were 2 and 6 ml, respectively. $^{45}CaCl_2$ was added to one of the aqueous phases, and the protein was added to the

organic phase. The organic phase was stirred gently by a stirring bar. The nonradioactive aqueous side was then assayed for the appearance of radioactivity.

Phosphorus determination.

Phosphorus was determined by a modified method of Chen et al. (1956). Samples containing 10-40 nmol of phosphorus in 13 x 100 mm pyrex test tubes were mixed with 40 µl 10% $Mg(NO_3)_2 \cdot 6\ H_2O$ in 95% ethanol (Ames and Dubin, 1960). Each tube was then evaporated to dryness over a flame with rapid shaking and further heated in the flame until the brown fumes had disappeared. After the tubes had cooled, 0.3 ml of 0.5 N HCl was added to each tube. The tubes were then capped with marbles and heated in a boiling water bath for 15 minutes to hydrolyze to inorganic phosphate any pyrophosphate formed in the ashing procedure. After cooling, 0.7 ml of ascorbic acid-molybdate mixture was added to each tube. This ascorbic acid-molybdate mixture , which was made up on the day of the experiment, contained 1 part of 10% ascorbic acid and 6 parts of 0.42% ammonium molybdate . $4H_2O$ in 1 N H_2SO_4. These tubes were then incubated at 45°C for 30 minutes and the absorbance was read at 820 nm. Standards (0-50 µl of 1 mM Na_2HPO_4) were treated in the same manner.

Partial delipidation of the isolated protein.

The phospholipids associated with the purified protein were partially removed by the following two methods:

(a) Methylene chloride extraction.

Protein sample containing 80 µg of purified protein was dried

under a stream of nitrogen gas in a test tube . The dry sample was then solubilized by adding 2 ml of methylene chloride and mixed with 2 ml of distilled water for 2 minutes on a vortex mixer. The two phases were separated by centrifugation in a Beckman TJ-6 table-top centrifuge at 1000 x g for 15 minutes at room temperature. The aqueous phase was carefully withdrawn and kept. The remaining organic phase was mixed with another 2 ml of distilled water and the same procedure repeated. The two aqueous phases were then combined, lyophilized, and solubilized in pyridine. The protein and phospholipid phosphorus concentrations were determined and the mole ratio of phospholipid:protein was calculated.

(b) Silicic acid column chromatography.

Silicic acid (Bio-Sil A, 100-200 mesh) was packed in a short glass column with a 1.2 cm diameter. The total bed-volume was 2.8 ml. The column was washed with 10 bed-volumes of methanol and then 10 bed-volumes of chloroform/methanol (C/M) 6:1 before the sample was applied. The sample containing 100 µg of protein in 200 µl of C/M 6:1 was eluted with 15 bed-volumes of C/M 6:1 and then with 10 bed-volumes of C/M 1:1. Three-milliliter fractions were collected. Each tube was dried under a stream of nitrogen gas and re-solubilized in 200 µl of sequanal grade pyridine. An aliquot (20 µl) from each tube was then analyzed by TLC on Silica gel G plates (250 µm) with chloroform-methanol-28% ammonium hydroxide (65:35:5, by volume) as the developing solvent (Meissner and Fleischer, 1971). The plates were charred at 100°C for one hour after spraying

with chromerge (Christie, 1976). Fractions containing phosphatidylethanolamine (PE) and cardiolipin (not separable by this method) and phosphatidylcholine (PC) as identified with the appropriate standards were pooled, respectively. Fractions which did not contain any phospholipids by visual analysis were also pooled. These combined fractions were then dried under nitrogen individually and each was re-solubilized in a small volume of sequanal grade pyridine. The protein and phospholipid phosphorus concentrations of each fraction were then determined and the mole ratio of phospholipid:protein was each calculated.

Phospholipase C digestion of phospholipids.

Phospholipids were digested by phospholipase C according to the method of Mavis et al. (1972). This digestion was carried out in a two-phase system. Two milliliters of diethyl ether containing 4.2 µmol of phospholipid phosphorus to be digested were added to a screw cap culture tube containing 0.5 ml of 0.05 M potassium phosphate buffer, pH 7.0. The assay was initiated by pipetting the phospholipase C directly into the aqueous phase. The tube was capped tightly and vortexed for 2 hours at room temperature. The digestion was terminated by removing the tube from the mixer and allowing the phases to separate. Aliquots of the ether phase were then analyzed by thin layer chromatography (TLC) on a 250 µm Silica gel G plate with petroleum ether-diethyl ether-acetic acid (80: 75: 1.5, by volume) as the developing solvent (Mavis, et al., 1978). The plate was sprayed with chromerge (Christie, 1976) and baked at 100°C

for one hour. Diglycerides, the products of phospholipase C hydrolysis, migrated in this solvent combination, whereas undigested phospholipids stayed at the origin (Mavis, et al., 1978).

RESULTS

EPR assay of high-affinity Mn^{2+} binding.

The EPR spectrum of Mn^{2+} in distilled water shows six hyperfine lines (Fig. 1) due to the interaction of unpaired manganese electrons with its $I = \frac{5}{2}$ nuclear spin. The peak-to-peak separation in the sextet spectrum of $Mn^{2+}(H_2O)_6$ was determined to be 95 Oe. The proportionality constant K, mentioned in the Methods section, was determined to be 18.73 (results not shown). The EPR spectra, demonstrating how the Mg^{2+}-irreplaceable high-affinity Mn^{2+}-binding sites were determined, are shown in Fig. 2. In this figure only the first two peaks of the $Mn^{2+}(H_2O)_6$ sextet spectrum are shown. The height of the first peak is proportional to the free Mn^{2+} concentration in the solution. The total Mn^{2+} concentration was the same in every sample. Fig. 2a shows the Mn^{2+} spectrum in the buffer solution. The sample used in Fig. 2b was a protein fraction which contained only low-affinity Mn^{2+}-binding sites. Upon flushing with excess Mg^{2+}, all of the bound Mn^{2+} was released into the medium in the form of free Mn^{2+}. This resulted in an increase in the intensity of the spectrum. Fig. 2c shows the same experiment for a protein fraction containing high-affinity Mg^{2+}-irreplaceable Mn^{2+}-binding sites as well as low-affinity Mn^{2+}-binding sites. There was a small increase in the free Mn^{2+} intensity due to Mn^{2+} released from the low-affinity binding sites when the solution was flushed with excess Mg^{2+}. The sites which Mn^{2+} remained bound to, even after the addition of Mg^{2+}, were referred to as the high-affinity Mn^{2+}-binding sites.

Figure 1: The first derivative of the EPR absorption curve versus magnetic field. The sample was 37 μM of Mn^{2+} in distilled water. The total scan was 1000 Oe, and the peak-to-peak separation was determined to be 95 Oe. The central vertical line corresponds to the absorption peak of a g = 2 standard.

Figure 2: EPR spectra demonstrating the identification of Mg^{2+}-irreplaceable high-affinity Mn^{2+}-binding sites. Only two peaks of the hyperfine $Mn^{2+}(H_2O)_6$ sextet are shown here. Total Mn^{2+} added to each sample was identical. (a) shows the spectrum of 37 μM free Mn^{2+} in 10 mM Tris.HCl, pH 7.8. The superimposed spectra in (b) and (c) were taken before and after the addition of Mg^{2+}. After the addition of Mn^{2+}, the decreased intensities in (b) and (c) compared to (a) were due to Mn^{2+} bound to either low (L_1+L_2) - or high (H_1+H_2) - affinity sites. T_1 + T_2 represents the total Mn^{2+} binding. Mg^{2+} was added at 50 times higher concentration than Mn^{2+}. Fractions which contained only low-affinity Mn^{2+}-binding sites showed bound Mn^{2+} replaced by Mg^{2+} and released into free solution. This resulted in an increase of the spectrum (b). Fractions enriched in high-affinity Mn^{2+}-binding sites showed that the intensity of the free Mn^{2+} spectrum was still small even after the addition of excess Mg^{2+} (c).

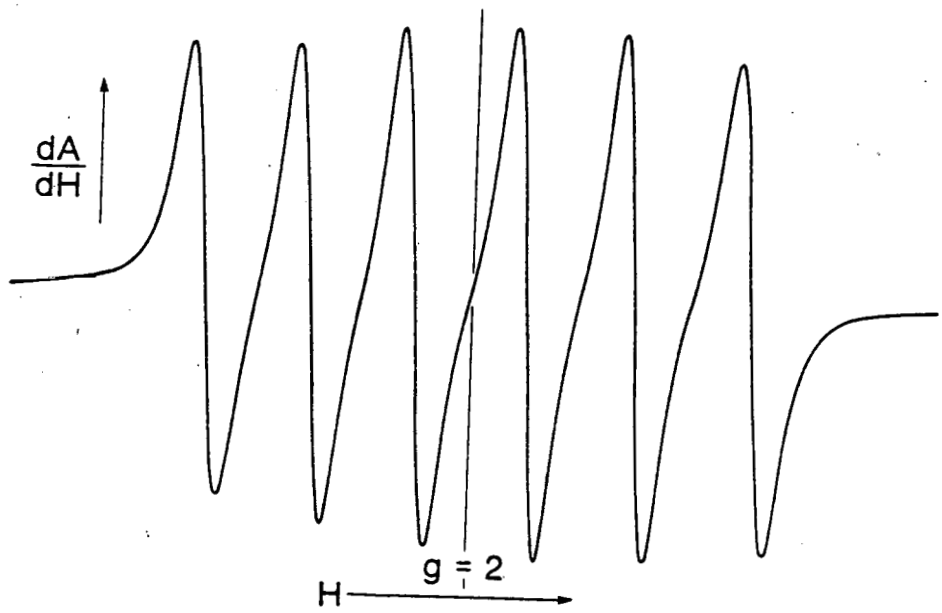


Figure 1

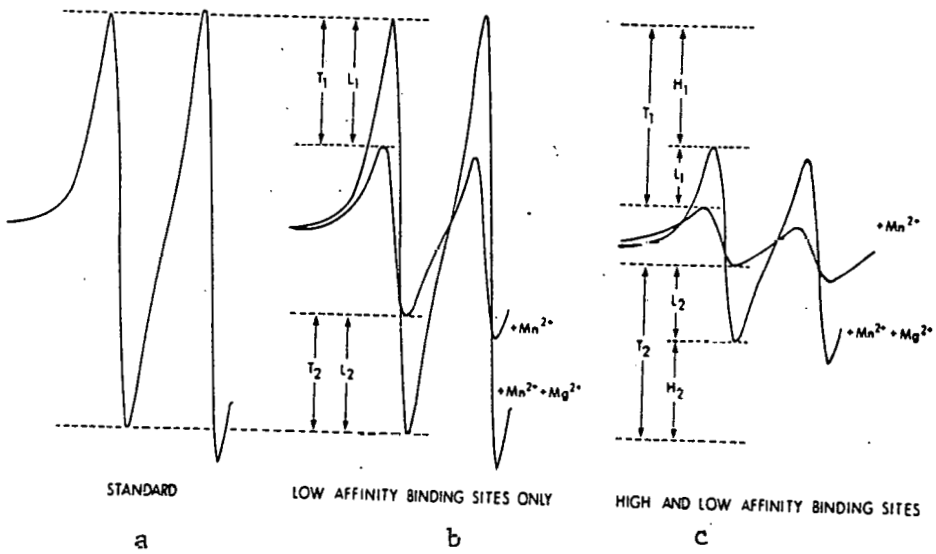


Figure 2

Protein purification.

High-affinity Mn^{2+} binding was measured starting with intact mitochondria. It was found that the high-affinity Mn^{2+}-binding sites reside in mitoplasts (mitochondria devoid of outer membranes), inner mitochondrial membranes, and DOC-soluble inner mitochondrial membrane proteins (results not shown). Since DOC was reported to be able to bind cations (Hofmann and Small, 1967), the effect of DOC on the Mn^{2+} binding was thus tested. Table 1 shows that when 37 μM of Mn^{2+} was added to solution containing 1% K DOC, about one-third of the total Mn^{2+} added was bound to DOC. Thus, whenever samples containing DOC were used, they were dialyzed against 10 mM Tris.HCl, pH 7.8, for at least 72 hours before the EPR assay of high-affinity Mn^{2+} binding was performed.

The DOC-soluble inner mitochondrial membrane proteins were dialyzed against 1% K DOC, 0.02% NaN_3, and 10 mM Tris.HCl, pH 7.8, for 2 hours at room temperature and the dialysate was centrifuged. The supernatant was found to be enriched with high-affinity Mn^{2+}-binding sites.

The molecular exclusion chromatography of the supernatant on a Sephadex G-50 column in DOC yielded a 280 nm absorbance elution profile consisting of 6 major peaks (Fig. 3). The fractions under each peak were pooled and dialyzed against 10 mM Tris.HCl, pH 7.8 for 72 hours at 4°C to remove the bulk of DOC before the EPR assay. Table 2 shows the result of the Mn^{2+}-binding assay for each absorption peak from the Sephadex G-50 column. This table shows that the pellet obtained from the previous centrifugation prior to the

TABLE 1

The effect of DOC on the EPR assay of Mn^{2+} binding

Solution	Free Mn^{2+} concentration in solution (μM)	% Mn^{2+} bound
Standard	36.9	0
Isolation buffer + 1 M KCl	34.5	6.5
Isolation buffer + 1 M KCl + 1% K DOC	24.5	34

The standard solution was $MnCl_2$ in distilled water. The isolation buffer contained 0.25 M sucrose and 10 mM Tris.HCl, pH 7.8. The amount of Mn^{2+} added to all of the three solutions was identical (36.9 μM). EPR spectra were taken for each solution. The free Mn^{2+} concentration in each solution was calculated using the equation shown in the Methods section.

Figure 3: Elution profile of the DOC-soluble inner mitochondrial membrane proteins chromatographed on a Sephadex G-50 column. The column was pre-equilibrated with 1% K DOC, 0.02% NaN_3, and 10 mM Tris.HCl, pH 7.8, and was eluted with the same buffer. The elution profile was monitored by 280 nm absorbance.

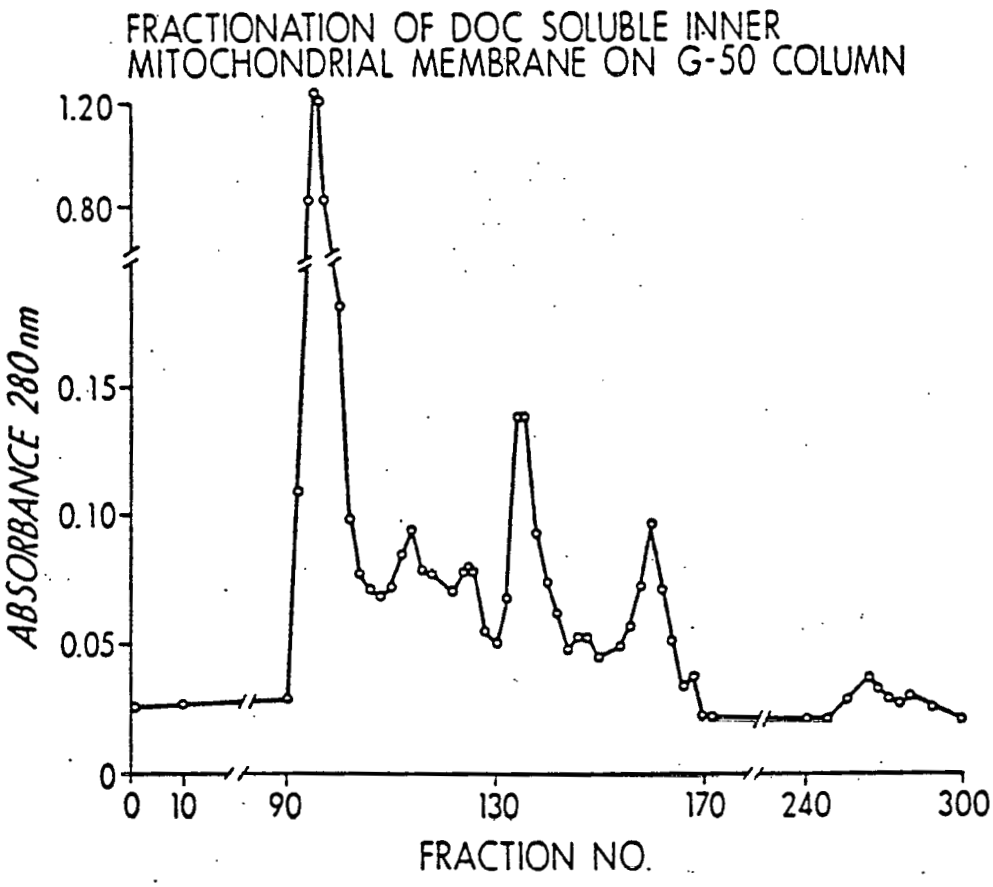

FRACTIONATION OF DOC SOLUBLE INNER MITOCHONDRIAL MEMBRANE ON G-50 COLUMN
ABSORBANCE 280nm
1.20
0.80
0.15
0.10
0.05
0
0
10
90
130
170
240
300
FRACTION NO.

TABLE 2

EPR assay of Mn^{2+} binding for samples from each absorption peak of the Sephadex G-50 column chromatography

Sample	Mn^{2+} binding sites (nmol/mg protein)		
	Total	Mg^{2+}-replaceable	Mg^{2+}-irreplaceable
Pellet after dialysis	2.0	1.7	0.3
Peak 1	9.2	5.7	3.5
Peaks 2 and 3 combined	19.0	19.0	0
Peak 4	81.0	71.8	9.2
Peak 5	112.2	66.3	45.9
Peak 6	211.1	211.1	0

The DOC-soluble inner mitochondrial membrane proteins were dialyzed against the column buffer (1% K DOC, 0.02% NaN_3, and 10 mM Tris.HCl, pH 7.8) for 2 hours and the dialysate was centrifuged. The supernatant was chromatographed on a Sephadex G-50 column. The samples eluted from the column were dialyzed against 10 mM Tris.HCl, pH 7.8, for 72 hours at 4°C to remove the bulk of DOC before the EPR assay. All of the Mn^{2+}-binding sites were calculated by comparing with a standard. The standard was obtained by dialyzing column buffer against 10 mM Tris.HCl, pH 7.8, for 72 hours at 4°C. The Mg^{2+}-replaceable and Mg^{2+}- irreplaceable Mn^{2+}-binding sites are referred to as the low- and high-affinity Mn^{2+}-binding sites, respectively.

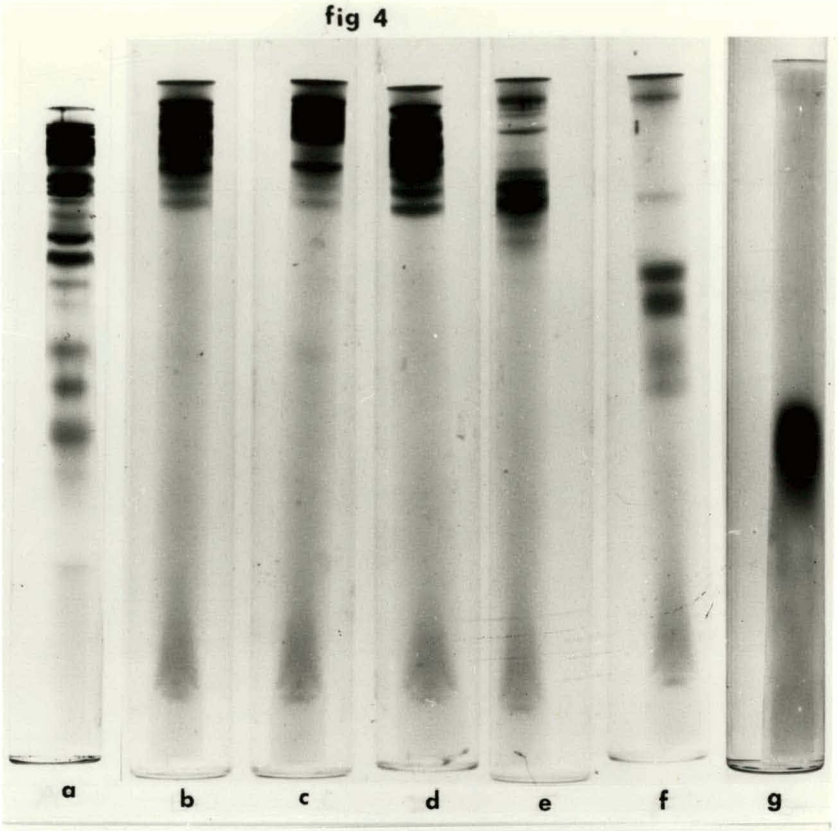
column chromatography had few Mn^{2+}-binding sites. The first, the second, and the third peaks also had few Mn^{2+}-binding sites. From the same table, it can be seen that the fourth, the fifth, and the sixth peaks had a large number of Mn^{2+}-binding sites. While most of the Mn^{2+}-binding sites of the fourth and the sixth peaks were Mg^{2+}-replaceable (low affinity), at least 40% of the Mn^{2+}- binding sites of the fifth peak were Mg^{2+}-irreplaceable (high affinity). Thus, the fifth peak, eluted at volume/total column volume = 0.53, was found to contain the highest number of Mg^{2+}-irreplaceable Mn^{2+}-binding sites per mg of protein. Urea/SDS polyacrylamide gel electrophoresis of protein from this peak showed a single band (Fig. 4).

By comparison with low molecular weight standards, this protein has an apparent molecular weight of about 3000 daltons (Fig. 5). The molecular weight was further confirmed from the amino acid analysis (Table 3). The minimum molecular weight calculated from Table 3 is 2883. The isolated protein gave an intensive pink color when stained with the periodic acid/Schiffs base reagent (Fig. 6, gels b and d), an indication that it might be a glycoprotein. Figure 7 summarizes the isolation procedure. The percent yield of protein at each step of the isolation procedure is shown in Table 4. The purified protein only accounts for 0.05% of the total mitochondrial protein.

Figure 4: Urea/SDS gels of protein samples from each peak of the Sephadex G-50 column chromatography. Gel (a) was the sample applied to the column. Gels (b) and (c) were early and late fractions from the first peak, respectively. Gels (d), (e), (f), and (g) were sample fractions from the second, the third, the fourth, and the fifth peak, respectively.

Figure 5: Urea/SDS gels of the isolated protein and the low molecular weight protein standards. Gel (a) was the isolated Mg^{2+}-irreplaceable Mn^{2+}-binding protein. Gels (b), (c), and (f) were soybean trypsin inhibitor (21,300), cytochrome c (11,700), and bacitracin (1,400), respectively. Gels (d) and (e) were insulin (5,700) in the absence and presence of mercaptoethanol, respectively. With the addition of mercaptoethanol, insulin was reduced to chain A (2,300) and chain B (3,400), which can not be resolved in this gel system.

fig 4



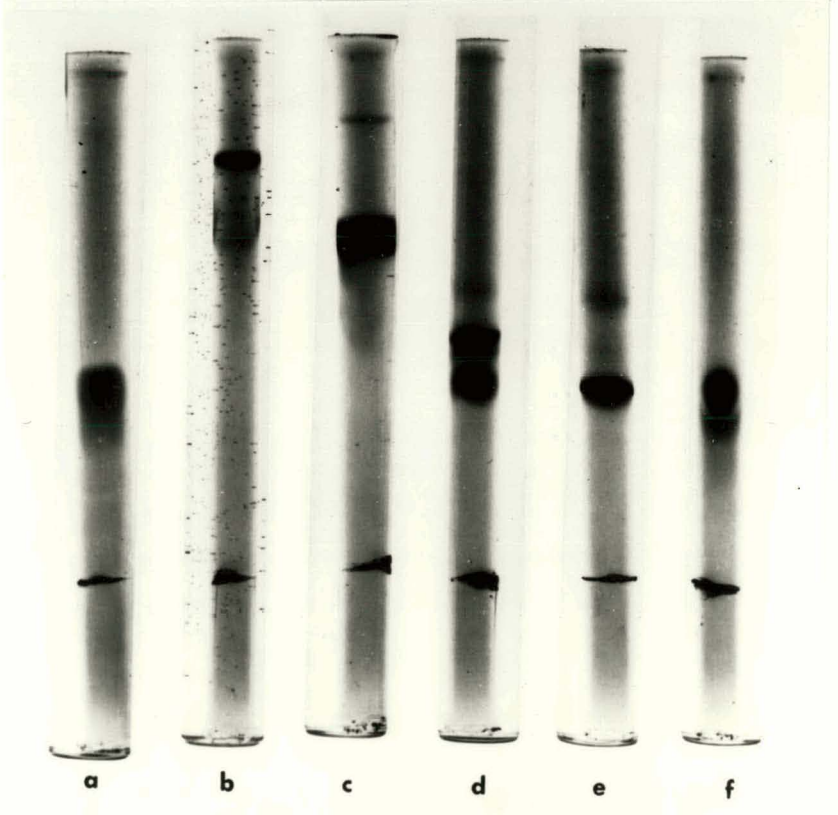


fig. 5

TABLE 3

Amino acid composition of the isolated protein

Amino acid	Content (mol/mol of protein)
Asx	2
Thr, Ser	4
Glx	4
Pro	1
Gly	4
Ala	2
Val	1
Ile	1
Leu	2
Tyr	1
Phe	1
His	2
Lys	1
Arg	1

Minimum molecular weight = 2883

Tryptophan content was not determined.

Figure 6: Urea/SDS gels of the isolated protein in different staining systems. Gel (a) contained 10 mg of DOC only. Gels (b) and (c) contained 13 µg of protein which was twice the amount of protein applied to gels (d) and (e). Gels (a), (b), and (d) were stained by the periodic acid/Schiff base method. Gels (c) and (e) were stained with Coomassie blue. The gels showing protein were run following dialysis to remove DOC.

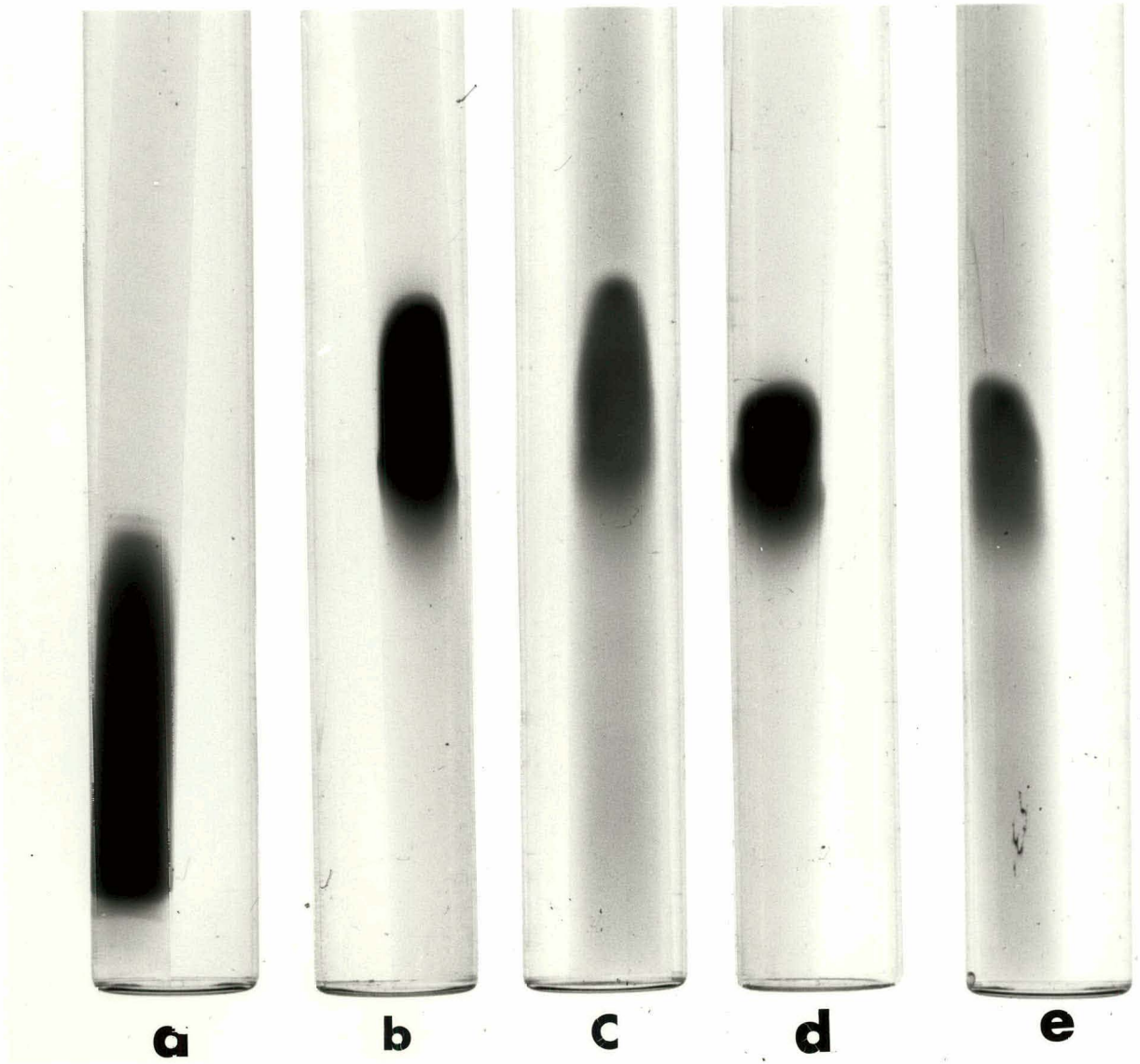

a
b
c
d
e

Figure 7: Flow chart of the isolation procedure. The single and double arrows after each centrifugation step represent supernatant and pellet, respectively.

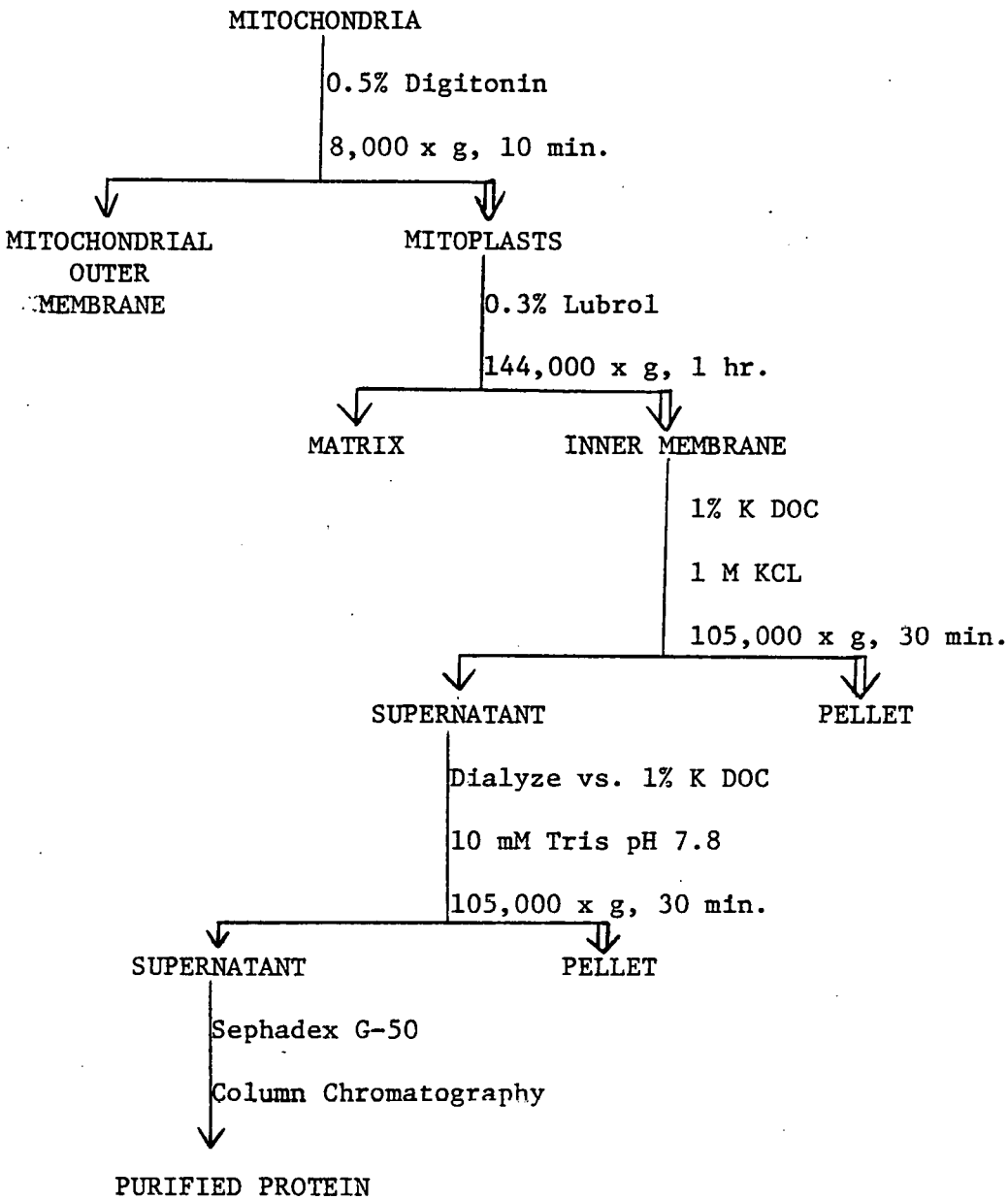

MITOCHONDRIA
0.5% Digitonin
8,000 x g, 10 min.
MITOCHONDRIAL OUTER MEMBRANE
MITOPLASTS
0.3% Lubrol
144,000 x g, 1 hr.
MATRIX
INNER MEMBRANE
1% K DOC
1 M KCL
105,000 x g, 30 min.
SUPERNATANT
PELLET
Dialyze vs. 1% K DOC
10 mM Tris pH 7.8
105,000 x g, 30 min.
SUPERNATANT
PELLET
Sephadex G-50
Column Chromatography
PURIFIED PROTEIN

TABLE 4

Percent protein obtained in each isolation step

Isolation step	Protein yield, %
Mitochondria	100
Mitoplasts	46
Inner mitochondrial membrane	38
DOC-soluble proteins	22
Purified protein	0.05

Characterization of the Ca^{2+}-binding properties

The dissociation constants of the isolated protein for Ca^{2+} were calculated from flow dialysis experiments. Fig. 8 is a Scatchard plot (Scatchard et al., 1957) of the binding data from a flow dialysis experiment. The plot is biphasic, which indicates two classes of binding sites. The high-affinity sites had a dissociation constant of 9.5 μM and bound 170 nmol Ca^{2+} per mg of protein. Using 3000 as the molecular weight, the stoichiometry was about 0.5 mol of Ca^{2+} bound per mol of protein. The low-affinity sites had a dissociation constant of 33 μM and bound 0.8 mol of Ca^{2+} per mol of protein.

Organic solvent extraction experiments

Table 5 shows data from the equilibrium extraction experiments with organic solvents of dielectric constants ranging from 2 (decane) to 17.8 (butanol). The concentration of protein in every sample was 25 μM. The pH of the aqueous $CaCl_2$ solution was 7.8. The amount of Ca^{2+} extracted into the organic phase was approximately 1.3 mol per mol of protein, using the assumption that all of the protein was in the organic phase. The addition of picrate, a lipid-soluble anion, showed little effect at pH 7.8 in increasing the amount of Ca^{2+} extracted in the presence of the protein. The hydrophobicity of the solvent also had little effect on the net amount of Ca^{2+} extracted. Table 6 shows the relative amounts of various monovalent and divalent cations extracted into methylene chloride in the presence of the protein. Divalent cations were greatly favored over monovalent cations.

Figure 8: Scatchard plot of Ca^{2+}-binding data constructed from flow diaylsis experiments. The protein sample used was dialyzed for 3 days against 10 mM Tris.HCl, pH 7.8, to remove DOC. For high-affinity binding, n = 170 nmol/mg of protein and K_d = 9.5 μM; for low-affinity binding, n = 264 nmol/mg of protein and K_d = 33 μM; n is the number of Ca^{2+}-binding sites and K_d is the dissociation constant. K_d values were calculated from the ratio of x intercepts to y intercepts. The number of low-affinity sites was calculated from the difference between the numbers of total Ca^{2+}-binding sites (434 nmol/mg of protein) and high-affinity Ca^{2+}-binding sites.

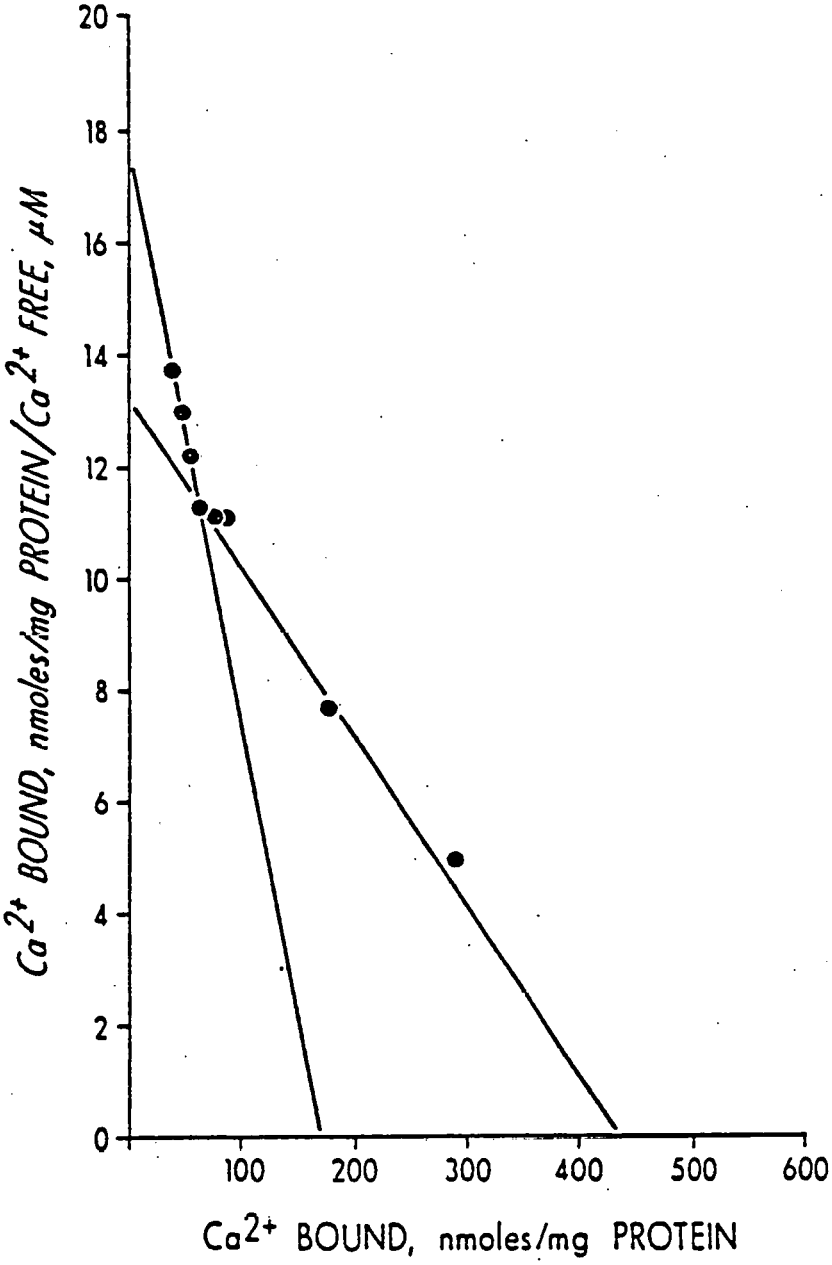

20
18
16
14
12
10
8
6
4
2
0
100
200
300
400
500
600
Ca2+ BOUND, nmoles/mg PROTEIN/Ca2+ FREE, μM
Ca2+ BOUND, nmoles/mg PROTEIN

TABLE 5

Protein-mediated Ca^{2+} extraction into different organic phases

	Ca^{2+} extracted, μM			
	Decane	Methylene Chloride	30% butanol/ 70% toluene	Butanol
Protein	35	32	29	60
Pyridine	0	0	0	33
Protein + picrate	34	38	35	104
Pyridine + picrate	0	0	2	81

Ca^{2+} extracted from aqueous phase containing 10 mM Ca^{2+} and 10 mM Tris.HCl, pH 7.8, into various organic solvents with and without the addition of protein. Pyridine-solubilized protein was added to various organic solvents. The final concentration of the protein was 25 μM. Picrate (500 μM) was added to see if a lipid-soluble anion facilitated Ca^{2+} extraction.

TABLE 6

Cation selectivity

Sequence	Zn	>	Sr	>	Ca	>	Mn	>	Na	>	Rb
Ratio	1.9		1.3		1.0		0.2		0.17		0.02

The cation selectivity was determined by the protein-mediated equilibrium extraction into a methylene chloride phase. The starting cation concentration in the aqueous phase was 1 mM buffered by 4 mM Tris.HCl, pH 7.0. The protein concentration was 0.7 μM. Cations extracted into the organic phases were measured by direct isotope counting. The amount of Ca^{2+} extracted was set at 1.0. The ratios shown were normalized to that of the Ca^{2+} extracted.

Ca^{2+} was favored over Mn^{2+} by 5:1, but Zn^{2+} and Sr^{2+} were both favored over Ca^{2+}.

Competition studies.

Cation competition and inhibition experiments were also conducted by the organic solvent extraction procedure. As expected from the previous data (Table 6), Zn^{2+} inhibited Ca^{2+} extraction into the organic phase more strongly than any other divalent cations tested (Fig. 9). Monovalent cations (K^+, Na^+) inhibited Ca^{2+} extraction only slightly. Ba^{2+}, Sr^{2+}, and Mg^{2+} demonstrated inhibition intermediate between K^+ , Na^+ and that of Zn^{2+} and Mn^{2+}. La^{3+} and ruthenium red showed the strongest inhibition of Ca^{2+} extraction.

Bulk phase transport.

The protein-mediated transport of Ca^{2+} through a bulk hydrophobic phase is shown in Fig. 10. In Fig. 10a, the starting Ca^{2+} concentration on both sides was 1 mM. Experiments were run with a pH gradient from 8.4 on the donor side to 6.0 on the receiver side. When the pH gradient was reversed, to 5.0 on the donor side and 7.8 on the receiver side, no Ca^{2+} translocation occurred. Also, no Ca^{2+} translocation was observed in the absence of a pH gradient (both sides pH 8.0 or pH 5.0). There was also no Ca^{2+} translocation when a gradient of Na^+, K^+, or Ca^{2+} was substituted for the H^+ gradient. In the presence of a Ca^{2+} gradient (10 mM on the donor side and no Ca^{2+} on the receiver side) in addition to the pH gradient,

Figure 9: Inhibition of protein-mediated Ca^{2+} extraction by various cations and ruthenium red (RR) into a methylene chloride phase. Experiments were conducted by the equilibrium extraction procedure. Starting Ca^{2+} concentration in the aqueous phase as 1 mM in 5 mM Tris.HCl, pH 7.0. Protein concentration in the organic phase was 2.5 μM. The amount of Ca^{2+} extracted in the absence of any other cations was set at 100%.

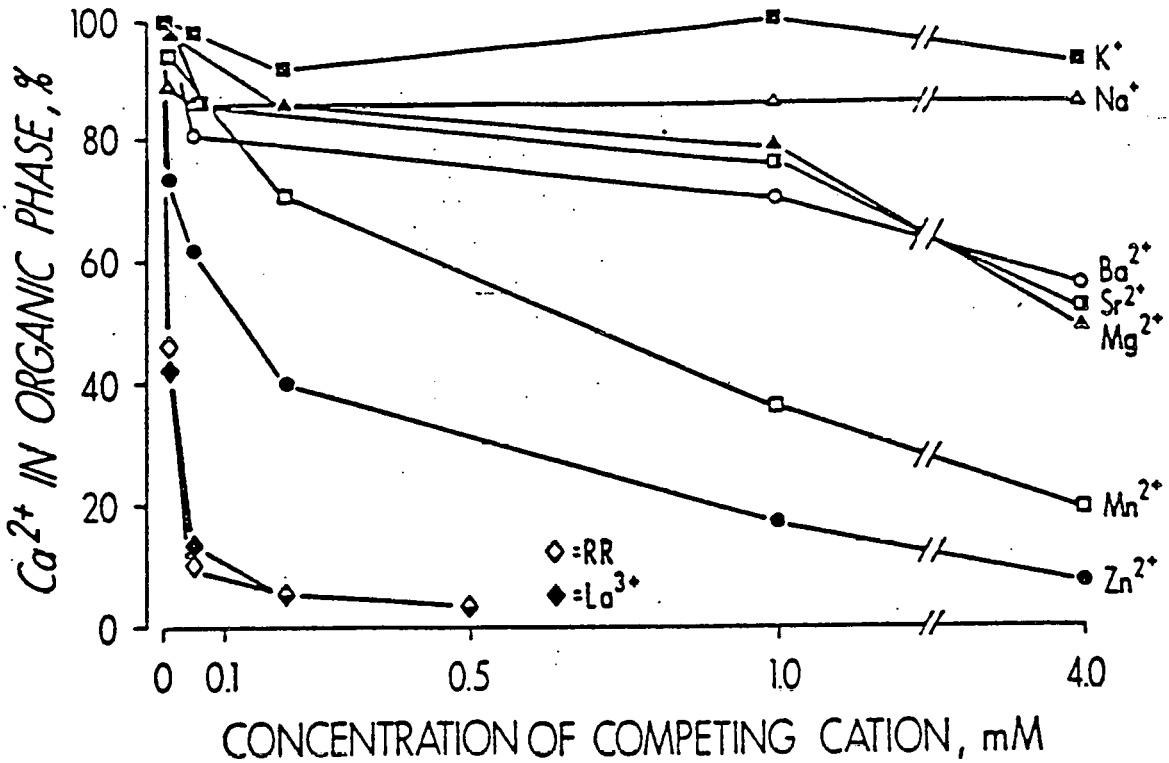
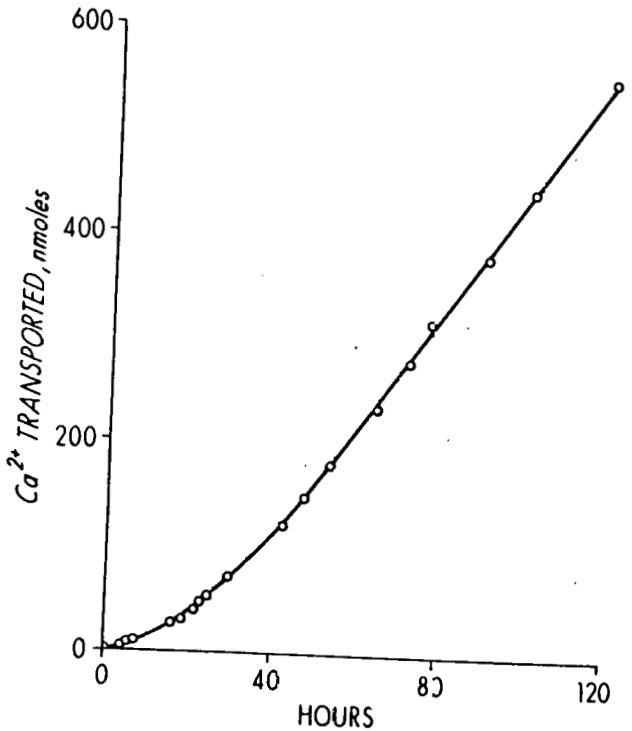
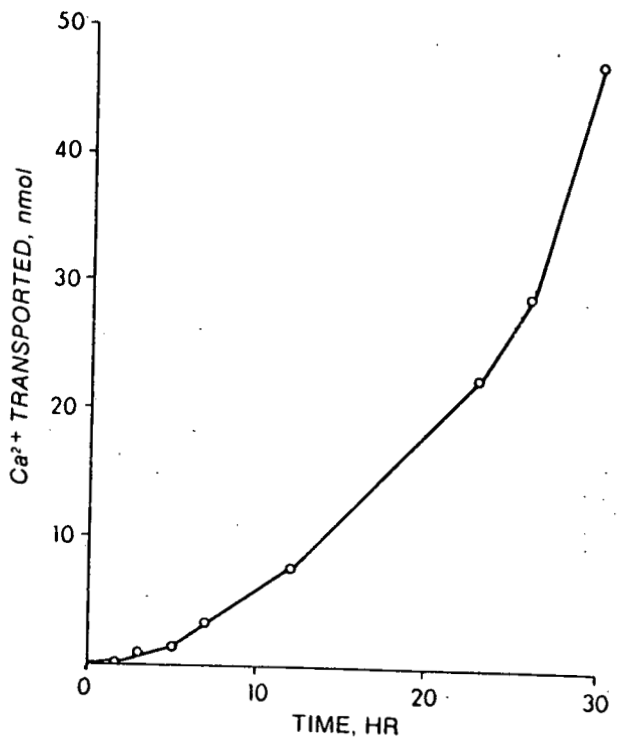

100
80
60
40
20
0
Ca^{2+} IN ORGANIC PHASE, %
0
0.1
0.5
1.0
4.0
CONCENTRATION OF COMPETING CATION, mM
K^+
Na^+
Ba^{2+}
Sr^{2+}
Mg^{2+}
Mn^{2+}
Zn^{2+}
◇=RR
◆=La^{3+}

Figure 10: Protein-mediated Ca^{2+} transport from a donor aqueous phase through an organic phase (methylene chloride) to a receiver aqueous phase. (a) $^{45}Ca^{2+}$ was transported from pH 8.4 on the donor aqueous phase to pH 6.0 on the receiver aqueous phase. Protein was initially added to the methylene chloride phase. The final concentration of the protein was 2.8 μM. Starting Ca^{2+} concentrations on both donor and receiver sides were 1 mM. The donor and receiver sides were buffered with 5 mM Tris (pH adjusted by HCl) and 5 mM MES (pH adjusted by Tris), respectively. Experiments were conducted by using Pressman cells. The control experiments with no protein added and experiments with reversed H^+ gradient showed zero transport. (b) Pressman cell experiments in the presence of a Ca^{2+} gradient as well as a pH gradient. The donor side was 10 mM $^{45}CaCl_2$ buffered with 10 mM Tris.HCl, pH 8.5 and the receiver side was 10 mM MES, pH 6.5 adjusted with Tris. The protein concentration was 4.3 μM.


600
400
200
0
Ca^{2+} TRANSPORTED, nmoles
0
40
80
120
HOURS

a


50
40
30
20
10
0
Ca^{2+} TRANSPORTED, nmol
0
10
20
30
TIME, HR

b

the Ca^{2+} transport rate through the organic phase was not enhanced (Fig. 10b). Thus, the Ca^{2+} translocation properties of the protein are extremely dependent on a pH gradient.

Lipids associated with the protein.

The ethanol-precipitated protein used in the previously mentioned organic solvent extraction and bulk phase transport experiments was contaminated with lipids. Approximately 150 moles of phosphorus was found to be associated with each mole of protein (Table 7, below). Visual analysis of the lipids with approximate standards by TLC indicated that the lipid composition was about 50% PE, 30% PC, and less than 5% cardiolipin (Fig. 11).

Partial delipidation.

The work of Tyson et al. (1976) indicated that cardiolipin might contribute to some of the Ca^{2+} transport properties reported here. In fact, cardiolipin-mediated Ca^{2+} transport through a bulk organic phase under the same condition as that shown in Fig. 10b was confirmed (Fig. 12). Thus, it was necessary to delipidate the protein to see if the ionophorous properties were still associated with the protein.

Blondin et al. (1977) reported a rapid delipidation procedure by using diethyl ether to solubilize the majority of phospholipids leaving proteins precipitated. However, this approach was not successful. Almost 100% of the protein was found in the ether-soluble phase and the amount of phospholipids associated with the protein was not

Figure 11: Phospholipids associated with the protein. The composition was analyzed on a TLC plate by comparing with the phospholipid standards.

(a) 65 µg of the isolated protein.

(b) 35 µg of cardiolipin.

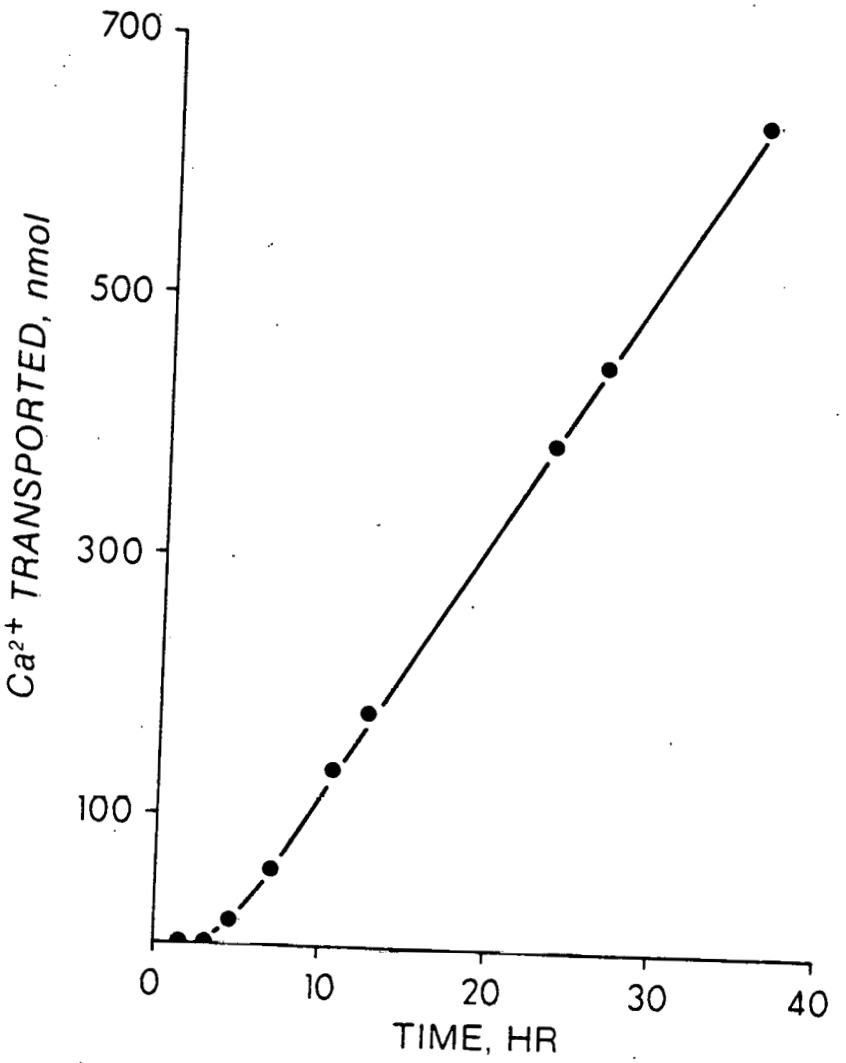
(c) 35 µg of PE.

(d) 35 µg of PC.

The developing solvent was chloroform-methanol-28% ammonium hydroxyde (65: 35: 5:, by volume).


a
b
c
d

Figure 12: Cardiolipin-mediated Ca^{2+} translocation in the Pressman cell assay. The donor side was 10 mM $^{45}CaCl_2$ buffered with 10 mM Tris.HCl, pH 8.5 and the receiver side was 10 mM MES, pH 6.5 adjusted with Tris. The concentration of cardiolipin was 0.42 mM. Methylene chloride was used as the organic phase.


700
500
300
100
Ca^{2+} TRANSPORTED, nmol
0
10
20
30
40
TIME, HR

changed. By using a methylene chloride extraction procedure, the amount of phospholipid was reduced to 36 mol per mol of protein (Table 7).

A better method of dilipidation was to use silicic acid column chromatography. Cardiolipin and PE were eluted first from the column with chloroform/methanol (C/M) 6:1. Protein with no phospholipid contamination by visual analysis on a TLC plate followed as a peak behind the major phospholipid contamination. PC could be eluted from the column with C/M 1:1. The phospholipids associated with the protein were found to decrease down to 13 mol per mol of protein (Table 7).

Bulk phase transport experiments after partial delipidation

Fig. 13 compares Pressman cell experiments of the partial delipidated protein and the major constituents of the contaminating phospholipids. In the experiments shown in Fig. 13, the amount of each of the phospholipid standards used was identical to the total amount of phospholipids associated with the protein after partial delipidation. The protein samples used in Fig. 13a and 13b were partially delipidated by using methylene chloride extraction and silicic acid column chromatography, respectively. In Fig. 13a, the protein-mediated Ca^{2+} translocation was the slowest. When the phospholipid content associated with the protein was reduced further, the amount of protein-mediated Ca^{2+} translocation was increased whereas the Ca^{2+} translocations mediated by phospholipids were decreased (compare Fig. 13b with 13a).

TABLE 7

Partial delipidation of the protein

Methods used in delipidation	Concentration		Mole ratio P_i: protein
	Protein (mg/ml)	P_i* (mol/ml)	
None	1.3	64.4	149
Organic solvent extraction	0.12	1.45	36
Silicic acid column	0.26	1.08	13

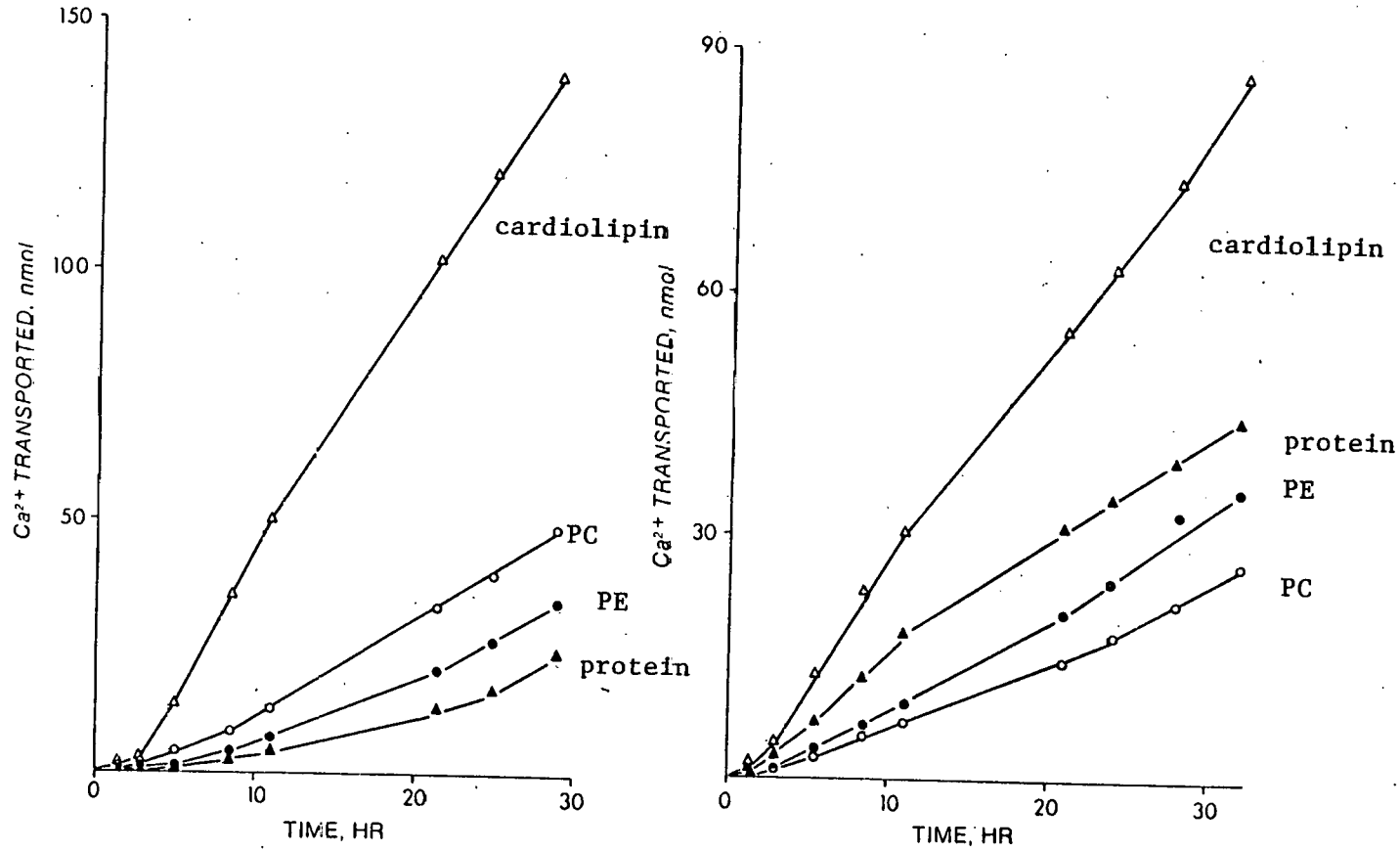
*P_i = phospholipid phosphorus.

The molecular weight of the isolated protein was taken as 3000 in calculating the mole ratios. The organic solvent used in the organic solvent extraction delipidation procedure was methylene chloride.

Figure 13: Pressman cell experiments with partially delipidated protein. The donor side was 10 mM $^{45}CaCl_2$ buffered with 10 mM Tris.HCl, pH 8.5, and the receiver side was 10 mM MES, pH 6.5 adjusted with Tris.

(a) Protein was delipidated by using methylene chloride extraction procedure. The concentration of the protein in the organic phase was 0.82 μM, and the concentration of the phospholipid phosphorus associated with the protein was 29 μM. The concentrations of the phospholipids used: PE, 29 μM; PC, 29 μM; cardiolipin, 14.5 μM (contained 29 μM of phosphorus).

(b) Protein was delipidated by silicic acid column chromatography. The concentration of the protein was 0.72 μM, and the concentration of the protein-associated phosphorus was 9 μM. The concentrations of the phospholipids used: PE, 9 μM; PC, 9 μM; cardiolipin, 4.5 μM.


150
100
50
0
10
20
30
Ca2+ TRANSPORTED, nmol
TIME, HR
cardiolipin
PC
PE
protein
a
90
60
30
0
10
20
30
Ca2+ TRANSPORTED, nmol
TIME, HR
cardiolipin
protein
PE
PC
b

Cardiolipin was reported to be able to transport Rb^+ faster than Ca^{2+} (Tyson et al., 1976) by Pressman cell assay. The same situation was also observed (Fig. 14), but it was opposite for the isolated protein.

Phospholipase C digestion.

Another method used to distinguish the protein-mediated Ca^{2+} transport properties from those of the contaminating phospholipids was carried out by phospholipase C digestion of phospholipids. The products of the phospholipase C digestion, diglycerides, can be resolved from the undigested phospholipids by using TLC with petroleum ether-diethyl ether-acetic acid (80: 75: 1.5:, by volume) as the developing solvent (Mavis et al., 1978). Figure 15 shows the results of phospholipase C digestion of the isolated protein, cardiolipin, PE, and PC. It can be seen that both PE and the phospholipids associated with the protein were almost completely digested. The majority of PC was also digested whereas cardiolipin was very resistant to the phospholipase C digestion. Table 8 compares the Ca^{2+} transport properties associated with PE and the protein before and after the performance of phospholipase C digestion. The amount of PE-mediated Ca^{2+} translocation was decreased while that mediated by the protein remained unchanged after the phospholipase C digestion.

Figure 14: Comparison between Rb^+ and Ca^{2+} transports in the Pressman cell experiments mediated by the protein and cardiolipin. The protein was partially delipidated by using silicic acid column chromatography. The concentrations of the protein and cardiolipin were 0.72 μM and 4.5 μM, respectively. The donor side contained 10 mM radioactive cation and 10 mM Tris.HCl, pH 8.5. The receiver side contained 10 mM MES, pH 6.5 adjusted with Tris.

● : cardiolipin-mediated Rb^+ transport.

O : cardiolipin-mediated Ca^{2+} transport.

▲ : protein-mediated Rb^+ transport.

Δ : protein-mediated Ca^{2+} transport.

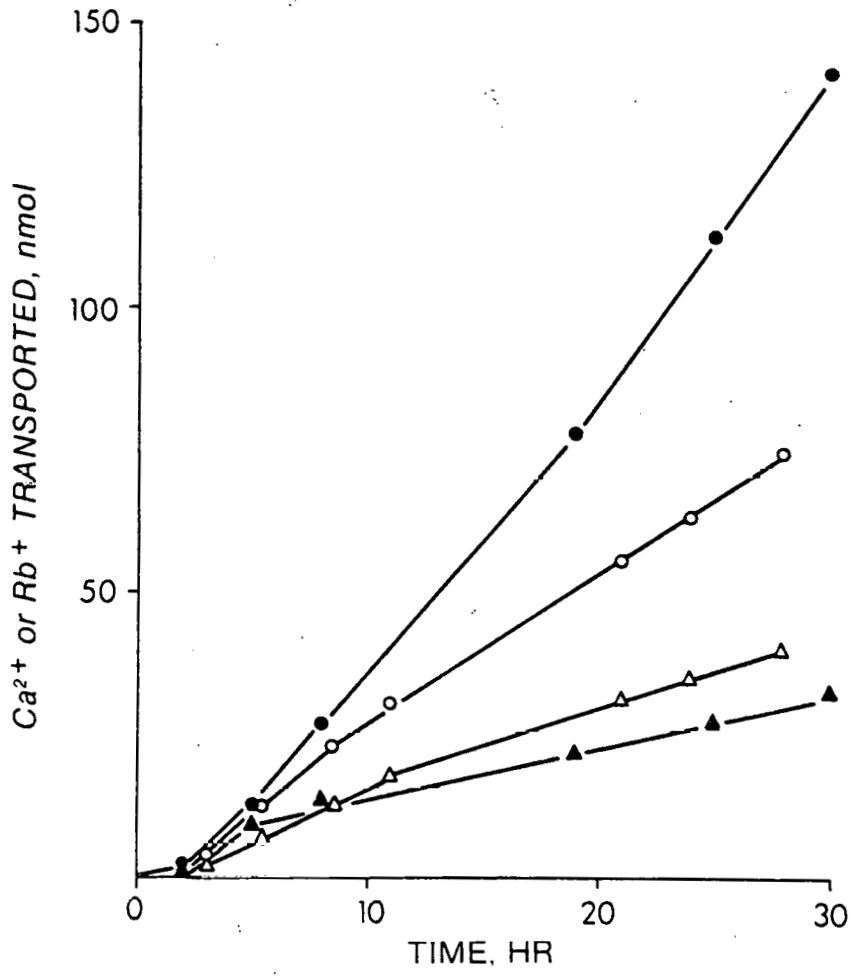

150
100
50
0
10
20
30
TIME, HR
Ca2+ or Rb+ TRANSPORTED, nmol

Figure 15: Phospholipase C digestion of the protein and the phospholipid standards.

(a), (c), (e), and (g) were undigested protein, cardiolipin, PC, and PE, respectively, while (b), (d), (f), and (h) were the corresponding digested samples.

The developing solvent was petroleum ether-diethyl ether-acetic acid (80: 75: 1.5, by volume).

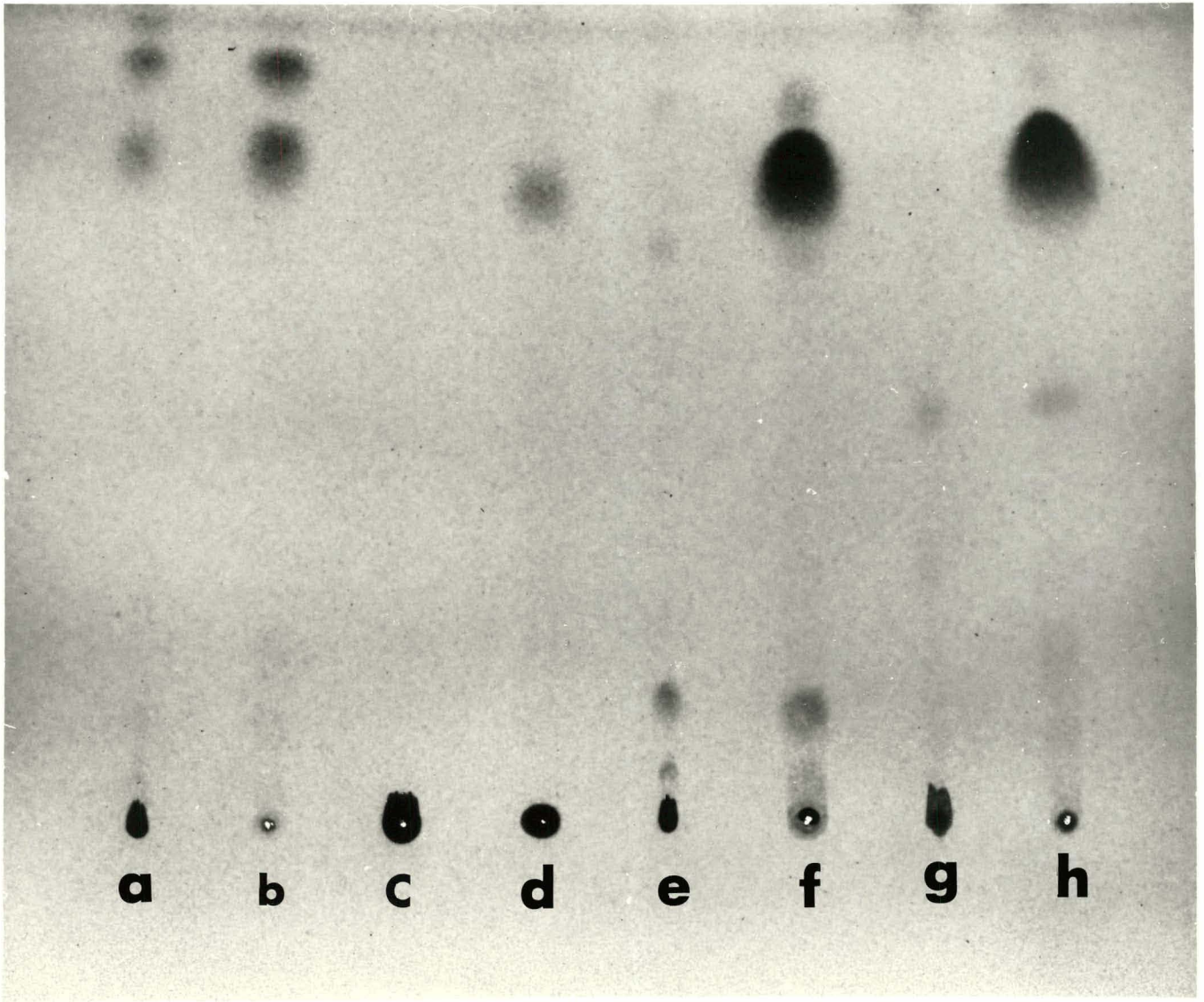

a
b
C
d
e
f
g
h

TABLE 8

The effect of phospholipase C digestion on the Ca^{2+} translocation in the Pressman cell experiments

Treatment	Ca^{2+} translocated in 30 hours (nmol)	
	Protein-mediated	PE-mediated
none	47	105
Phospholipase C	50	44

The donor side was 10 mM $^{45}CaCl_2$ and 10 mM Tris.HCl, pH 8.5. The receiver side was 10 mM MES, pH 6.5 adjusted with Tris. Protein concentration was 4.3 μM. The concentration of the phospholipid phosphorus associated with the protein was 0.64 mM. PE concentration was 0.64 mM.

DISCUSSION

The molecular mechanism of Ca^{2+} transport in mitochondria is essentially unsolved after more than 15 years of extensive study. Several completely dissimilar mechanisms have been proposed. Among these are the uniport mechanism proposed by Lehninger (Lehninger, 1974; Lehninger and Brand, 1976) and Selwyn et al. (1970), the antiport mechanism of Crompton et al, (1976), the symport mechanism of Moyle and Mitchell (1977), and the theory of more than one mechanism for Ca^{2+} transport in mitochondria proposed by Puskin et al. (1976). Despite the disagreement over the mechanism of Ca^{2+} transport, it is generally accepted that the transport of Ca^{2+} across the inner mitochondrial membrane is a carrier-mediated process (Carafoli and Sottocasa, 1974; Lehninger, 1970; Reed and Bygrave, 1974; Selwyn et al., 1970). One way to study the mechanism of Ca^{2+} transport in mitochondria is to isolate this carrier in its active form and study the mechanism of Ca^{2+} transport in reconstituted systems.

There are several properties associated with this specific Ca^{2+} carrier. The minimum criteria required to identify any isolated protein as the specific Ca^{2+} carrier should consist of: its localization in the inner mitochondrial membranes (Pedersen and Coty, 1972), high specificity for Ca^{2+} (Lehninger et al., 1967), transport of Sr^{2+} and Mn^{2+} as well (Lehninger and Carafoli, 1971; Vinogradov and Scarpa, 1973), and high sensitivity to inhibitors of mitochondrial Ca^{2+} transport systems (Mela, 1968; 1969; Moore, 1971; Reed and

Bygrave, 1974).

Using the facts that Mn^{2+} is a good analog of Ca^{2+} transport in mitochondria(Lehninger et al., 1967; Puskin and Gunter, 1972) and free Mn^{2+} can be distinguished from bound Mn^{2+} by EPR spectroscopy (Gunter and Puskin, 1972), together with the assumption that there exist high-affinity Mn^{2+}-binding sites in mitochondria, a protein has been isolated from inner mitochondrial membrane that is able to bind Mn^{2+} with high affinity. The yield of the isolated protein was determined as 3 μg per mg of mitochondrial protein before the removal of DOC, which was used to equilibrate the column (Jeng et al. 1978). Since DOC was shown to interfere with the Lowry protein determination (Gaál and Holczinger, 1975), the purified protein was dialyzed against 50% ethanol to remove DOC. The adjusted yield of this purified protein (Table 4), 0.17 nmol per mg of mitochondrial protein assuming the molecular weight of the protein is 3000, is between the two estimated numbers of Ca^{2+} carrier sites reported: 1.2 nmol/mg of mitochondrial protein (Reynafarje and Lehninger, 1969) and 0.06 nmol/mg of mitochondrial protein (Mela, 1969), and is closer to the number arrived at by Mela (1969). This Mn^{2+}-binding protein can also bind Ca^{2+} with high affinity, as can be seen from Fig. 8. Also, from the selectivity sequence obtained by organic solvent extraction experiments (Table 6), the isolated protein is more specific for Ca^{2+} than for Mn^{2+} and also favors Ca^{2+} over Mg^{2+} as inferred from the EPR assay of the isolation procedure. The amount of Zn^{2+} extracted into an organic phase is almost twice as much as Ca^{2+}. In the presence of

equal concentrations of Zn^{2+} and Ca^{2+}, the amount of Ca^{2+} extracted into the organic phase is decreased by 83% (Fig 9). Zn^{2+} has been reported to be an inhibitor of Ca^{2+} uptake by mitochondria of frog skeletal muscle (Batra, 1973). It is likely that Zn^{2+} competes for the Ca^{2+} binding site on the protein. Other divalent cations (Mn^{2+}, Sr^{2+}, Ba^{2+}, and Mg^{2+}) have been found to be able to compete for the same site of Ca^{2+} binding (Fig. 9),whereas monovalent cations (Na^{+} and K^{+})cannot. La^{3+} and ruthenium red, inhibitors of mitochondrial Ca^{2+} transport (Reed and Bygrave, 1974), inhibit Ca^{2+} extraction at very low concentrations. The mole ratio of total La^{3+} or ruthenium red to protein for 90% inhibition of the protein-mediated Ca^{2+} extraction into the methylene chloride phase was 10:1. Hence, La^{3+} and ruthenium red are potent inhibitors of this Ca^{2+} ionophore. An unexpected result in comparison with the data shown in Fig. 9 is the large inhibitory effect shown by Mn^{2+}. The previously mentioned work (Table 6) showed that only a small amount of Mn^{2+} was extracted into the organic phase in comparison with Ca^{2+}. This inhibitory effect may be due to the interaction between Mn^{2+} and Ca^{2+}, which is consistent with the Ca^{2+}-stimulated Mn^{2+} uptake observed in mitochondria (Vinogradov and Scarpa, 1973).

Recently, Blondin et al. (1977) have reported the isolation of a low molecular weight Ca^{2+} ionophore from beef heart mitochondria. The ionophore is released from a large molecular weight protein by using prolonged tryptic digestion. This procedure is totally different from the non-proteolytic isolation of the intact Ca^{2+} ionophore reported here. Also, the amino acid composition of their ionophore

is considerably different from that shown in Table 3.

A major problem in the characterization of the transport properties of this protein is the lipid contamination. Tyson et al. (1976) have shown that phospholipids may function as ionophores in the Pressman cell assay under certain conditions. Their data show that cardiolipin transports Ca^{2+} in the Pressman cell assay. This cardiolipin-mediated Ca^{2+} translocation in the Pressman cell assay was also confirmed (Fig. 12) under the same condition that was used to assay that of the isolated protein (Fig. 10b). This fact prompted a further investigation into whether part or all of the Ca^{2+} transport properties of the isolated protein are due to co-purified phospholipids. The following results strongly indicate that the Ca^{2+} transport properties associated with the protein are not due to the contaminating cardiolipin: (a) The rate of Ca^{2+} transport with cardiolipin was reported to be insensitive to pH over the range 5.0 to 8.5 (Tyson et al., 1976), while Ca^{2+} transport with the isolated protein is very pH dependent, showing zero Ca^{2+} transport rate with no pH gradient. (b) When the mole ratio of associated phospholipids to protein was reduced to 36:1 by methylene chloride extraction, the Ca^{2+} transport rate by the isolated protein was still slower than those of cardiolipin, PE, and PC, assuming all the associated phospholipids were cardiolipin, PE, and PC, respectively (Fig. 13a). After the mole ratio of associated phospholipids to protein was decreased to 13:1 by silicic acid gel chromatography (Table 7), the amount of Ca^{2+} transported by cardiolipin, PE, and PC were all reduced (Fig. 13b), whereas that of the isolated protein increased. These results imply that the protein, not the associated phospholipids, plays the

major role in the Ca^{2+} translocation in the Pressman cell assay. (c) Tyson et al.(1976) found that cardiolipin could translocate Rb^{+} faster than Ca^{2+}. This observation was confirmed under the condition of the experiments reported here (Fig. 14). However, the isolated protein transported Rb^{+} slower than Ca^{2+} (Fig. 14). The phospholipase C digestion experiments tend to eliminate the possibility that PE plays a major role in the protein-mediated Ca^{2+} translocation. As can be seen from Fig. 15, most of the phospholipids associated with the protein were digested by phospholipase C. While digested PE decreases its ability to translocate Ca^{2+} through a bulk organic phase, the phospholipase C digestion leaves the protein-mediate Ca^{2+} translocation unchanged (Table 8). Thus, the Ca^{2+} transport properties of this 3000-dalton protein are proven not due to the contaminating phospholipids by the indirect methods mentioned above. A more direct method of showing the independence of the protein-mediated transport property from those of phospholipids is to delipidate the protein completely or to an insignificant amount. This approach has been successful and will be shown in Chapter 2.

The isolated protein stains pink in periodic acid/Schiff base stain (Fairbanks, 1971), an indication that this protein might be a glycoprotein. However, after delipidation, the protein is no longer stained with the glycoprotein stain (results will be shown in Chapter 2). This fact together with the insignificant amount of carbohydrates detected in the delipidated protein (see Chapter 2 for details) assures that the isolated protein is not a glycoprotein.

Probably the main objection against this isolated protein being the specific Ca^{2+} carrier comes from the results of organic solvent extraction experiments summarized in Table 5. It is generally agreed that the driving force for Ca^{2+} transport in mitochondria is the negative-inside membrane potential across the inner mitochondria (Scarpa and Azzone, 1970). This implies that the Ca^{2+}-carrier complex is positively charged. Thus, this specific carrier-mediated Ca^{2+} extraction into the organic phase should require lipophilic anions, e.g. picrate, to neutralize the complex as in the case of valinomycin-mediated K^{+} extraction into the organic phase (Haynes and Pressman, 1974). Table 5 shows that the addition of picrate had little effect in increasing the net amount of Ca^{2+} extraction into all of the organic phases tested. These experiments may indicate either that the Ca^{2+}-protein complex is neutral or that the contaminating phospholipids act as lipophilic anions. In Chapter 2, it will be shown that after complete delipidation, the protein-mediated Ca^{2+} extraction into the organic phase absolutely requires picrate. Thus, this 3000-dalton protein seems to be an electrogenic calcium ionophore protein and is therefore named "calciphorin". The exact role of calciphorin in the overall mitochondrial Ca^{2+} transport system is not certain. It could act as either the carrier for the inward transport of Ca^{2+} or for the efflux mechanism (Crompton, et al. 1976; Gunter et al., 1977) in the mitochondrial Ca^{2+} transport system.

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CHAPTER 2

THE ELECTROGENICITY OF THE DELIPIDATED CALCIUM IONOPHORE

SUMMARY

The isolated calcium ionophore protein (named calciphorin) is delipidated by using Sephadex LH-20 column chromatography. The mole ratio of phospholipids to calciphorin can be reduced to 0.1 mole of phospholipids per mole of calciphorin. There are no free fatty acids, hexosamines, or sialic acids associated with the delipidated calciphorin. The extraction of Ca^{2+} into an organic phase mediated by the delipidated calciphorin requires the presence of a lipophilic anion, picrate. Picrate also enhances the rate of calciphorin-mediated Ca^{2+} translocation through a bulk organic phase. Thus, calciphorin seems to be an electrogenic Ca^{2+} ionophore.

INTRODUCTION

Since the first introduction by Pressman (Moore and Pressman, 1964;Pressman, 1965), the ion transport properties of the ionophores have created a new field of research. Ionophores, meaning ion bearers, can provide not only a valuable tool for studies on the linkage between metabolism and ion transport,but also present perfect models for studies on the molecular mechanism of the ion selectivity (Pressman, 1976;Gomez-Puyou and Gomez-Lojero, 1977). In general, ionophores include carriers and channels. Carriers are assumed to be lipophilic molecules that bind substrates on one side of the membrane, diffuse through the lipid bilayer, and dissociate the bound substrates on the other side of the membrane. Channels are aqueous filled pathways spanning the membrane (Shamoo and Goldstein, 1977). Among all the model carriers reported, valinomycin (Moore and Pressman, 1964;Pressman, 1965), A23187 (Reed and Lardy, 1972), and X-537A (Pressman, 1973) are the most commonly used in experiments, whereas gramicidin (Bodanszky and Perlman, 1969) and alamethicin (Mueller and Rudin, 1968) are the representatives of model channels.

There are two distinct classes of model carriers. The first class is the electrogenic carriers, and valinomycin is the best known example. Valinomycin is electrically neutral and forms a positively charged complex with K^+. The K^+-valinomycin complex can respond to an externally applied potential across the artificial bilayer membrane causing an increase in membrane conductance (Eisenman et al., 1973). The extrac-

tion of the ion-carrier complex of this class into a bulk organic phase requires the presence of a lipophilic anion to neutralize the complex (Haynes and Pressman, 1974). The second class is the electroneutral carriers. A23187 and X-537A belong to this class. The ion-carrier complex is electrically neutral. This complex cannot respond to an externally applied potential across the bilayer membrane to increase the membrane conductance (Wulf and Pohl, 1977), and the extraction of the ion-carrier complex into an organic phase does not require a lipophilic anion (Pfeiffer and Lardy, 1976).

Despite the fact that these model carriers and channel-formers act as ion transport mediators when introduced into natural membranes or artificial bilayer membranes, they have no known in vivo transport function (Shamoo and Goldstein, 1977). A logical extension to the model ionophores would be the isolation of ionophores from biological membranes of higher organisms which have known ion-transport properties (Shamoo and Goldstein, 1977).

Ca^{2+} transport in mitochondria has attracted much interest recently (Mela, 1977;Bygrave, 1977;Bygrave, 1978; Carafoli and Crompton, 1978). The reason is at least two-fold. Physiologically, there are lines of evidence that Ca^{2+} transport in mitochondria plays an important role in regulating the intracellular Ca^{2+} concentration (Bygrave, 1978; Carafoli and Crompton, 1978). Kinetically, Ca^{2+} transport in mitochondria appears to be a carrier-mediated process (Carafoli and Sottocasa, 1974; Lehninger, 1970; Reed and Bygrave, 1974; Selwyn et al.,1970), and this Ca^{2+}-carrier complex responds to a negative-inside membrane potential across the inner mitochondrial membrane (Scarpa and Azzone, 1970). The

isolation of this carrier would therefore provide a better model for carrier-mediated transport studies. Indeed, there have been numerous attempts to isolate this specific carrier (Lehninger, 1971; Gomez-Puyou et al., 1972; Sottocasa et al., 1972; Carafoli and Sottocasa, 1974; Utsumi and Oda, 1974; Carafoli, 1975). However, all of the isolated fractions or pure proteins appear to be hydrophilic, extrinsic membrane binding proteins rather than the Ca^{2+} carrier (Carafoli, 1976;Prestipino et al., 1974).

In Chapter 1, a 3000-dalton calcium ionophore protein (named calciphorin) has been isolated by using a novel approach (Jeng et al., 1978;Shamoo and Jeng, 1978; Jeng and Shamoo, 1978). Calciphorin has shown high affinity and high specificity for Ca^{2+}, and the calciphorin-mediated Ca^{2+} extraction into the organic phase is inhibited by low concentrations of ruthenium red and La^{3+}. Also, calciphorin can translocate Ca^{2+} through a bulk organic phase in Pressman cell experiments in the presence of a pH step gradient between the aqueous phases, with high pH on the donor side. However, large amounts of phospholipids were found to be associated with calciphorin. Indirect experiments with the partially delipidated calciphorin indicate that the transport properties found are associated with the protein and not the phospholipids. Another objection against calciphorin being the specific Ca^{2+} carrier is that the addition of a lipophilic anion had little effect on the net amount of calciphorin-mediated Ca^{2+} extraction into all of the organic phases tested. These experiments may indicate either that the Ca^{2+}-calciphorin complex is neutral or that the contaminating phospholipids act as lipophilic anions.

In this chapter, a complete delipidation procedure is described and the question as to the electrogenicity of calciphorin is answered.

Some of the results described in this chapter have been reported previously (Shamoo, et al., 1978).

EXPERIMENTAL PROCEDURE

MATERIALS

$^{45}CaCl_2$ was purchased from Amersham-Searle Corp. Sephadex LH-20 (25-100μm)was obtained from Pharmacia. Silica gel G plates (250 μm) were products of Fisher Scientific. Sequanal grade pyridine was purchased from Pierce. Methylene chloride, mannose, galactosamine hydrochloride, N-acetylneuraminic acid were obtained from Sigma. All other reagents used were of the highest commercial purity available.

METHODS

Isolation of calciphorin

Calciphorin was isolated from calf heart mitochondria as detailed in Chapter 1. Mitochondria were prepared as previously described (Crane et al., 1956). Inner mitochondrial membranes,isolated according to the method of Schnaitman and Greenawalt (1968), were partially solubilized with DOC. The DOC-soluble inner mitochondrial membrane was chromatographed on a Sephadex G-50 column equilibrated with 1% K DOC, 0.02% NaN_3, and 10 mM Tris.HCl,pH 7.8. The elution profile was monitored by 280 nm absorbance and the fifth major peak was identified as calciphorin.

Delipidation of calciphorin by Sephadex LH-20 column chromatography.

Sephadex LH-20 beads in chloroform/methanol (C/M, 6:1) were packed in a Bio-Rad Econo-column with 0.7 cm in inner diameter. The total bed-volume was 10 ml. The column was washed with 2 bed-volumes of C/M 6:1 before the sample was applied. The protein sample used in this procedure came from the fifth major peak of the Sephadex G-50 column chromatography (Fig. 3, Chapter 1) and had been dialyzed against 50% (vol/vol) ethanol at 4°C for 5 days. The resulting cloudy dialysate was centrifuged at 228,000 x g for 30 minutes. The yellowish pellet was dissolved in a small volume of C/M 6:1. The sample containing 200 µg of protein in 200 µl of C/M 6:1 was eluted with 3 bed-volumes each C/M 6:1, C/M 1:1, and methanol. Three-milliliter fractions were collected. Each tube was dried under a stream of N_2. The first 12 fractions were re-solubilized in 200 µl of sequenal grade pyridine while the rest of the fractions were in the same volumes of methanol. An aliquot (20 µl) from each tube was then analyzed by TLC on Silica Gel G plates (250 µm) with chloroform-methanol-28% ammonium hydroxide (65:35:5, by volume) as the developing solvent (Meissner and Fleischer, 1971). The plates were charred at 100°C for one hour after spraying with chromerge. Fractions containing no phospholipids by visual analysis (Fraction C) were pooled. The rest of the fractions (Fraction B) other than the void volume (Fraction A) were also pooled. These combined fractions were then dried under nitrogen individually. Fractions A and B were resolubilized in small volumes of sequanal grade pyridine and Fraction C in methanol. The

protein and phospholipid phosphorus concentrations of each combined fraction was then determined and the mole ratio of phospholipid: protein was calculated.

Fatty Acid Analysis.

The total fatty acid content associated with the delipidated calciphorin was determined using gas chromatography. Before the application to the gas chromatograph, the fatty acids were converted to fatty acid methyl esters according to the method or Farquhar (1962) modified by Salem et al.(1976). Delipidated calciphorin (100 µg) was taken to dryness with a stream of N_2 and was re-solubilized in 2 ml of HCl-methanol solution containing 5% HCl (w/v) and 0.01% butylated hydroxytoluene (BHT) in anhydrous methanol. The tube was flushed with nitrogen, capped tightly, placed in a water bath at 60°C for 1 hour, and vortexed occasionally. After removal from the water bath, 4 ml of water and 6 ml of petroleum ether with 0.01% BHT were added to the tube. The tube was vortexed and the resulting mixed phases were separated by centrifugation at 1000 x g for 5 minutes. The petroleum ether extraction was repeated twice and tne extracts were pooled and concentrated. The final concentration of the fatty acid methyl esters was about 1 mg/ml of petroleum ether and 3 µl was injected using a Hamilton lambda syringe to a Hewlett Packard Model 5830A gas chromatograph equipped with dual flame ionization detectors. The solid phase of this gas chromatograph was 10% diethylene glycol succinate on chromosorb W, and helium was used as tne carrier gas at a flow rate of 24 ml/min. The temperatures of the injector and the

detector were 200 and 250°C, respectively. A Hewlett Packard Model 18850A gas chromatography terminal was used to plot the elution pattern of the fatty acid methyl esters as a function of the retention time and to compute the area under each peak. The total amount of fatty acids in the sample was estimated by comparison with the total peak areas of the standards. The standards used were NHI-F, GLC-60 (both from Supelco), and GLC-006 (Analabs). The free fatty acid content of the sample was calculated by subtracting 2 times the amount of phospholipid phosphorus associated with the delipidated calciphorin from the total amount of fatty acids obtained above.

Neutral sugar determination

The total amount of neutral sugars (glucose, galactose, mannose, fucose, and xylose) associated with the delipidated calciphorin was determined by the method of Dubois et al. (1956) using mannose as the standard. Samples containing 20 µg of delipidated calciphorin in methanol were dried in test tubes under a stream of nitrogen and were resuspended in 200 µl of water by vortexing. Five microliters of 80% redistilled reagent grade phenol immediately followed by 500 microliters of concentrated sulfuric acid were then added to each tube. The stream of sulfuric acid was directed against the liquid surface rather than against the side of the test tube in order to obtain a good mixing. The tubes were allowed to stand for 10 minutes, vortexed, and then placed in a water bath at 25°C for 15 minutes. The absorbance at 490 nm was then read. The standards containing 0.5 to 10 µg of mannose in 200 µl of water were treated in the same manner.

All of the standards and samples were prepared at least in triplicate to minimize errors resulting from contaminations.

Determination of hexosamines

The total amount of hexosamines (glucosamine and galactosamine) associated with the delipidated calciphorin was measured according to the method of Dische and Borenfreund (1950). Galactosamine hydrochloride was used as the standard. Duplicate samples containing 10 µg of delipidated calciphorin in methanol were dried in test tubes under N_2 and were resuspended in 62.5 µl of water by vortexing. Equal volumes (62.5 µl) of 5% sodium nitrite and 33% acetic acid were then added to each tube to deaminate the hexosamines and to convert them to 2,5-hexose anhydrides. The excess nitrous acid was then removed by adding 62.5 µl of 12.5% ammonium sulfamate to each tube, and the tubes were shaken for 30 minutes at room temperature. For control experiments without deamination, 187.5 µl of a mixture of equal volumes of 5% sodium nitrite, 33% acetic acid, and 12.5% ammonium sulfamate were each added to another pair of duplicated samples containing 10 µg of delipidated calciphorin in 62.5 µl of water. After shaking, 250 µl of 5% hydrochloric acid and 25 µl of 1% indole in ethanol were added to each of the deaminated and non-deaminated samples. The tubes were then placed in a heating block for 5 minutes at 100°C. After cooling, 250 µl of ethanol were added to each tube to remove the slight turbidity which appeared after heating. The tubes were vortexed and the absorbances at both 492 and 520 nm were recorded. The standards containing 1 to 50 nmoles of galactosamine hydrochloride were treated in

the same manner. The difference of the absorbance at 492 and 520 nm ($A_{492} - A_{520}$) for the non-deaminated solutions was subtracted from the corresponding values for the deaminated samples or standard solutions. The amount of galactosamine in the sample was estimated from the ($A_{492} - A_{520}$) vs. galactosamine hydrochloride plot for the standard solutions.

Determination of sialic acids.

The total content of sialic acids (N-acetyl-, N-glycolyl-, and N,O-diacetylneuraminic acid) associated with the delipidated calciphorin was determined according to the method of Warren (1959). N-acetylneuraminic acid was used as the standard. Samples containing 10 µg of delipidated calciphorin in methanol were dried in test tubes under N_2, and were resuspended in 50 µl of water by vortexing. Periodate solution (25 µl) containing 0.2M sodium periodate (meta) in 9M phosphoric acid was then added to each tube. The tubes were then vortexed and allowed to stand at room temperature for 20 minutes. Arsenite solution (250 µl) which contained 10% sodium arsenite, 0.5 M sodium sulfate and 0.1N H_2SO_4 was added next, and the tubes were vortexed until a yellow-brown color had disappeared. Finally, 750 µl of 0.6% thiobarbituric acid in 0.5 M sodium sulfate was added, the tubes vortexed and capped with marbles, and then heated in a heating block at 100°C for 15 minutes. The tubes were then removed and placed in cold water for 5 minutes. After cooling, 1.075 ml of cyclohexanone was added. Each tube was vortexed for 30 seconds and the phases were separated by centrifugation in a Beckman TJ-6 table-top

centrifuge at 900 x g for 5 minutes. The clear upper cyclohexanone phase was red and the absorbance at 549 nm was read. The standards containing 1 to 10 nmoles of N-acetylneuraminic acid were treated in the same manner.

Other methods.

The concentrations of calciphorin and the phosphorus associated with the delipidated calciphorin were determined by the methods of Lowry et al. (1951) and Ames and Dubin (1960), respectively. Methylene chloride was used as the organic phase in the organic solvent extraction experiments. Ca^{2+} transport through a bulk organic phase was measured by using Pressman cells (Pressman, 1973). Urea/SDS polyacrylamide gel electrophoresis was carried out according to the method of Swank and Munkres (1971). These methods were described in detail in Chapter 1.

RESULTS

Delipidation of Calciphorin

Calciphorin was delipidated completely by Sephadex LH-20 column chromatography. Fig. 1a shows the elution pattern from the Sephadex LH-20 column run on a TLC plate. The numbers on the plate correspond to the actual fraction numbers collected from the column. The zeroth spot was the sample before fractionation. Fractions 1 to 10 were eluted with C/M 6:1, fractions 11-20 with C/M 1:1, and the rest of the fractions with methanol. It can be seen that the bulk of the phospholipids were eluted in the first two fractions (Fraction A). Fractions 13-30, containing no phospholipids by visual analysis, were combined(Fraction C). Other fractions (3-12) containing only little phospholipids, were also pooled (Fraction B). Figure 1b shows the elution profile of DOC on the Sephadex LH-20 column eluted under the same condition as that shown in Fig. 1a. By comparison with Fig. 1a and 1b, it can be concluded that there was an insignificant amount, if any, of DOC present in the Fraction C.

The mole ratios of phospholipid phosphorus to calciphorin in Fractions A, B, and C are shown in Table 1. The mole ratio of Fraction A shown in this table was an underestimation. Because the presence of large amounts of phospholipids was found to interfere with the Lowry protein determination giving a high absorbance at 750 nm (unpublished observation), the concentration of calciphorin in Fraction A should have been lower than the measured value. Thus, the mole ratio

Figure 1. TLC plates showing the elution profile from the Sephadex LH-20 column chromatography. The numbers on the plates correspond to the actual fraction numbers collected from the column. The zeroth spots were samples before applying to the column. Fractions were eluted by the following solvents: 1 to 10,C/M 6:1; 11 to 20, C/M 1:1; 21 to 30, methanol. The developing solvent was chloroform-methanol-28% ammonium hydroxide (65:35:5 by volume).

a) The sample applied to the column was 200 μg of calciphorin. The zeroth spot was 10 μg of calciphorin.

b) The sample applied to the column was 6.4 mg of DOC. The zeroth spot was 130 μg of DOC.

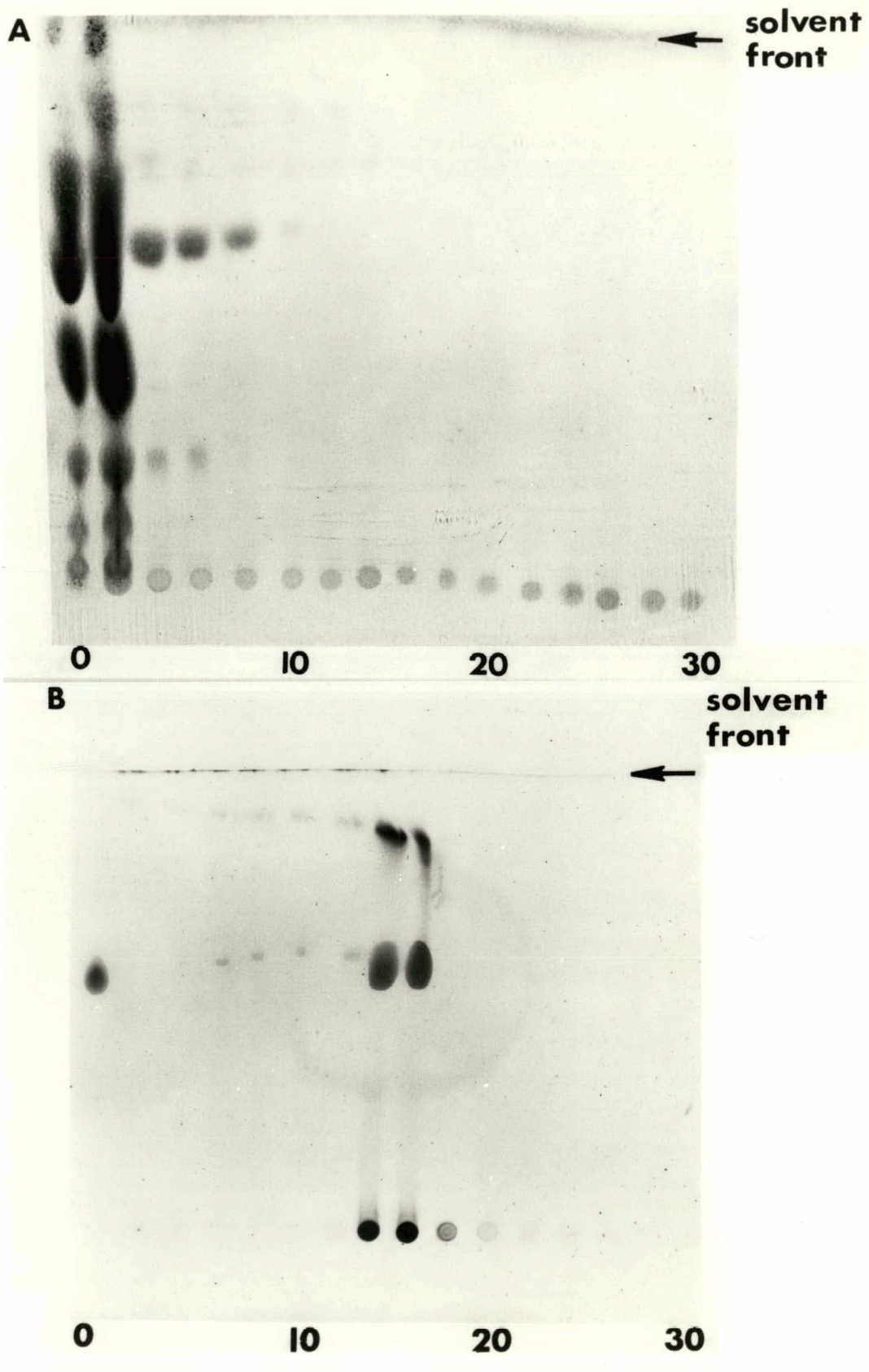

A
solvent front
0
10
20
30
B
solvent front
0
10
20
30

TABLE 1

Phospholipid phosphorus associated with calciphorin in fractions eluted from the Sephadex LH-20 column

Sample	mole ratio phosphorus:calciphorin
Calciphorin before delipidation	150
Fraction A	410
Fraction B	5
Fraction C	0.3

In calculating the mole ratio, 3000 was taken as the molecular weight of calciphorin. Thirty fractions were collected from the Sephadex LH-20 column during the delipidation procedure. These fractions were combined into 3 groups according to the visual analysis of the phospholipid content on TLC plates. Fraction A, Fraction B, and Fraction C were combined from fraction numbers 1 to 2, 3 to 12, and 13 to 30, respectively.

in Fraction A should be much higher than 410. The mole ratio of phospholipid phosphorus to calciphorin in Fraction C could be reduced to about 0.1 by combining only the fractions eluted with methanol from the Sephadex LH-20 column. It will be seen that the presence of phospholipids at a mole ratio of 0.3 does not contribute to the characteristics associated with calciphorin (Table 3 and Fig. 3, below). Thus, in order to obtain a higher yield of the delipidated calciphorin, more fractions eluted from the Sephadex LH-20 column were combined in Fraction C. The mole ratio of phospholipid phosphorus to calciphorin in Fraction C used in the subsequent experiments was 0.3. The delipidated calciphorin was not soluble in water, but could be solubilized in methanol.

Fig. 2 shows the result of the urea/SDS polyacrylamide gel electrophoresis of calciphorin before and after delipidation. After delipidation, calciphorin migrated faster in the urea/SDS polyacrylamide gels. The mobilities of calciphorin before and after delipidation were 0.54 and 0.68, respectively. The amino acid composition of the delipidated calciphorin was still the same as that shown in Table 3 of Chapter 1 and the delipidated calciphorin lost its pink color in periodic acid/Schiff base stain (results not shown).

Prosthetic groups associated with the delipidated calciphorin.

The contents of phospholipid phosphorus, free fatty acids, and carbohydrates associated with the delipidated calciphorin are shown in Table 2. Since there are two fatty acid chains associated with each head group of the phospholipids, the content of free fatty acids

Figure 2. Urea/SDS gels and gel scans of calciphorin before and after delipidation. Gels were scanned at 560 nm.

Top Gel: 7 µg calciphorin before delipidation

Bottom Gel: 3 µg calciphorin after delipidation

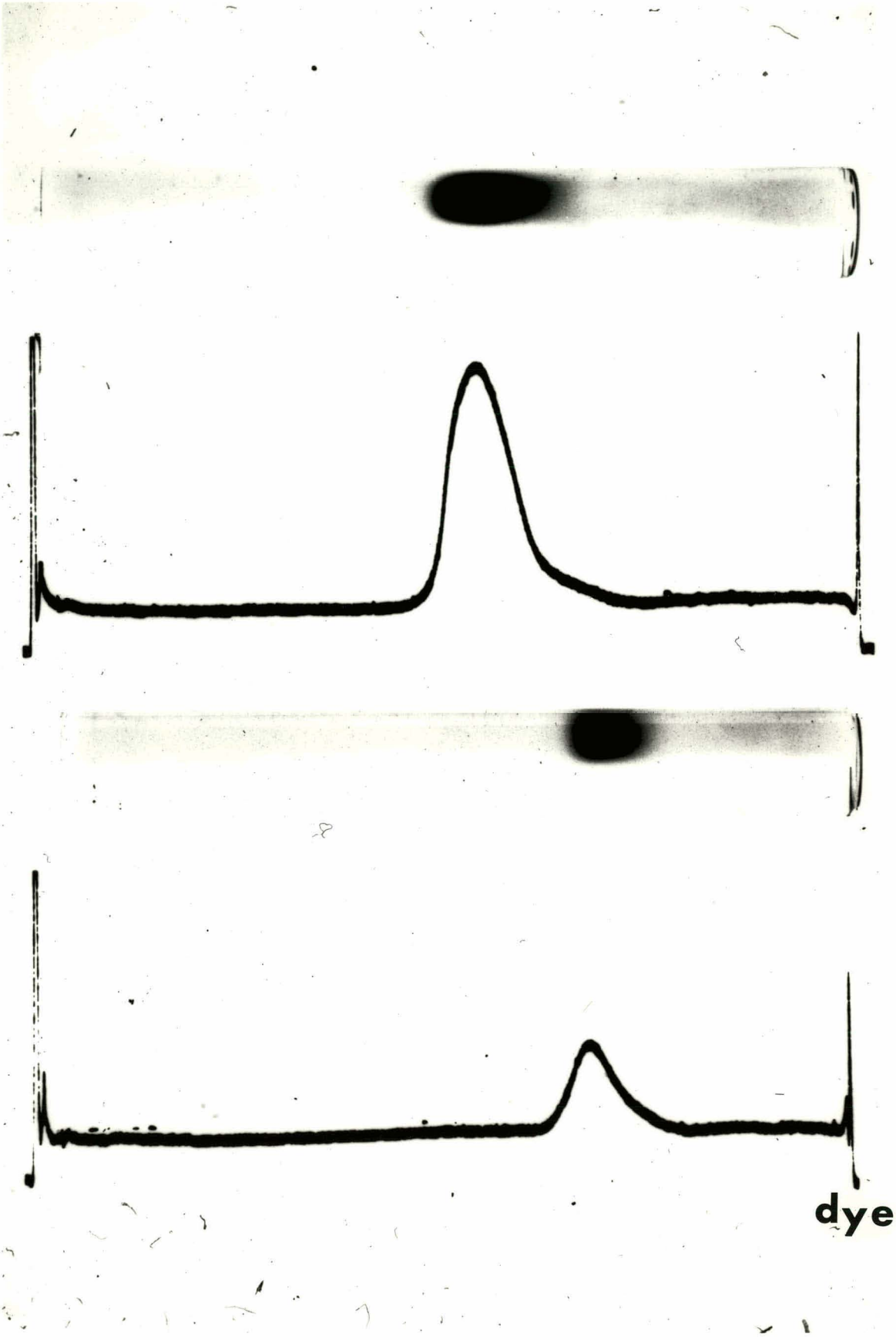

dye

TABLE 2

Prosthetic groups associated with the delipidated calciphorin

Prosthetic group	Mole ratio prosthetic group:calciphorin
Phospholipid phosphorus	0.1
Free fatty acids	0
Neutral sugars (glucose, galactose, mannose, fucose, xylose)	0.5
Hexosamines (glucosamine, galactosamine)	0
Sialic acids	0

was calculated by subtracting 2 times the amount of phospholipid phosphorus associated with the delipidated calciphorin from the total amount of fatty acids obtained by gas chromatography. Except for neutral sugars, the samples used in the determination of other prosthetic groups were obtained from the Sephadex LH-20 column delipidation procedure. By using calciphorin delipidated from Sephadex LH - 20 column, the mole ratio of the neutral sugars to calciphorin was 14:1. None of the other components of the carbohydrates (hexosamines and sialic acids) were found in the sample of delipidated calciphorin. Since the same mole ratio of neutral sugar to calciphorin was also found in other fractions containing higher phospholipid content eluted from the Sephadex LH-20 column (result not shown) and there are no known glycoproteins containing neutral sugars as the sole carbohydrate component, the measured content of neutral sugars was probably due to contamination. Therefore, the possible sources of interferring substances such as various phospholipids and high concentrations of DOC were tested. None of the above substances showed any interference on the determination of the neutral sugars (results not shown) carried out by the method of Dubois et al. (1956). Because calcipnorin was delipidated from tne Sephadex LH-20 column and the Sephadex beads were prepared by cross-linking selected dextran (anhydroglucose polymer) fractions with epichlorohydrin, it is likely that the neutral sugars measured in the sample of delipidated calciphorin came from the degradation products of the Sephadex beads. Indeed, control experiments with Sephadex LH-20 beads gave extremely high readings at 490 nm.

By using methylene chloride as the delipidation procedure, it was found that the mole ratio of neutral sugars to calciphorin was 0.5. (Table 2). Thus, the total carbohydrate content in calciphorin is insignificant and calciphorin is therefore not a glycoprotein.

Organic solvent extraction experiments.

The results of calciphorin-mediated Ca^{2+} extraction into the methylene chloride phase are shown in Table 3. In the absence of any lipophilic ions, the amount of Ca^{2+} extracted into the organic phase was only 0.01 mole per mole of calciphorin under the assumption that all of the protein was in the organic phase. The addition of picrate, a lipophilic anion, enhanced the mole ratio to 0.25, whereas the addition of tetramethylammonium, a lipid-soluble cation, had no effect on the amount of calciphorin-mediated Ca^{2+} extraction. Control experiments with pyridine, in which calciphorin was solubilized, showed zero effects on the Ca^{2+} extraction under all of the conditions. The protein sample used in the organic solvent extraction experiments had 0.3 moles of phospholipids associated with each mole of delipidated calciphorin. The effects of phospholipids on Ca^{2+} extraction were therefore tested. In the experiments shown in Table 3, the amount of each of the phospholipid standards used was identical to the total amount of phospholipids associated with calciphorin. For all the experiments carried out under this condition, phospholipid standards were found not to be able to extract Ca^{2+} into the organic phase in the presence or absence of a lipophilic anion, except the small effect of cardiolipin in the presence of picrate. Since cardiolipin only accounted for less than 5%

TABLE 3

Effects of lipophilic ions and contaminating phospholipids on calciphorin-mediated Ca^{2+} extraction into the organic phase

Addition	Mole ratio, Ca^{2+} extracted/protein				
	calciphorin	pyridine	cardiolipin	PE	PC
None	0.01	0	0	0	0
Picric acid	0.25	0	0.02	0	0
Tetramethyl-ammonium chloride	0.01	0	-	-	-

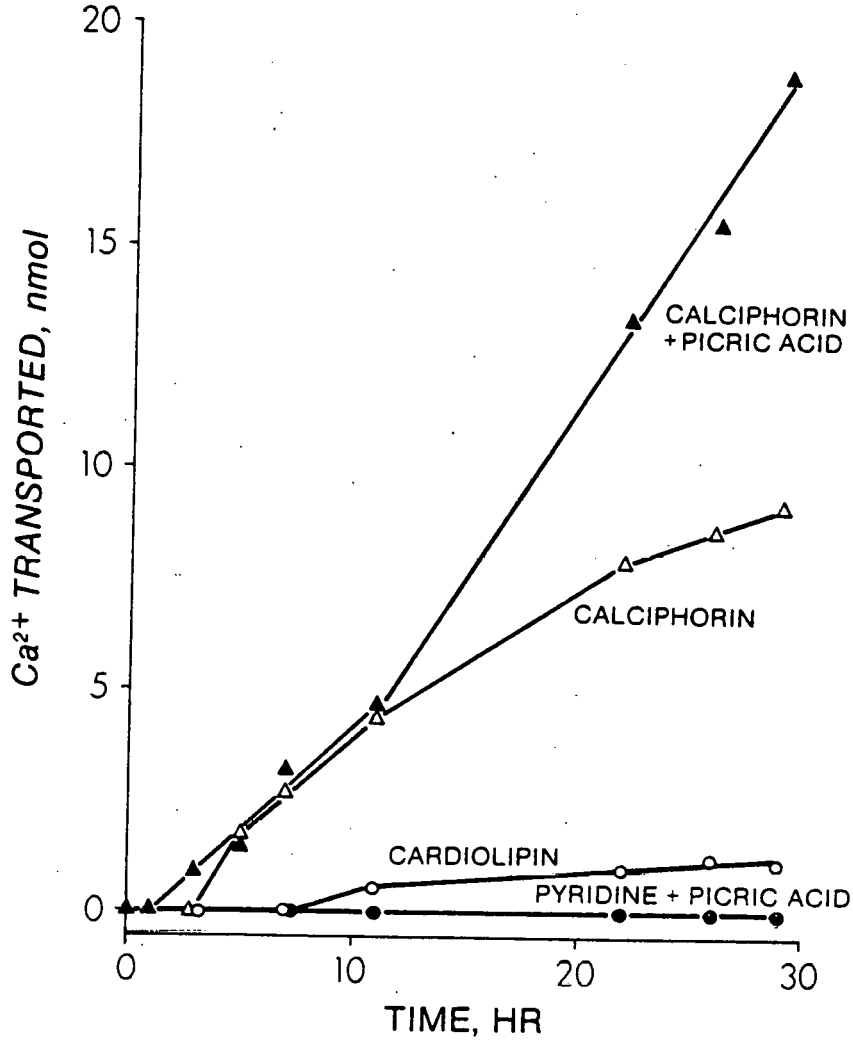
The aqueous phase contained 1 ml of 1 mM $^{45}CaCl_2$ and 5 mM Tris.HCl, pH 7.8. The organic phase was 2 ml of methylene chloride. When used, 50 μM picric acid or tetramethylammonium chloride was added to the aqueous phase. Calciphorin and phospholipid standards in pyridine were added to the organic phase. Calciphorin was delipidated by Sephadex LH-20 column chromatography. There were 0.3 moles of phospholipids associated with each mole of delipidated calciphorin. The concentrations of calciphorin and the phospholipid standards used were 1.3 and 0.4 μM, respectively. The molecular weight of calciphorin was taken as 3000 daltons in calculating the mole ratios. The mole ratio of the cardiolipin-mediated Ca^{2+} extraction in the presence of picrate was calculated in the same manner in order to make a direct comparison with the extraction mediated by calciphorin.

of the total contaminating phospholipids before the delipidation of calciphorin (Fig. 11, Chapter 1) and almost 100% of the cardiolipin could be eluted in the void volume during the Sephadex LH-20 column delipidation procedure (Fig. 1a), the small effect of Ca^{2+} extracted due to cardiolipin in the presence of picrate can be neglected.

Bulk phase transport experiments.

The rate of Ca^{2+} translocation from a donor aqueous phase through a bulk organic phase to a receiver aqueous phase mediated by the delipidated calciphorin was also enhanced by the addition of picrate. Fig. 3 shows the results of the bulk phase transport experiments. In these experiments, the donor aqueous phase was 10 mM $^{45}CaCl_2$ and 10 mM Tris.HCl, pH 8.5 and the receiver aqueous phase was 10 mM MES, pH 6.5 adjusted with Tris. The rate of calciphorin-mediated Ca^{2+} translocation was enhanced at least twice by the addition of picric acid to the donor side. Cardiolipin, at the same concentration as that of the phospholipids associated with the delipidated calciphorin, had little effect on Ca^{2+} translocation, while the presence of picric acid and pyridine showed no effect.

Figure 3. Bulk phase transport experiments with delipidated calciphorin. The donor aqueous phase was 2 ml of 10 mM $^{45}CaCl_2$ and 10 mM Tris.HCl, pH 8.5, and the receiver aqueous phase was 2 ml of 10 mM MES, pH 6.5 adjusted with Tris. The organic phase used was 6 ml of methylene chloride. When used, 50 μM of picric acid was added to the donor aqueous phase. Calciphorin and cardiolipin were added directly to the organic phase. The mole ratio of the contaminating phospholipids associated with calciphorin was 0.3. The concentrations of calciphorin and cardiolipin in the organic phase were 1.4 and 0.4 μM, respectively.


20
15
10
5
0
Ca^{2+} TRANSPORTED, nmol
CALCIPHORIN
+PICRIC ACID
CALCIPHORIN
CARDIOLIPIN
PYRIDINE + PICRIC ACID
0
10
20
30
TIME, HR

DISCUSSION

As mentioned in Chapter 1, a major problem in the characterization of the transport properties of calciphorin is the lipid contamination. It has been shown that the transport properties are associated with calciphorin rather than the contaminating phospholipids by indirect approaches with the partially delipidated calciphorin. A more direct method is to use Sephadex LH-20 column chromatography to delipidate calciphorin (Fig. 1a). The mole ratio of phospholipids to calciphorin was reduced to an insignificant amount, 0.1 mole of phospholipids per mole of calciphorin. However, a mole ratio of 0.3 was found to be satisfactory in the subsequent study of calciphoin-associated transport properties. At this mole ratio, phospholipids did not contribute to the calciphorin mediated Ca^{2+} extraction into the organic phase (Table 3). Tyson et al. (1976) have shown that cardiolipin can function as an ionophore in the Pressman cell experiments. Even when the phospholipid content of calciphorin was reduced down to a mole ratio of 13 by silicic acid column chromatography, the rate of Ca^{2+} transport medicated by calciphorin in the Pressman cell experiments was still much slower than that mediated by cardiolipin assuming that all of the phospholipids associated with calciphorin were cardiolipin (Fig. 13b, Chapter 1). However, when the mole ratio of phospholipids to calciphorin was reduced to 0.3 by Sephadex LH-20 column chromatography, cardiolipin was no longer active as compared with the Ca^{2+} transport rate associated with calciphorin in the Pressman cell

experiments (Fig.3). Thus it can be concluded that the transport properties are associated with calciphorin and not the contaminating phospholipids.

Both the integer numbers of residues in the amino acid composition of calciphorin (Table 3, Chapter 1) and the single band in the urea/SDS polyacrylamide gel electrophoresis of calciphorin before delipidation (Fig. 6, Chapter 1) give indications that calciphorin is a pure polypeptide. Although it is not likely, it is still possible that more than one polypeptide migrated with the same mobility in the gel as a single band. However, after delipidation, calciphorin migrates to a spot different from that of calciphorin before delipidation(Fig. 2). The chance of more than one polypeptide having similar amino acid composition, migrating to the same spot in the gel before delipidation, being solubilized with the same organic solvent when eluted from the Sephadex LH-20 column during the delipidation procedure and moving with the same mobility again after delipidation is extremely small. Thus, the urea/SDS polyacrylamide gel electrophoresis of the delipidated calciphorin once again assures the purity of calciphorin.

It has been shown that the driving force for Ca^{2+} transport in mitochondria is the negative-inside membrane potential across the inner mitochondrial membrane (Scarpa and Azzone, 1970). This means the Ca^{2+} - carrier complex is positively charged and the extraction of this complex into an organic phase requires a lipophilic anion to neutralize the positive charge of the complex. However, before delipidation, the addition of picrate had little effect in increasing the net amount of

calciphorin-mediated Ca^{2+} extraction into all of the organic phases tested (Table 5, Chapter 1). These experiments may indicate either that the Ca^{2+}-calciphorin complex is neutral or that the contaminating phospholipids act as lipophilic anions. The latter explanation is true, since after delipidation the presence of a lipophilic anion has been shown to be able to enhance the extraction of the Ca^{2+}-calciphorin complex into the organic phase significantly (Table 3). In the same table, a lipophilic cation shows no effect in enhancing the calciphorin-mediated Ca^{2+} extraction into the organic phase. Thus, the Ca^{2+}-calciphorin complex is positively charged. Another piece of evidence to support this point comes from the Pressman cell experiments. In Fig. 3, it can be seen that the rate of calciphorin-mediated Ca^{2+} translocation through a bulk organic phase is also enhanced by the presence of picrate.

Probably the most distinct property associated with calciphorin is its hydrophobicity and its insignificant contents in the prosthetic groups like phospholipids and carbohydrates. There are several reports of the isolation of high-affinity Ca^{2+}-binding proteins from mitochondria (Lehninger, 1971; Sottocasa et al., 1972; Carafoli and Sottocasa, 1974; Utsumi and Oda, 1974; Carafoli, 1975). However, all of these isolated fractions or pure proteins are water-soluble glycoproteins. It has been shown that the Ca^{2+} carrier is localized in the inner mitochondrial membrane (Pedersen and Coty, 1972). Thus, the requirement of hydrophobicity for the Ca^{2+} carrier eliminates those glycoproteins as candidates for being the carrier. On the other hand, calciphorin

is very hydrophobic. As mentioned in Chapter 1, calciphorin is soluble in diethyl ether before the delipidation procedure. After delipidation, calciphorin is still not water-soluble. The mole ratio of phospholipids associated with each mole of calciphorin after delipidation can be reduced to 0.1 and there is no free fatty acid associated with calciphorin (Table 2). Although there are some contaminating neutral sugars in the delipidated calciphorin due to the degradation products of the Sephadex LH-20 beads eluted during the delipidation procedure, these contaminating sugars do not partition into the organic phase in the organic solvent extraction experiments. Therefore, the presences of these neutral sugars does not interfere with the physical-chemical properties associated with calciphorin. A similar situation has also been reported on the content of neutral sugars associated with the nicotinic acetylcholine receptor where the various amounts of neutral sugars detected were ascribed to the contamination from the agarose column used during preparation (Briley and Changeux 1977). The lack of carbohydrate content of calciphorin is also consistent with the negative result from the periodic acid/Schiff base glycoprotein stain in gels of the delipidated calciphorin. Thus, its hydrophobicity and its insignificant content in the prosthetic groups render calciphorin to be a unique protein.

The mole ratio of Ca^{2+} extracted by delipidated calciphorin (stoichiometry) in the presence of picrate is 0.25 (Table 3). However, these organic solvent extraction experiments were not performed under the optimal conditions. It will be shown in Chapter 3 that the vor-

texing time, the ratio of picrate to protein, and the pH and concentration of Ca^{2+} in the aqueous solution are all important factors to achieve the optimal stoichiometry of the Ca^{2+} extraction. Similarly, the calciphorin-mediated Ca^{2+} transport rate through a bulk organic phase in the presence of picrate can be enhanced by investigating the optimal condition.

The selectivity of this delipidated calciphorin for cations, the effects of inhibitors of Ca^{2+} transport on the calciphorin-mediated Ca^{2+} extraction into the organic phase, and other physical and chemical properties associated with calciphorin will be studied in Chapter 3.

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CHAPTER 3

FURTHER CHARACTERIZATION OF THE DELIPIDATED CALCIUM IONOPHORE

SUMMARY

The physical and chemical properties of the delipidated calciphorin were investigated. It was found that delipidated calciphorin becomes more hydrophobic in the presence of Ca^{2+} and alkaline pH in the organic solvent extraction experiments. The pH profile of the mole ratio of Ca^{2+} to calciphorin exhibited a typical titration curve, showing a pK_a of 8.0-8.1. The relative cation selectivity of calciphorin determined from the organic solvent extraction experiments was $Zn^{2+} > Ca^{2+}$, $Sr^{2+} > Rb^{2+}$, $Na^{+} > Mn^{2+}$. Ruthenium red and La^{3+} were shown to inhibit calciphorin-mediated Ca^{2+} extraction into the organic phase. Respiratory inhibitors, oligomycin, and an uncoupling agent had no effect on the Ca^{2+} extraction. Phosphate did not stimulate calciphorin-mediated Ca^{2+} extraction. The Ca^{2+}-calciphorin complex appears to have two positive charges. The delipidated calciphorin only had one class of Ca^{2+}-binding sites as revealed from the flow dialysis studies. These Ca^{2+}-binding sites had a dissociation constant of 5.2 μM and bound 1 mole of Ca^{2+} per mole of calciphorin. Evidence suggests that calciphorin may be a strong candidate for the Ca^{2+} carrier responsible for the influx mechanism in mitochondrial Ca^{2+} transport system. Two possible models of calciphorin-mediated Ca^{2+} transport in mitochondria are proposed.

INTRODUCTION

One of the most important problems in mitochondrial Ca^{2+} transport that remains to be resolved is the mechanism of Ca^{2+} transport. Selwyn et al. (1970) investigated the passive Ca^{2+} movement into non-respiring mitochondria down an electrochemical gradient of various Ca^{2+} salts. They concluded that the Ca^{2+} transport mechanism is either a Ca^{2+} uniporter or a Ca^{2+}/K^{+} antiporter. Moyle and Mitchell (1977) estimated the electric charge stoichiometry of Ca^{2+} translocation from the number of acid equivalents that were exported through the respiratory chain per Ca^{2+} imported through the La^{3+}-sensitive Ca^{2+} carrier. They suggested that the specific Ca^{2+} carrier is not a Ca^{2+} uniporter, but is a Ca^{2+}/phosphate symporter with a net charge transfer of +1. However serious doubts on the validity of the antiport mechanism have been raised (Azzone et al., 1977;Reynafarje and Lehninger, 1977).

The distributions of Ca^{2+} and Rb^{+}(in the presence of valinomycin) across the mitochondrial membrane have been measured by Rottenberg and Scarpa (1974). It was found that the distribution ratio for Ca^{2+} was about the same as the square of the distribution ratio for Rb^{+}. Assuming that Rb^{+} distribution in the presence of valinomycin was governed by membrane potential obeying the Nernst equation, Rottenberg and Scarpa (1974) interpreted their results as evidence that Ca^{2+} uptake in mitochondria is an electrogenic process driven by a membrane potential with a net charge transfer of +2.

There have been many attempts at measuring the membrane potential across the inner mitochondrial membrane. The measured values vary from 130 to 200 mV (Mitchell and Moyle, 1969; Nicholls, 1974; Azzone et al.,1976). Assuming that the uniporter is the sole mechanism reponsible for the Ca^{2+} transport in mitochondria, a membrane potential of 180 mV would suggest that the equilibrium gradient of ionized Ca^{2+} across the inner mitochondrial membrane is 10^6. The maximal Ca^{2+} concentration in the matrix compartment is about 1 mM (Puskin et al., 1976). The uniport mechanism thus implies that the cytoplasmic Ca^{2+} concentration is almost 10^{-9}M. At such a low Ca^{2+} concentration, many cytoplasmic enzyme systems will be inactivated (for a summary of these enzymes, see Carafoli and Crompton, 1978). Therefore, it is not likely that uniport mechanism is the only functional process in regulating Ca^{2+} transport in mitochondria.

Puskin et al.(1976) used electron paramagnetic resonance (EPR) spectroscopy to measure the distribution of Mn^{2+} across the inner mitochondrial membrane. Since free Mn^{2+} can be measured by EPR (Gunter and Puskin, 1972), this approach in calculating the membrane potential is probably more accurate. However, the membrane potential calculated by using Mn^{2+} is usually 40-50 mV lower than that calculated from the K^+ distribution in the presence of valinomycin (Puskin et al., 1976). This finding, together with the fact that the addition of ruthenium red to Ca^{2+} loaded mitochondria causes an efflux of Ca^{2+} from mitochondria, has led Puskin et al.(1976) to suggest that there

may be more than one Ca^{2+} mechanism in mitochondria. One of the Ca^{2+} transport mechanisms is ruthenium red sensitive (presumably, the Ca^{2+} uniporter) and the other is "less ruthenium red sensitive."

Ca^{2+} efflux from mitochondria has also been studied by other groups (Akerman, 1978; Crompton et al., 1977, 1978; Pozzan et al., 1977; Ramachandran and Bygrave, 1978). It appears that both influx and efflux mechanisms are involved in regulating Ca^{2+} transport in mitochondria. Whether these mechanisms are separate entities is not clear. While Puskin et al. (1976) and Crompton et al. (1977, 1978) favor separate mechanisms, Pozzan et al. (1977) suggest that Ca^{2+} efflux occurs always through the native carrier responsible for Ca^{2+} influx in mitochondria. The isolation of the Ca^{2+} transport system in mitochondria will provide a possible way to resolve the disagreements.

In the previous chapters, a Ca^{2+} ionophore (calciphorin) has been isolated from the inner mitochondrial membrane (Jeng and Shamoo, 1978; Jeng et al., 1978; Shamoo and Jeng, 1978; Shamoo et al., 1978). The molecular weight of calciphorin is 3000. Calciphorin is hydrophobic and does not contain any prosthetic groups like phosphate and carbohydrates. Also, calciphorin is electrogenic. The extraction of the Ca^{2+}-calciphorin complex into a methylene chloride phase requires the presence of a lipophilic anion. These properties suggest that calciphorin is a likely candidate for the Ca^{2+} carrier.

In this chapter, the physical and chemical properties of calciphorin are further investigated. Possible mechanisms for cal-

ciphorin-mediated Ca^{2+} transport in mitochondria are discussed.

Some of the results described in this chapter have been reported previously (Jeng and Shamoo, 1979).

EXPERIMENTAL PROCEDURE

MATERIALS

$^{86}RbCl$, $^{22}NaCl$, $^{54}MnCl_2$, $^{65}ZnCl_2$, and $^{85}SrCl_2$ were purchased from New England Nuclear. $^{45}CaCl_2$ was obtained from Amersham. Sephadex LH-20 (25-100 μm) was purchased from Pharmacia. Silica gel G plates (250 μm) were products of Fisher Sci. Polypropylene tubes were obtained from Falcon. Methylene chloride, oligomycin, rotenone, antimycin A, carbonyl cyanide m-chlorophenylhydrazone (CCCP) were purchased from Sigma. All other reagents were of the highest commercial purity available.

Purified ruthenium red was a generous gift from Dr. Jerome S. Puskin.

METHODS

Isolation and delipidation of calciphorin.

Calciphorin was isolated from calf heart mitochondria as detailed in Chapter 1. Mitochondria were prepared as previously described (Crane et al., 1956). Inner mitochondrial membranes, isolated according to the method of Schnaitman and Greenawalt(1968), were partially solubilized with DOC. The DOC-soluble inner mitochondrial membrane proteins were chromatographed on a Sephadex G-50 column equilibrated with 1% KDOC, 0.02% NaN_3, and 10 mM Tris.HCl,

pH 7.8. The elution profile was monitored by 280 nm absorbance and the fifth major peak was identified as calciphorin.

Calciphorin was delipidated using Sephadex LH-20 column chromatography as described in Chapter 2. The phospholipid content associated with the delipidated calciphorin was less than 0.3 mole per mole of protein. Delipidated calciphorin was solubilized in methanol at a concentration of about 0.5 mg/ml, and kept in the freezer.

Investigation of the optimal conditions in the organic solvent extraction experiments.

The maximal amount of calciphorin-mediated Ca^{2+} extraction was investigated both as a function of extraction time and as a function of the picric acid to calciphorin ratio. In both types of experiments, the organic phase used was 1 ml of methylene chloride, and the aqueous phase was 0.5 ml of 1 mM $^{45}CaCl_2$, buffered by 5 mM Tris.HCl, pH 7.8. The concentration of calciphorin was 2 μM in the organic phase. These phases were mixed in 12 x 75 mm polypropylene tubes by vortexing and were separated by centrifugation in a Beckman TJ-6 table-top centrifuge at 1000 x g for 15 minutes. The tubes were then punctured from the bottom and the methylene chloride phase was collected. Aliquots (250 μl, duplicated) of the organic phase were then counted in a liquid scintillation counter.

In investigating the amount of Ca^{2+} extracted as a function of vortexing time, the concentration of picric acid was 100 μM (picric acid to calciphorin ratio was 25:1) in aqueous phase. When

the experiments of Ca^{2+} extraction as a function of picric acid to calciphorin ratio were performed, the vortex time was 2 minutes.

The effect of pH on the amount of calciphorin partitioned in the organic phase.

The amount of calciphorin partitioned in the organic phase as a function of the pH in the aqueous phase was measured by the organic solvent extraction procedure. The concentration of calciphorin was 2 μM in the organic phase. The aqueous phase contained 1 mM $CaCl_2$ buffered with 5 mM Tris or MES depending on the pH used. For pH ranging from 5.0 to 6.5, MES was used as the buffer and the pH's were adjusted by Tris. Tris.HCl was used in the pH range between 6.5 and 9.5. The organic solvent extraction experiments were carried out under the optimal extraction conditions investigated above. An aliquot (600 μl) of the methylene chloride phase from each tube was dried under N_2, and the amount of protein was determined by the method of Lowry et al. (1951).

Ca^{2+}-binding studies.

Ca^{2+}-binding was measured either by organic solvent extraction procedure or by flow dialysis experiments at room temperature. When the organic solvent extraction procedure was used, the concentration of calciphorin was 2 μM in the methylene chloride phase, and the concentrations of $^{45}CaCl_2$ in the aqueous phases ranged between 2.5 and 500 μM. The extraction was performed under the optimal conditions described above. In constructing the Scatchard plots (Scatchard et al., 1957), the bound and the

free Ca^{2+} were assumed to be those that appeared in the organic phase and remained in the aqueous phase, respectively, after the phases were separated by centrifugation.

The flow dialysis experiments (Colowick and Womack, 1969) were detailed in Chapter 1. The concentration of calciphorin in the upper chamber was 9 μM buffered by 25 mM Tris.HCl, pH. 7.4. The dialysis buffer was 25 mM Tris.HCl,pH 7.4. Spectrapor 2000-dalton molecular weight cutoff dialysis membranes were used.

Determination of the charge on the Ca^{2+}-calciphorin complex.

The charge of the Ca^{2+}-calciphorin complex was determined by measuring the amount of picrate extracted into the organic phase in the organic solvent extraction experiments. The amount of picrate extracted was measured spectrophotometrically. In preparing the standard absorbance versus picrate concentration plot, 50 μM picric acid in distilled water was scanned over the visible wavelength in a spectrophotometer, and the wavelength of maximal absorbance was identified. The absorbance at this identified wavelength (355 nm) versus picric acid concentration in distilled water plot was then constructed.

The organic solvent extraction experiments were performed under the optimal Ca^{2+} extraction conditions described above. The concentration of calciphorin used in these experiments varied from 0.5 μM to 5 μM in 1 ml of methylene chloride. The aqueous phase contained 1 mM $CaCl_2$ and 5 mM Tris.HCl, pH 8.5. In each sample, the total picric acid to calciphorin ratio was 50:1. After

the separation of the two phases, 700 µl of the methylene chloride phase was withdrawn from each sample. Among this, 200 µl of the methylene chloride phase was counted in a scintillation counter to measure the amount of Ca^{2+} extracted. The rest of the methylene chloride phase (500 µl) was dried under N_2, resuspended in 750 µl of distilled water, and the absorbance was read at the wavelength of maximal picric acid absorption identified above. In calculating the amount of picrate in the organic phase, two types of background absorption were subtracted. The first type was the partition of picrate into the organic phase in the absence of calciphorin, and the second type was the scattering effect due to the presence of calciphorin in distilled water.

Other methods.

The concentrations of calciphorin and the phosphorus associated with the delipidated calciphorin were determined by the methods of Lowry et al.(1951) and Ames and Dubin (1960), respectively. The purity of calciphorin was identified by urea/SDS polyacrylamide gel electrophoresis (Swank and Munkres, 1971). These methods were detailed in Chapter 1.

RESULTS

The optimal conditions in the organic solvent extraction experiments.

The maximal amount of calciphorin-mediated Ca^{2+} extraction was investigated both as a function of the vortexing time and as a function of the picric acid to calciphorin ratio. Fig. 1 shows the results of Ca^{2+} extracted as a function of the vortexing time. In these experiments, the picric acid to calciphorin ratio used was 25:1, which was not yet the optimal condition for the Ca^{2+} extraction (See Fig. 2, below). From Fig. 1, it can be seen that at least 2 minutes of vortexing time is required to achieve a maximal Ca^{2+} extraction into the organic phase.

Fig. 2 shows the results of Ca^{2+} extracted as a function of the mole ratio of picric acid to calciphorin. The mole ratio of picric acid to calciphorin required to optimize the Ca^{2+} extraction was found to be 50:1. Therefore, in all of the subsequent organic solvent extraction experiments, the amount of picric acid added to the aqueous phase was 50 times that of calciphorin added to the organic phase, and the two phases were vortexed for 2 minutes.

Factors favoring the partition of calciphorin into the organic phase.

As mentioned in Chapter 1, calciphorin was very hydrophobic before the delipidation procedure. It could be solubilized in diethyl ether in the absence of Ca^{2+}. After delipidation, although calciphorin was not water-soluble, it became less hydrophobic. The pH and concentration of Ca^{2+} in the aqueous phase were found to be critical in the partition of calciphorin into the organic phase.

Figure 1. The effect of vortexing time on the calciphorin-mediated Ca^{2+} extraction into the organic phase. The aqueous phase was 0.5 ml of 1 mM $^{45}CaCl_2$ buffered with 5 mM Tris.HCl, pH 7.8, and the organic phase was 1 ml of methylene chloride. Delipidated calciphorin (2 nmoles) was added to the methylene chloride phase and picric acid (50 nmoles) to the aqueous phase. In calculating the mole ratio of Ca^{2+} extracted to calciphorin added, all of the delipidated calciphorin was assumed to be in the organic phase and the molecular weight of calciphorin was taken as 3000.

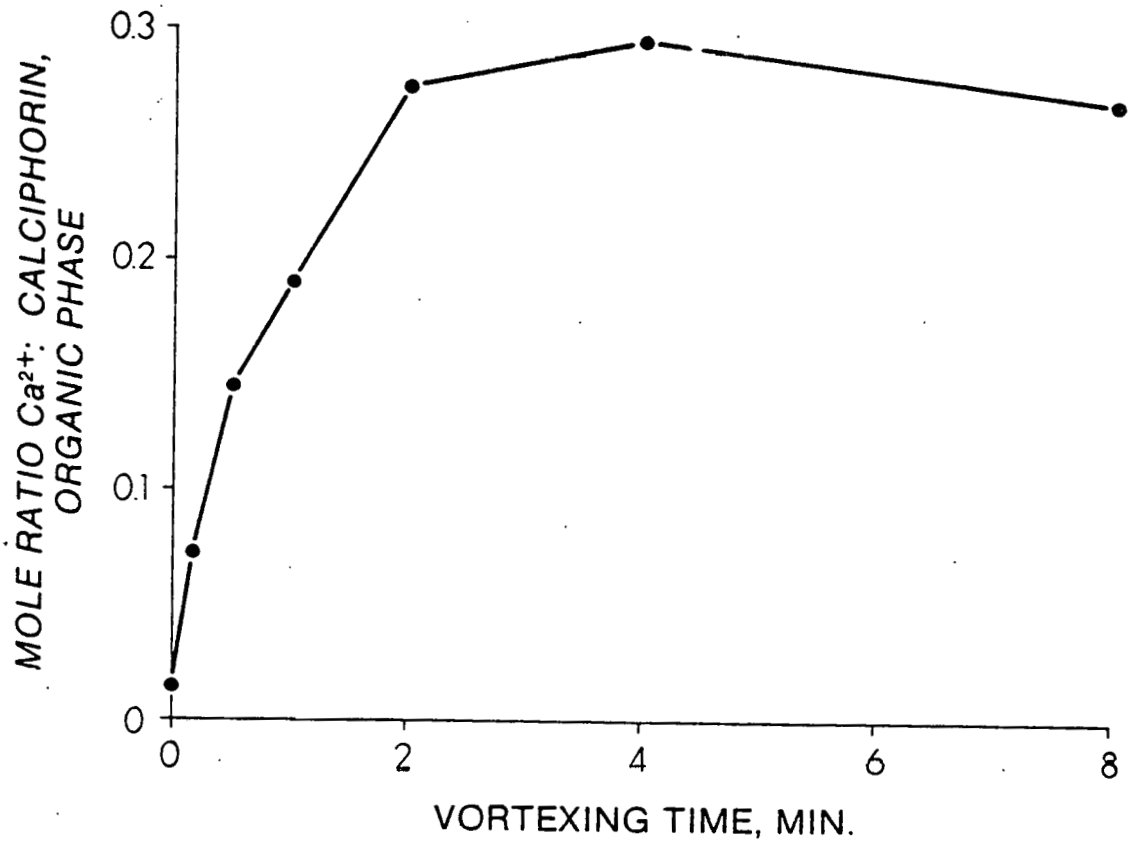

MOLE RATIO Ca^{2+}: CALCIPHORIN, ORGANIC PHASE
0.3
0.2
0.1
0
0
2
4
6
8
VORTEXING TIME, MIN.

Figure 2. The effect of picric acid on the calciphorin-mediated Ca^{2+} extraction into the organic phase. The aqueous phase was 0.5 ml of 1 mM $^{45}CaCl_2$ buffered with 5 mM Tris.HCl, pH 7.8, and the organic phase was 1 ml of methylene chloride. Delipidated calciphorin (2 nmoles) was added to the methylene chloride phase, and the indicated amount of picric acid to the aqueous phase. The vortexing time was 2 minutes. In calculating the mole ratio of Ca^{2+} extracted to calciphorin added, all of the delipidated calciphorin was assumed to be in the organic phase and the molecular weight of calciphorin was taken as 3000. The error bars shown in the figure are standard deviations.

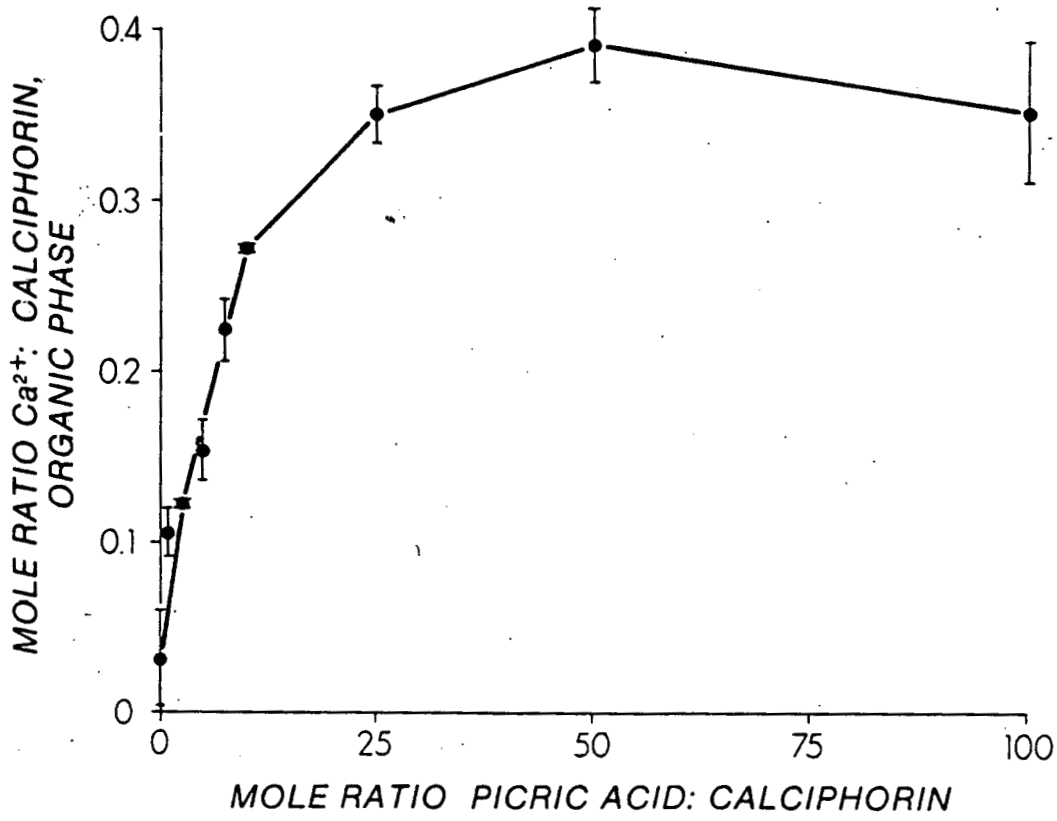

MOLE RATIO Ca^{2+}: CALCIPHORIN, ORGANIC PHASE
0.4
0.3
0.2
0.1
0
0
25
50
75
100
MOLE RATIO PICRIC ACID: CALCIPHORIN

Fig. 3 shows the effect of pH on the partition of calciphorin into the methylene chloride phase. When the pH of the aqueous phase was above 7.5,all of the calciphorin added in the organic solvent extraction experiments was found in the methylene chloride phase. At pH 6.5, both MES and Tris buffers were used. The amount of calciphorin found in the organic phase was the same with the two buffer systems. Thus,the pH profile of calciphorin partitioned in the organic phase was due to the pH of the aqueous phase and not to the buffer chosen. Fig.3 also shows that the amount of picric acid has little effect on the partition of calciphorin into the organic phase.

The effect of Ca^{2+} on the partition of calciphorin into the organic phase is shown in Table 1. At low pH (5.7), the hydrophobicity of the delipidated calciphorin was not affected by the presence of Ca^{2+}. At higher pH's (6.7 and 8.7), the partition of calciphorin was greatly favored in the organic phase in the presence of Ca^{2+}. Thus, at pH higher than 6.7, the effect of Ca^{2+} is more important than pH in facilitating calciphorin partioned into the organic phase.

Cation selectivity.

Table 2 shows the cation selectivity of the delipidated calciphorin by organic solvent extraction experiments. The pH of the aqueous solution was 7.4 and the concentrations of the cations used were 1 mM. Both Ca^{2+} and Sr^{2+} were favored over monovalent cations and Mn^{2+}. But Zn^{2+} was favored over Ca^{2+}.

Figure 3. The effect of pH on the partition of calciphorin into the organic phase. The experiments were performed by the organic solvent extraction procedure. Calciphorin (2 nmoles) was added to the organic phase and picric acid to the aqueous phase. The vortexing time was 2 minutes. The aqueous phase contained 1 mM $CaCl_2$ buffered with 5 mM Tris or MES depending on the pH used. In the pH range between 5.5 and 6.5, MES was used as the buffer, and the pH's were adjusted by Tris. For pH ranging from 6.5 to 9.5, Tris.HCl was used. Different amounts of picric acid were used to see if the partition of calciphorin was affected. The amount of calciphorin in the organic phase was determined by the method of Lowry et al.(1951).

O : MES buffer; picric acid, 50 nmol

Δ : MES buffer; picric acid, 100 nmol

● : Tris buffer; picric acid, 50 nmol

▲ : Tris buffer; picric acid, 100 nmol

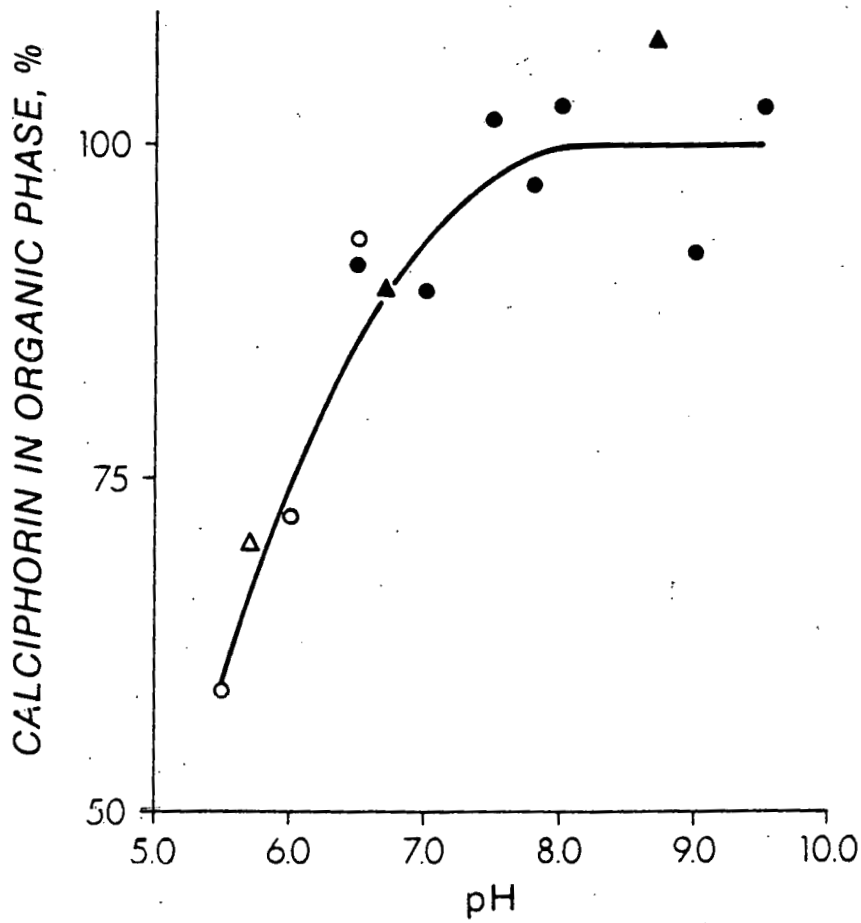

CALCIPHORIN IN ORGANIC PHASE, %
100
75
50
5.0
6.0
7.0
8.0
9.0
10.0
pH

TABLE 1

The effect of Ca^{2+} on the partition of calciphorin into the organic phase

pH	calciphorin in organic phase, %	
	no Ca^{2+}	1 mM Ca^{2+}
5.7	70	70
6.7	40	89
8.7	50	108

The experiments were performed by the organic solvent extraction procedure. Calciphorin (2 nmoles) was added to the organic phase and picric acid (50 nmoles) to the aqueous phase. The vortexing time was 2 minutes. When added, the concentration of $CaCl_2$ was 1 mM in the aqueous phase. At pH 5.7, the buffer was 5 mM MES, pH adjusted by Tris. For pH 6.7 and 8.7, 5 mM Tris.HCl was used as the buffer. The amount of calciphorin in the organic phase was determined by the method of Lowry et al. (1951).

TABLE 2

Cation selectivity of the delipidated calciphorin

Sequence	Zn	>	Ca	=	Sr	>	Rb	=	Na	>	Mn
Ratio	16.7		1.0		1.0		0.6		0.6		0.4

The cation selectivity was determined by the delipidated calciphorin-mediated equilibrium extraction into a methylene chloride phase. The cation concentration in the aqueous phase was 1 mM buffered by 5 mM Tris.HCl, pH 7.4. Calciphorin (2 nmoles) was added to the methylene chloride phase and picric acid (100 nmoles) to the aqueous phase. The vortexing time was 2 minutes. Cations extracted into the organic phase were measured by direct isotope counting. The amount of Ca^{2+} extracted was set at 1.0. The ratios shown were normalized to that of the Ca^{2+} extracted.

pH profile of Ca^{2+} extraction.

Fig. 4 shows calciphorin-mediated Ca^{2+} extraction as a function of pH of the aqueous phase. The amount of Ca^{2+} extracted increased monotonically between pH 5.5 and 8.5 and started showing saturation at pH higher than 8.5. The amounts of Ca^{2+} extracted, at pH's where both MES and Tris buffer were used, were about the same at each pH. Thus, the pH variation of the Ca^{2+} extraction was due to the pH of the aqueous solution and not due to the different buffers used. Fig. 4 also shows the mole ratio of Ca^{2+} extracted to calciphorin present in the organic phase. As shown in Fig. 3, all of the calciphorin added in the organic solvent extraction experiments was found in the methylene chloride phase, provided that the pH of the aqueous phase was higher than 7.5. Thus, for pH higher than 7.5, the curve obtained for the mole ratio paralleled that of the Ca^{2+} extracted. When the pH of the aqueous phase was lower than 7.5, the amount of calciphorin present in the organic phase was calibrated according to Fig. 3. The mole ratio versus pH curve thus obtained showed a pK_a of 8.0-8.1.

Ca^{2+}-binding studies by organic solvent extraction experiments.

Fig. 5 shows Scatchard plots of Ca^{2+}-binding data constructed from the organic solvent extraction experiments for different pH's of the aqueous phases. In these experiments, the bound Ca^{2+} was assumed to be that which appeared in the organic phase after the two phases were separated by centrifugation. The concentration of Ca^{2+} remaining in the aqueous phase was taken as the concentration of free Ca^{2+}. The plots at both pH's are biphasic, which indicates

Figure 4. Calciphorin -mediated Ca^{2+} extraction and mole ratio of Ca^{2+} to calciphorin in the organic phase as a function of pH of the aqueous phase. The experiments were performed by the organic solvent extraction procedure. Calciphorin (2 nmoles) was added to the organic phase and picric acid (150 nmoles) to the aqueous phase. The vortexing time was 2 minutes. The aqueous phase contained 1 mM $CaCl_2$ buffered with 5 mM Tris or MES depending on the pH used. In the pH range between 5.5 and 6.5, MES was used as the buffer, and the pH's were adjusted by Tris. For pH ranging from 6.0 to 9.2, Tris.HCl was used. Dashed line shows the total amount of Ca^{2+} extracted. The mole ratios of Ca^{2+} extracted to calciphorin present in the organic phase were obtained by dividing the amount of calciphorin in the organic phase into the total amount of Ca^{2+} extracted at each pH. The amount of calciphorin in the organic phase was calibrated using Fig. 3.

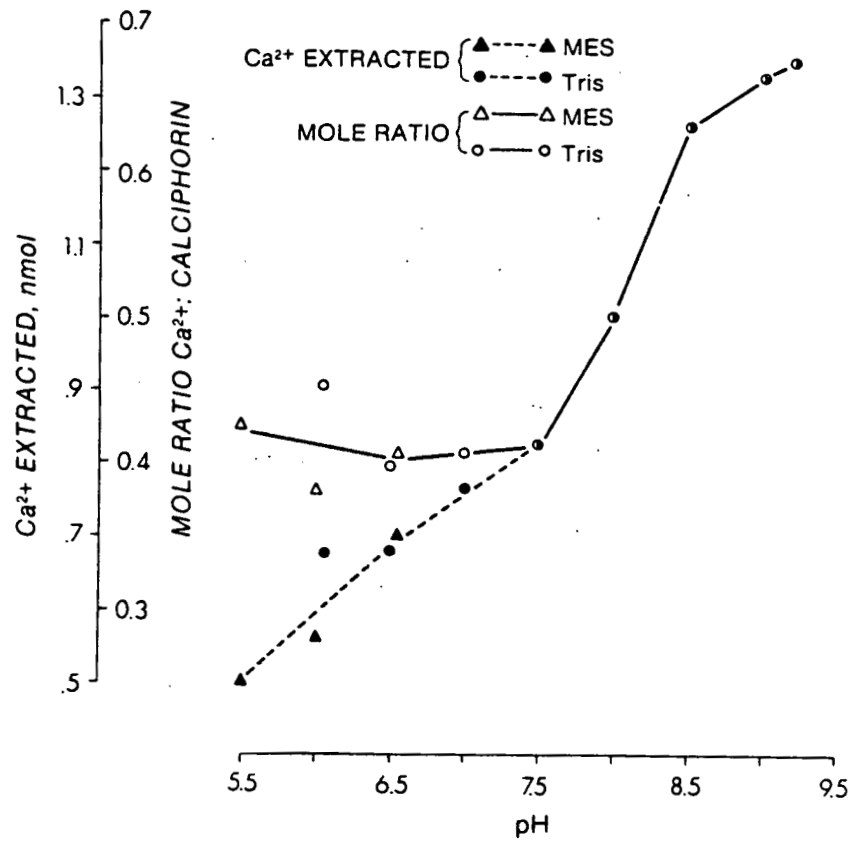
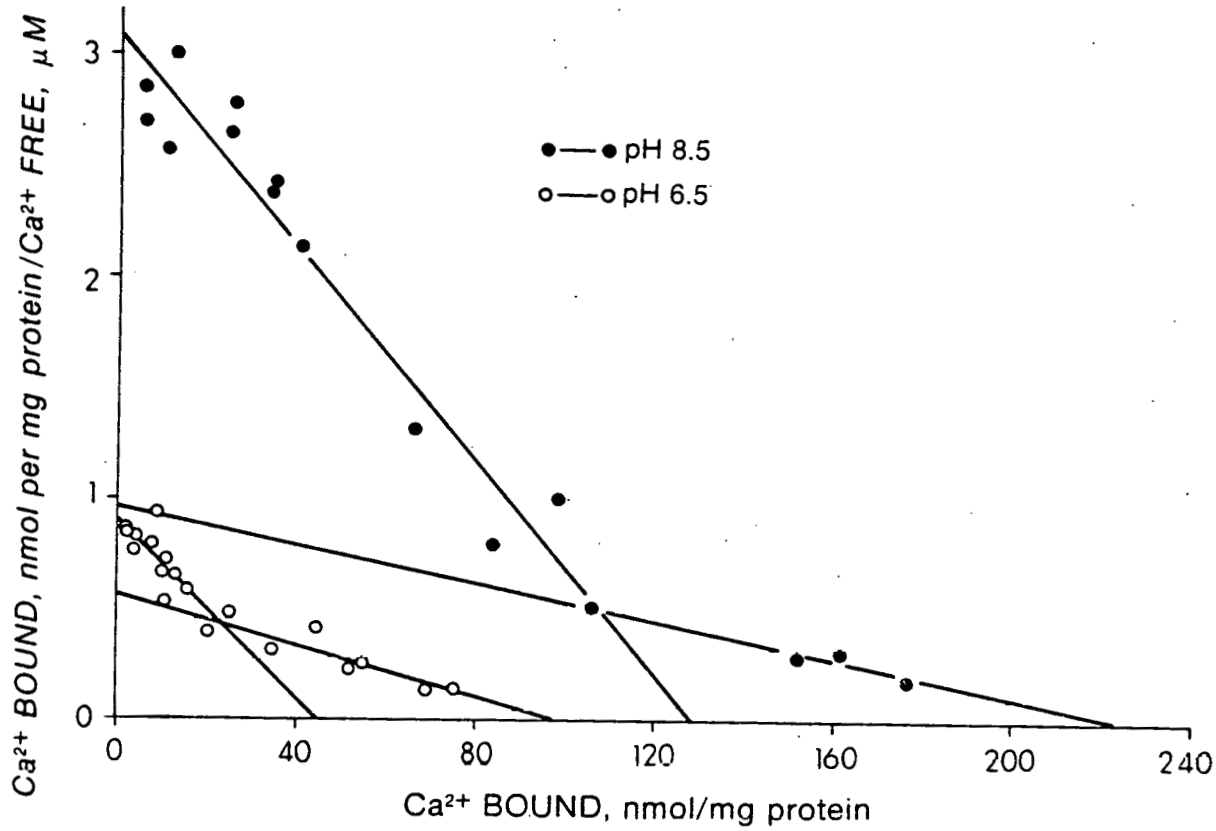

Ca²⁺ EXTRACTED
MES
Tris
MOLE RATIO
MES
Tris
Ca²⁺ EXTRACTED, nmol
MOLE RATIO Ca²⁺: CALCIPHORIN
1.3
1.1
.9
.7
.5
0.7
0.6
0.5
0.4
0.3
5.5
6.5
7.5
8.5
9.5
pH

Figure 5. Scatchard plots of Ca^{2+}-binding data constructed from the organic solvent extraction experiments. The organic phase was 1 ml of methylene chloride containing 2 μM of calciphorin. The aqueous phase contained varied amounts of $^{45}CaCl_2$, 200 μM picric acid, and 5 mM buffer. The buffers used at pH 8.5 and 6.5 were Tris.HCl and MES (pH adjusted with Tris), respectively. The bound and the free Ca^{2+} were assumed to be those that appeared in the organic phase and remained in the aqueous phase, respectively, after the phases were separated by centrifugation.


pH 8.5
pH 6.5
Ca²⁺ BOUND, nmol per mg protein/Ca²⁺ FREE, μM
Ca²⁺ BOUND, nmol/mg protein
0
1
2
3
0
40
80
120
160
200
240

that there are two classes of binding sites at each pH. Table 3 shows the results of these binding studies. At pH 8.5, the high-affinity sites had a dissociation constant of 42 μM and bound 129 nmole Ca^{2+} per mg calciphorin. Using 3000 as the molecular weight, the stoichiometry was about 0.39 mole of Ca^{2+} bound per mole of calciphorin. The low-affinity sites had a dissociation constant of 234 μM and bound 0.29 mole of Ca^{2+} per mole of calciphorin. The total Ca^{2+} bound, at pH 8.5, was then 0.68 mole per mole of calciphorin. At pH 6.5, the dissociation constant of the high-affinity sites (50 μM) was not much different from that measured at pH 8.5, but the number of Ca^{2+}-binding sites was much lower. It only bound 0.14 mole of Ca^{2+} per mole of calciphorin at the high-affinity sites. The low-affinity sites had a dissociation constant of 174 μM and bound 0.16 mole Ca^{2+} per mole of calciphorin.

Competition studies.

Cation competition experiments were also carried out by the organic solvent extraction procedure. As shown in Table 3, the high-affinity sites had dissociation constants around 50 μM at both pH 6.5 and 8.5. In order to study the competition of other cations for the high-affinity Ca^{2+}-binding sites, the concentration of Ca^{2+} used in these experiments was 50 μM. Fig. 6 shows the results of the competition experiments. Monovalent cations (K^+ Na^+) inhibited Ca^{2+} extraction only slightly. Zn^{2+} and Mn^{2+} demonstrated stronger inhibition than any other divalent cations tested. Ba^{2+}, Sr^{2+}, and Mg^{2+} showed an inhibition intermediate between K^+, Na^+ and that

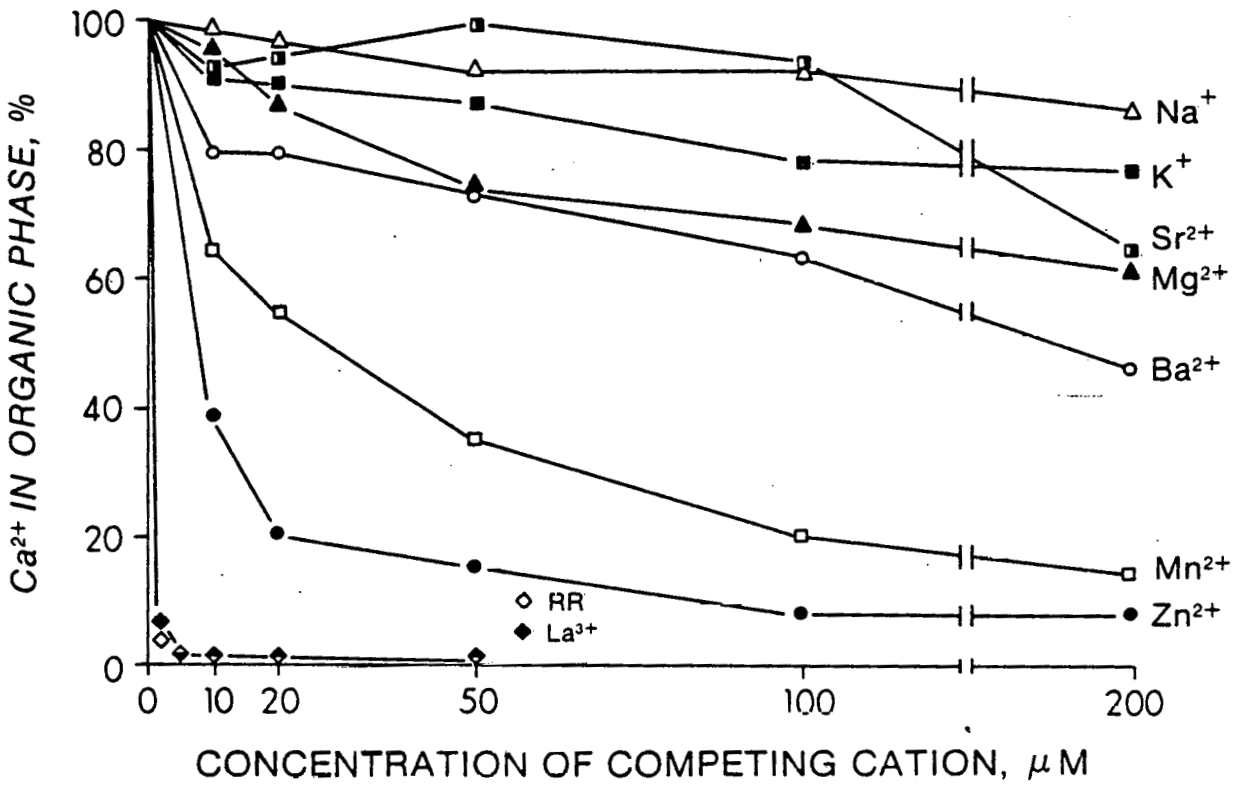
TABLE 3

The dissociation constants and numbers of Ca^{2+}-binding sites obtained from the Ca^{2+}-binding data using organic solvent extraction experiments

	High affinity		Low affinity	
pH	K_d (μM)	n($\frac{\text{nmol}}{\text{mg protein}}$)	K_d (μM)	n($\frac{\text{nmol}}{\text{mg protein}}$)
6.5	50	45	174	54
8.5	42	129	234	96

K_d and n are the dissociation constant and the number of Ca^{2+}-binding sites, respectively. These values were obtained from the Scatchard plots shown in Fig. 5. The x-intercepts are the numbers of Ca^{2+} binding sites. K_d values were calculated from the ratios of x intercepts to y intercepts. At each pH, the number of low-affinity sites was calculated from the difference between the numbers of total Ca^{2+}-binding sites (x-intercept of the low-affinity binding) and high- affinity binding sites (x-intercept of the high-affinity binding).

Figure 6. Inhibition of calciphorin-mediated Ca^{2+} extraction by various cations and ruthenium red (RR) into a methylene chloride phase. Experiments were conducted by the equilibrium extraction procedure. The aqueous phase contained 0.5 ml of 50 μM $CaCl_2$, 200 μM picric acid, 5 mM Tris.HCl, pH 7.4, and indicated concentrations of other cations. Calciphorin concentration was 2 μM in 1 ml of methylene chloride. The vortexing time was 2 minutes. The amount of Ca^{2+} extracted in the absence of any other cations was set at 100%.


100
80
60
40
20
0
Ca^{2+} IN ORGANIC PHASE, %
0
10
20
50
100
200
CONCENTRATION OF COMPETING CATION, μM
Na^+
K^+
Sr^{2+}
Mg^{2+}
Ba^{2+}
Mn^{2+}
Zn^{2+}
RR
La^{3+}

of Zn^{2+} and Mn^{2+}. The classic mitochondrial Ca^{2+} transport inhibitors, La^{3+} and ruthenium red, showed the strongest inhibition of Ca^{2+} extraction . Even when the amount of La^{3+} or ruthenium red added was half of the delipidated calciphorin, 95% inhibition was observed.

Table 4 shows the effects of respiratory inhibitors (rotenone, antimycin A), inhibitor of mitochondrial ATPase (oligomycin), uncoupler of oxidative phosphorylation (CCCP), and phosphate on calciphorin-mediated Ca^{2+} extraction into a methylene chloride phase. At a concentration of 10 μM (about 400 μg per mg of protein) rotenone, antimycin A, oligomycin, and CCCP had very little effect on Ca^{2+} extracted in the organic phase. Phosphate, at the same concentration as Ca^{2+}, also had little effect on the calciphorin-mediated Ca^{2+} extraction.

Determination of the charge on the Ca^{2+}-calciphorin complex.

The charge of the Ca^{2+}-calciphorin complex was determined spectrophotometrically by measuring the amount of picrate extracted into the organic phase in the organic solvent extraction experiments. Fig. 7 shows the absorbtion spectrum of 50 μM picric acid in distilled water. The absorbtion peak was found at 355 nm. The absorbance at 355 nm as a function of the concentration of picric acid is shown in Fig. 8. The plot is linear. The molar extinction coefficient calculated was 13,000 which is very close to the reported value, 13,700, arrived at by Eisenman et al. (1969). To determine the charge of the Ca^{2+}-calciphorin complex, Ca^{2+} and picric acid extracted were measured from the same sample. Fig. 9 shows the

TABLE 4

Effects of respiratory inhibitors, inhibitor of mitochondrial ATPase, uncoupler of oxidative phosphorylation, and phosphate on calciphorin-mediated Ca^{2+} extraction into a methylene chloride phase

Addition	% Extraction		
	2μM	10μM	50μM
None	100	100	100
Rotenone	97	90	--
Antimycin A	--	91	--
Oligomycin	94	100	--
CCCP	85	107	--
Phosphate	100	88	107

The experimental procedure was the same as shown in Fig. 6. The amount of Ca^{2+} extracted in the absence of any competing substance was set at 100%. Phosphate was added in the form of Na_2HPO_4. Rotenone, antimycin A, oligomycin, and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) were added in ethanolic solutions.

Figure 7. The absorption spectrum of picric acid in distilled water. The concentration of picric acid was 50 μM.

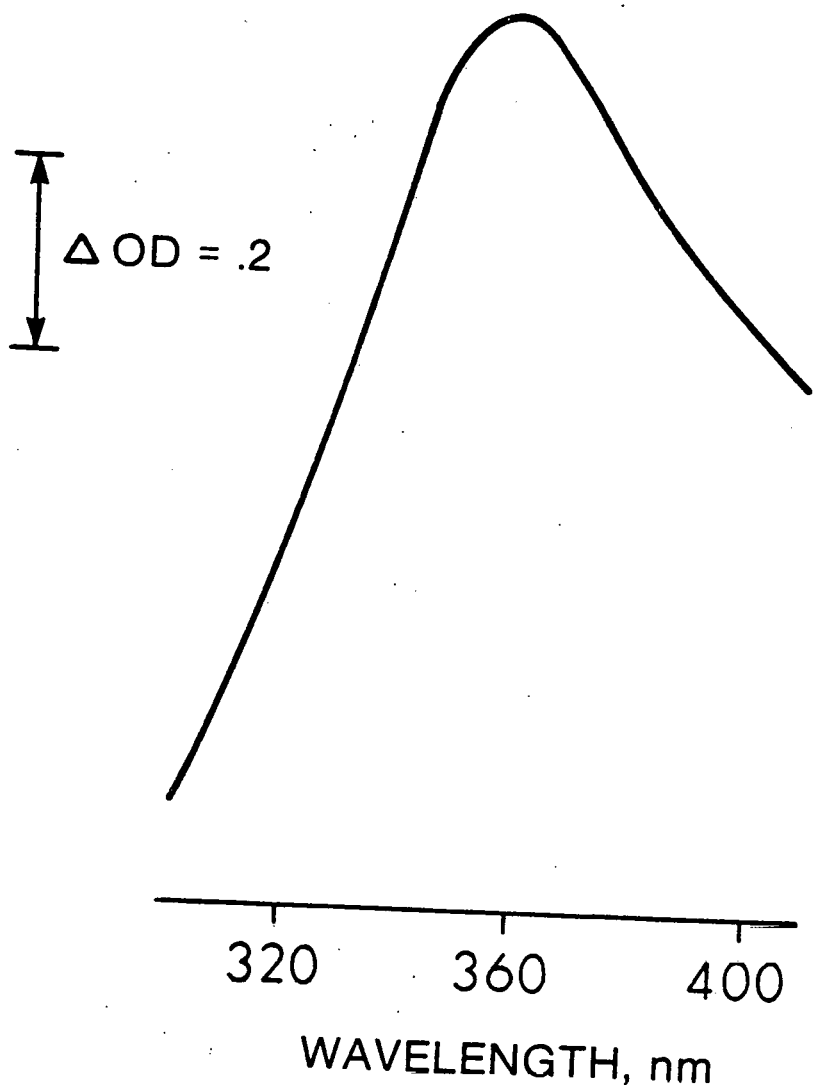

Δ OD = .2
320
360
400
WAVELENGTH, nm

Figure 8. The absorbance at 355 nm versus the concentration of picric acid. Picric acid was in distilled water. The light path was 1 cm.

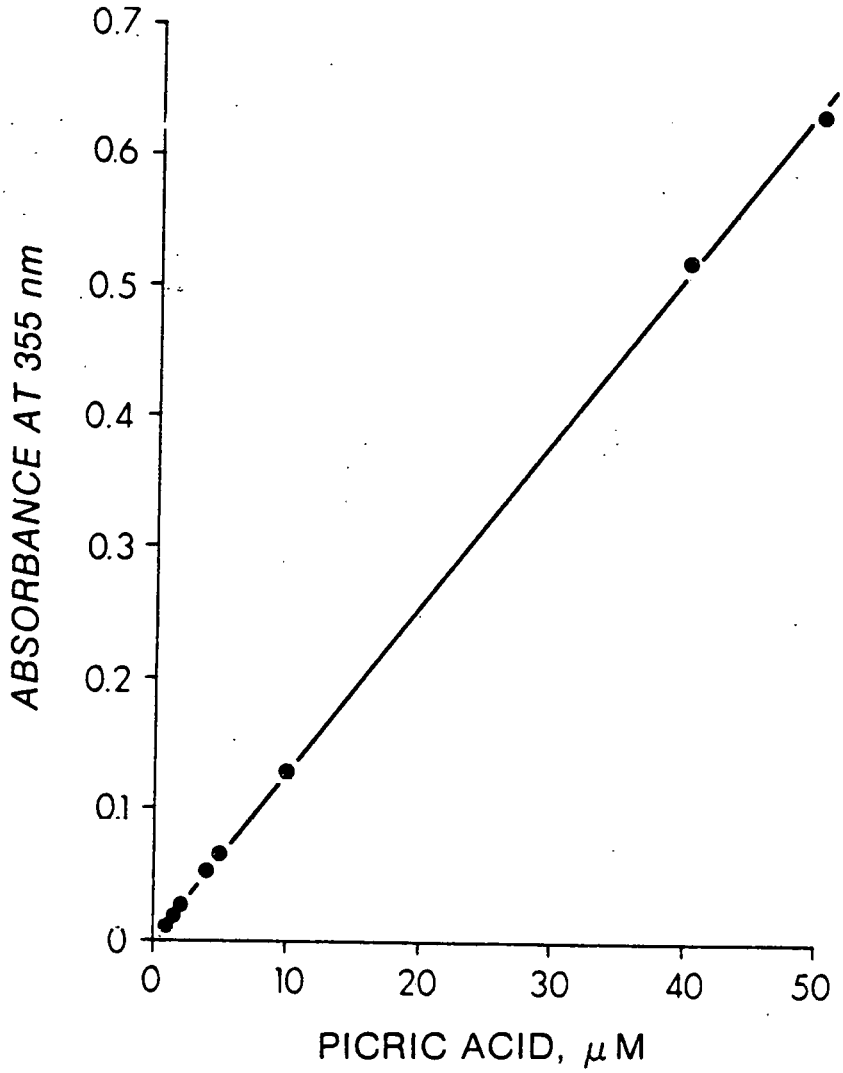
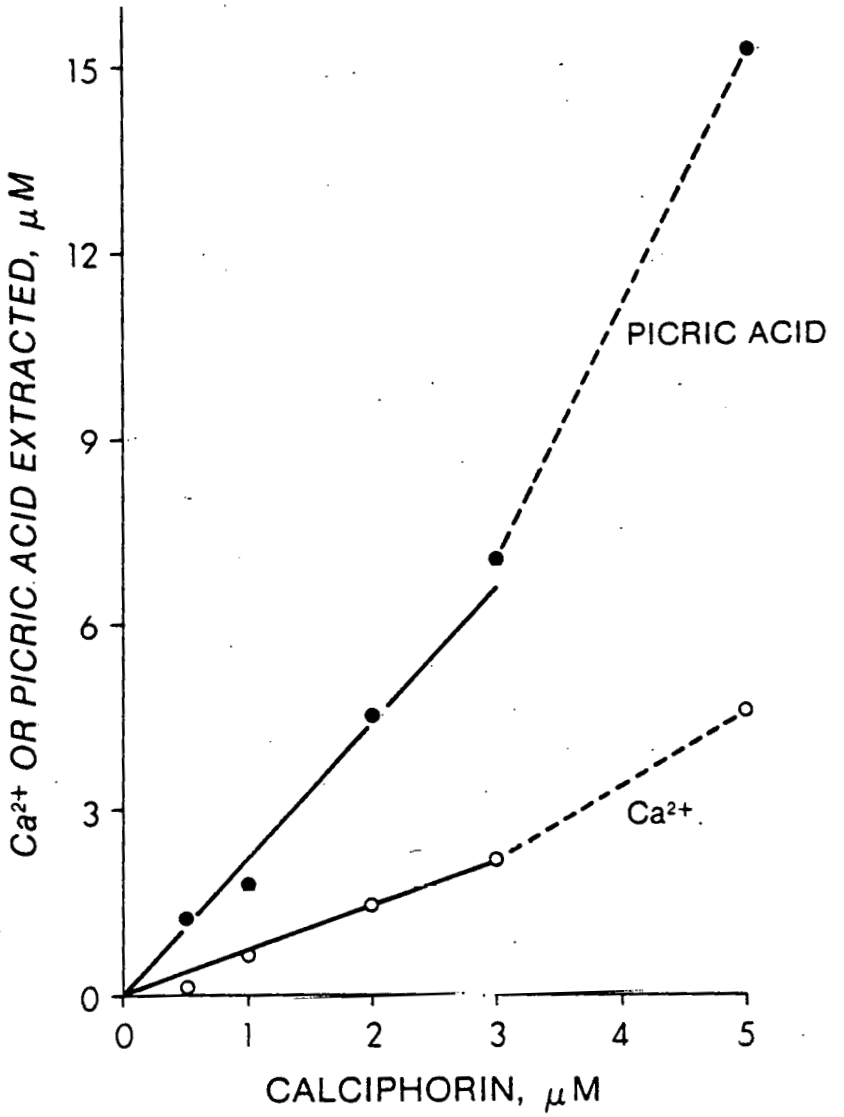

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE AT 355 nm
0
10
20
30
40
50
PICRIC ACID, μM

Figure 9. Ca^{2+} and picric acid extracted in the organic phase as a function of the concentration of calciphorin. The experiments were carried out by the organic solvent extraction procedure. The aqueous phase contained 1 mM $^{45}CaCl_2$, picric acid, and 5 mM Tris. HCl pH 8.5. The amount of picric acid added to the aqueous phase was always 50 times more than calciphorin indicated. Ca^{2+} extracted was obtained by radioactive counting. Picric acid extracted in the organic phase was dried under N_2, redissolved in distilled water, and the absorbance measured at 355 nm. The background readings due to the partition of picric acid in the organic phase in the absence of calciphorin and the turbidity of calciphorin in distilled water were corrected. Both Ca^{2+} and picric acid extracted were measured from the same sample. The solid lines were obtained by least-square fit and the broken lines were simply drawn by connecting two data points.


15
12
9
6
3
0
0
1
2
3
4
5
Ca2+ OR PICRIC ACID EXTRACTED, μM
CALCIPHORIN, μM
PICRIC ACID
Ca2+

results of the simultaneous measurements of Ca^{2+} and picrate extracted into the organic phase.In this figure, the solid lines were obtained by the least-square fit while the broken lines were simply drawn by connecting two data points. The stoichiometry of the Ca^{2+} extraction (mole of Ca^{2+} extracted per mole of calciphorin) was about 0.7 when 1 to 3 μM calciphorin was used, and the stoichiometry raised up to 0.92 at higher concentration of calciphorin added. The ratio of picrate to Ca^{2+} extracted in the entire range of calciphorin used was about 3:1.

Ca^{2+}-binding studies using flow dialysis.

The dissociation constant of calciphorin for Ca^{2+} in aqueous phase was measured by flow dialysis experiments. Fig. 10 shows a plot of Ca^{2+} bound to calciphorin as a function of total Ca^{2+} added. This plot shows a saturation phenomenon. When the total Ca^{2+} added to the medium was 18 μM, about 80% of the Ca^{2+}-binding sites were saturated. Fig. 11 shows a Scatchard plot of the same data. There is only one class of Ca^{2+} binding sites. These Ca^{2+}-binding sites had a dissociation constant of 5.2 μM and bound 343 nmoles of Ca^{2+} per mole of calciphorin. Using 3,000 as the molecular weight of calciphorin, the stoichiometry was about 1.0 mole of Ca^{2+} bound per mole of calciphorin. The same stoichiometry was also indicated by a Hill plot (Hill, 1910), where the slope was equal to 1.0 (Fig.12).

Figure 10. Ca^{2+}-binding data obtained from flow dialysis experiments. Delipidated calciphorin was 9 μM in 1 ml of 25 mM Tris.HCl,pH 7.4. The dialysis buffer was 25 mM Tris.HCl, pH 7.4. The abscissa is the concentration of total Ca^{2+} added.

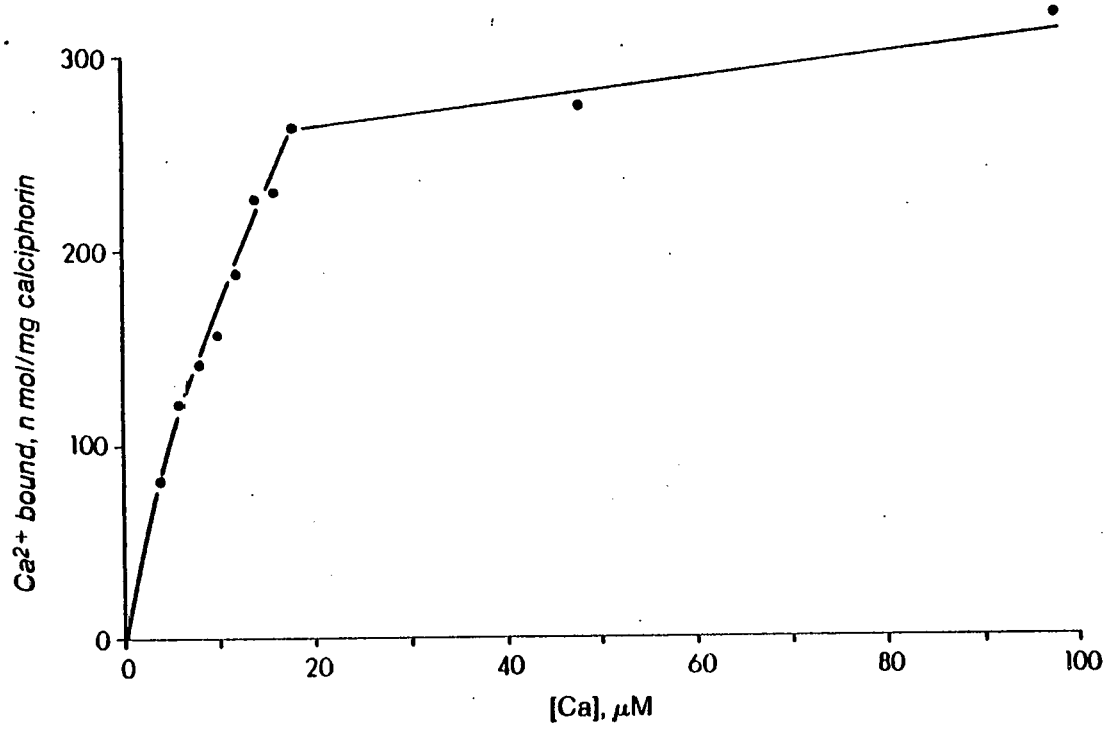

300
200
100
0
0
20
40
60
80
100
Ca2+ bound, n mol/mg calciphorin
[Ca], μM

Figure 11. Scatchard plot of Ca^{2+}-binding data constructed from flow dialysis experiments. The experimental conditions were the same as shown in Fig. 10. The dissociation constant obtained was 5.2 μM, and the number of Ca^{2+}-binding sites was 343 nmoles per mg of calciphorin.

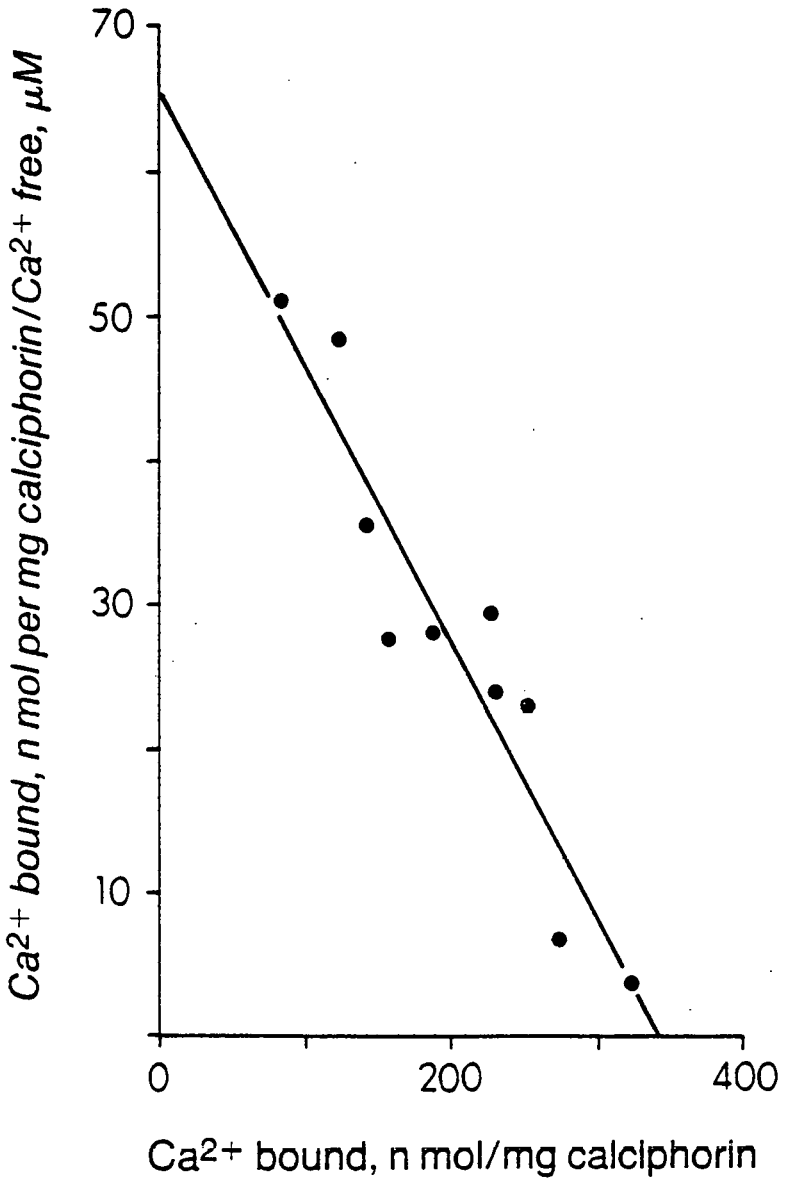
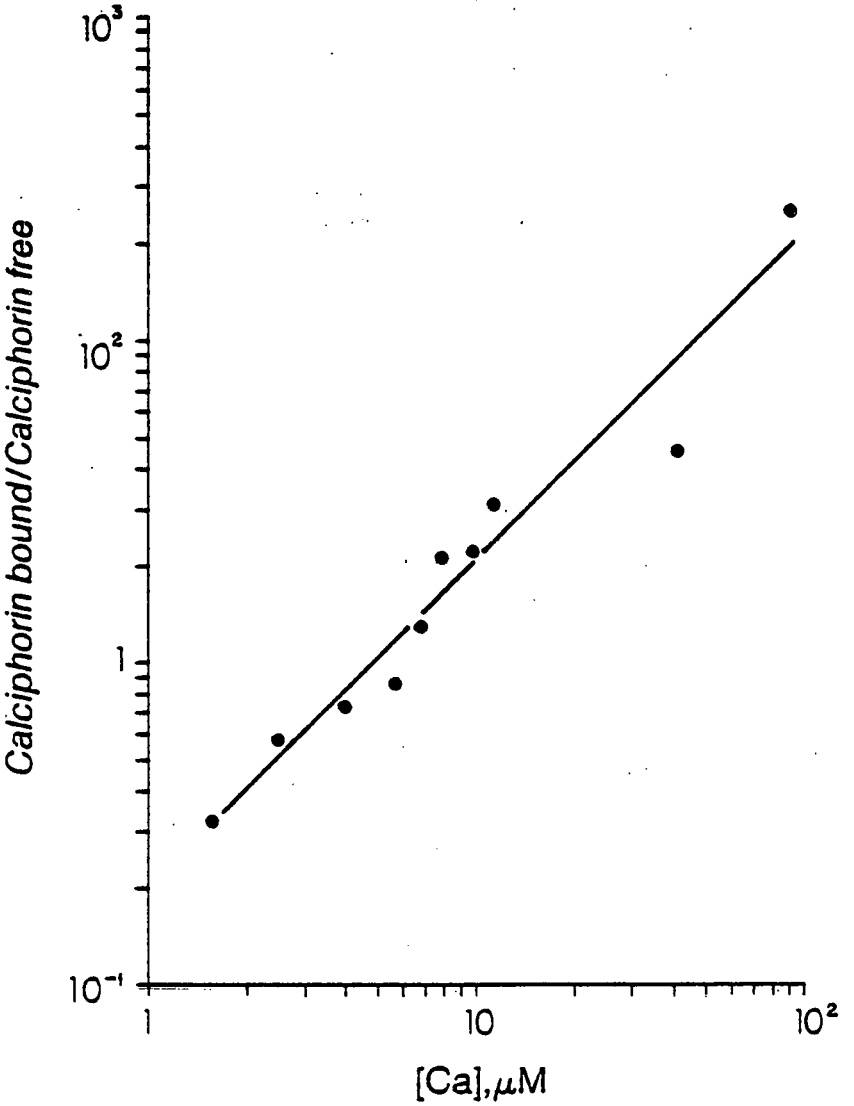

70
50
30
10
0
200
400
Ca^{2+} bound, n mol per mg calciphorin/Ca^{2+} free, μM
Ca^{2+} bound, n mol/mg calciphorin

Figure 12. Hill plot of Ca^{2+}-binding data from flow dialysis experiments. The experimental conditions were the same as shown in Fig. 10. The abscissa is the concentration of free Ca^{2+} in the solution. The slope of the plot is 1.0.


Calciphorin bound/Calciphorin free
10³
10²
1
10⁻¹
1
10
10²
[Ca],μM

DISCUSSION

The kinetics of Ca^{2+} transport in mitochondria are well documented (for review, see Mela,1977;Bygrave, 1977,1978;Carafoli and Crompton, 1978),. but the mechanism of Ca^{2+} transport is essentially not solved. Recent studies of the mechanism of Ca^{2+} transport favor that the intramitochondrial Ca^{2+} is regulated by an influx and an efflux mechanisms(Sordahl, 1974; Puskin et al., 1976; Crompton et al., 1977, 1978). Whether the pathways for Ca^{2+} influx and efflux are identical is still debatable. While Pozzan et al. (1977) have shown that Ca^{2+} efflux occurs always through the native influx carrier, others suggest that the two mechanisms are separated (Puskin et al.,1976; Crompton et al., 1978). Despite the disagreement in the efflux mechanism, it is accepted that the efflux mechanism is either not sensitive to ruthenium red (Crompton et al., 1977) or ruthenium red is not directly interacting with the efflux pathway (Pozzan et al., 1977). On the other hand, the influx mechanism of Ca^{2+} transport is very sensitive to ruthenium red (Moore, 1971; Reed and Bygrave, 1974a). Calciphorin is very sensitive to ruthenium red. The calciphorin-mediated Ca^{2+} extraction into the organic phase can be inhibited completely by very low concentrations of ruthenium red (Fig. 6). The sensitivity to ruthenium red, together with the hydrophobicity and the electrogenicity lead calciphorin to be a likely candidate for the Ca^{2+} carrier reponsible for the influx mechanism of Ca^{2+} transport in mitochondria.

As mentioned in Chapter 1, calciphorin is very hydrophobic

before delipidation. In the presence of its native phospholipid environment, calciphorin can be solubilized in diethyl ether. After delipidation, calciphorin becomes less hydrophobic. But it can regain its hydrophobicity in the presence of Ca^{2+} and alkaline pH in the organic solvent extraction experiments (Fig. 3 and Table 1). It is possible that calciphorin undergoes a conformational change in the presence of Ca^{2+}, binding Ca^{2+} in its hydrophilic pocket, and orienting itself in such a manner that the hydrophobic groups face the lipid milieu.

The pH profile of Ca^{2+} extracted into the methylene chloride phase showed a pK_a of 8.0-8.1 (fig. 4). It has been reported by Pfeiffer et al. (1978) that the pK_a of A23187, a divalent cation ionophore, decreases as the solvent polarity increases. At the interface between the aqueous medium and the outer half leaflet of the inner mitochondrial membrane, the pK_a of calciphorin is probably slightly lower than the observed value from the organic solvent extraction experiments. Reed and Bygrave (1975) have found that the group(s) involved in the binding of Ca^{2+} to the carrier have a pK_a of 7.8, by studying the kinetics of Ca^{2+} transport in mitochondria. Thus, the groups responsible for the binding of Ca^{2+} to calciphorin seem to be identical to that of the Ca^{2+} carrier.

Delipidated calciphorin binds much more Zn^{2+} than Ca^{2+} (Table 2), and Zn^{2+} is also able to inhibit Ca^{2+} extraction into the organic phase (Fig. 6). Mitochondria have been reported to be the target of heavy metal poisoning (Aldridge and Cremer, 1955). It is possible that calciphorin is the

local target in mitochondria. Delipidated calciphorin favors Ca^{2+} and Sr^{2+} over Mn^{2+} (Table 2) as was shown for the undelipidated calciphorin (Table 6, Chapter 1). Table 2 also shows that delipidated calciphorin favors Ca^{2+} over monovalent cations (Na^{+} and Rb^{+}). Although the selectivity ratios found between Ca^{2+} and monovalent cations are not as drastic as they were before the removal of the contaminating phospholipids (Table 6, Chapter 1), monovalent cations (Na^{+} and K^{+}) are still not able to compete with Ca^{2+} for extraction into the organic phase (Fig. 6). Mn^{2+} demonstrates again a high ability in competing with Ca^{2+} for extraction (Fig. 6 in this chapter and Fig. 9 in Chapter 1). As explained in Chapter 1, the inhibitory effect may be due to the interaction between Mn^{2+} and Ca^{2+}, which is consistent with the Ca^{2+}-stimulated Mn^{2+} uptake observed in mitochondria (Vinogradov and Scarpa, 1973). Other divalent cations (Sr^{2+}, Mg^{2+}, and Ba^{2+}) show a moderate inhibition of Ca^{2+} extraction (Fig. 6). The most striking inhibitory effects are shown by La^{3+} and ruthenium red, classic inhibitors of Ca^{2+} transport in mitochondria. In the presence of only half the amount of ruthenium red or La^{3+} as compared with calciphorin, Ca^{2+} extraction mediated by calciphorin is decreased by 95%. The stoichiometry of ruthenium red or La^{3+} bound to calciphorin is therefore 1:2.

Other important findings, revealed from the competition studies, are shown in Table 4. At high concentrations, the respiratory inhibitors (rotenone, antimycin A), the inhibitor of mitochondrial ATPase (oligomycin), and the uncoupler of oxidative phosphorylation (CCCP) do not have signif-

icant effects on Ca^{2+} extracted into the organic phase. It has been shown that the driving force for Ca^{2+} transport in mitochondria is the negative-inside membrane potential (Scarpa and Azzone, 1970). Binding of Ca^{2+} would thus be expected to be insensitive to respiratory inhibitors and uncoupling agents (Reed and Bygrave, 1974b). In fact, one of the major criticisms to the high-affinity Ca^{2+}-binding in respiration-inhibited mitochondria reported by Reynafarje and Lehninger (1969) is that the high-affinity Ca^{2+}-binding sites disappear in the presence of 2,4-dinitrophenol, an uncoupling agent (Southard and Green, 1974; Akerman et al., 1974). Recently, Moyle and Mitchell (1977) suggested that the Ca^{2+} carrier may be a Ca^{2+}-phosphate symporter which translocates one positive charge to the matrix compartment of mitochondria. However, this hypothesis has been shown to be based on inadequate data and assumptions (Azzone et al., 1977; Reynafarje and Lehninger, 1977). The Ca^{2+} carrier is thus expected not to have a binding site for inorganic phosphate. The results shown in Table 4 are consistent with these findings. At equal concentrations of Ca^{2+} and phosphate, Ca^{2+} extracted into the organic phase is not enhanced significantly by the presence of phosphate.

Without picrate, calciphorin is not able to extract large amounts of Ca^{2+} either in the absence or presence of a lipophilic cation, tetramethylammonium (Table 3, Chapter 2). This eliminates the possibility that calciphorin alone has a net charge of -2 or is more negatively charged. Also picrate is not found to be extracted

by calciphorin in the absence of Ca^{2+} at pH 8.5 (unpublished observation). The possibility that calciphorin is positively charged is therefore eliminated. Thus, calciphorin can either be neutral or has one negative charge, and the Ca^{2+}-calciphorin complex is expected either to have 2 positive charges or one positive charge, respectively. In the former case, the amount of picrate extracted should be twice as much as Ca^{2+} extracted into the organic phase. In the latter situation, both picrate and Ca^{2+} should be extracted in the same amount. Fig. 9 shows that picrate extracted is three times more than that of Ca^{2+}. It is likely that Ca^{2+}-calciphorin has 2 positive charges, but the extra picrate extracted can not be explained. In the experiments shown in Fig. 9, the background readings due to the partition of picric acid into the organic phase in the absence of calciphorin and the turbidity of calciphorin in distilled water were corrected. It is possible that calciphorin in distilled water becomes more turbid in the presence of Ca^{2+} and picrate, but there is no strong evidence for it.

An interesting finding among the properties associated with the delipidated calciphorin is the affinity of calciphorin for Ca^{2+} determined by flow dialysis experiments. Fig. 11 shows that calciphorin has only one class of Ca^{2+}-binding sites. These sites have high affinity for Ca^{2+}. The dissociation constant is 5.2 μM. Before delipidation, calciphorin showed two classes of Ca^{2+}-binding sites. The dissociation constants of the high- and low-affinity Ca^{2+}-binding sites were 9.5 and 33 μM, respectively. After delipidation, calciphorin loses its low-affinity Ca^{2+}-binding sites. These low-

affinity binding sites are evidently due to phospholipids (Reed and Bygrave, 1974a). The saturation kinetics in the mitochondrial Ca^{2+} transport system is always taken to be the strongest evidence for the carrier-mediated Ca^{2+} transport (Vinogradov and Scarpa, 1973). Calciphorin also shows a saturation phenomenon in the Ca^{2+}-binding studies (Fig. 10). The observed saturation kinetics in mitochondria may be due to the limited Ca^{2+}-binding of calciphorin. From Fig. 11, it is obvious that each mole of calciphorin can bind one mole of Ca^{2+}. The proposed mechanism for the first step of calciphorin (C)- mediated Ca^{2+} transport is:

$$C + Ca^{2+} \rightleftarrows C.Ca^{2+}$$

The Ca^{2+}-calciphorin complex is then moved to the inner phase of the membrane in response to a negative-inside membrane potential and dissociates the bound Ca^{2+} to the matrix compartment. The slope of log (bound calciphorin/free calciphorin)versus the log [free Ca^{2+}] plot is expected to be 1. Indeed, this slope is obtained as shown in Fig. 12.

The stoichiometry of Ca^{2+} bound to calciphorin increases from a low value (0.11) to 0.92 when total calciphorin concentration increases from 0.5 to 5 μM in the organic solvent extraction experiments (Fig. 9). This fact may indicate that there is a "cooperative" effect between calciphorin molecules. Also, only half the amount of ruthenium red or La^{3+} as compared with calciphorin is needed to inhibit the total calciphorin-mediated Ca^{2+} extraction into the organic phase (Fig. 6). Although there is no strong evidence,

it is possible that calciphorin is in a dimeric form. In this case, the proposed mechanism for the binding of Ca^{2+} to dimerized calciphorin at the external phase of the inner mitochondrial membrane is:

$$C + C \rightleftarrows C_2$$

$$C_2 + nCa^{2+} \rightleftarrows C_2Ca_n^{2+}$$

where n is the number of Ca^{2+} bound to one calciphorin dimer. Assuming K_1 and K_2 are the equilibrium constants of the two binding steps, respectively, shown above. Then,

$$K_1 = \frac{(C_2)}{(C)^2}$$

$$K_2 = \frac{(C_2Ca_n^{2+})}{(C_2)\ (Ca^{2+})^n}$$

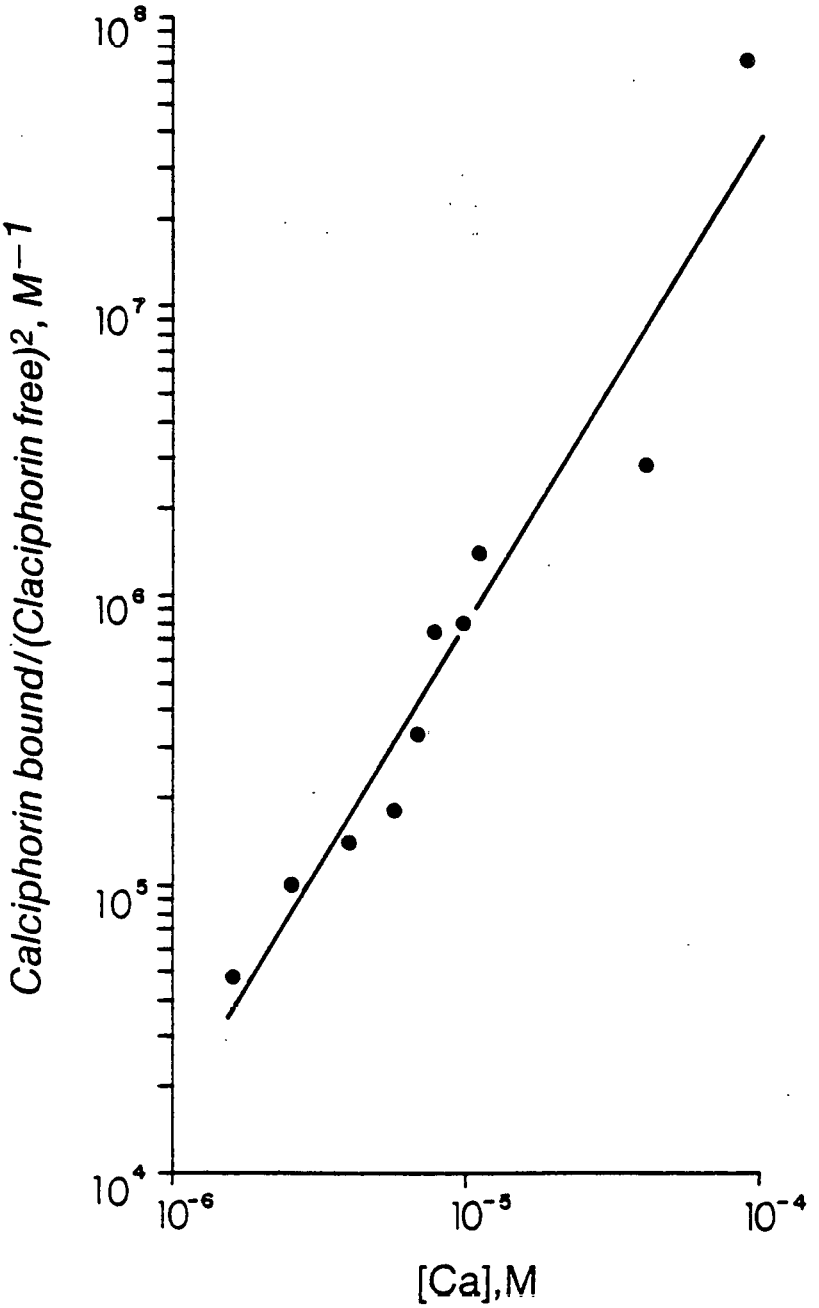
where the quantities in the parentheses represent activities. Therefore,

$$\frac{(C_2Ca_n^{2+})}{(C)^2} = K_1K_2(Ca^{2+})^n$$

The slope of log [bound calciphorin/(free calciphorin)^2] versus log (free Ca^{2+}) plot should yield n, which is expected to be 2. Fig. 13 shows such a plot, and the slope obtained is 1.7.

The two possible mechanisms of calciphorin-mediated Ca^{2+}

Figure 13. The plot of log [bound calciphorin/ (free calciphorin)^2] versus log (free Ca) constructed from flow dialysis experiments. The experimental conditions were shown in Fig. 10. The slope is 1.7.

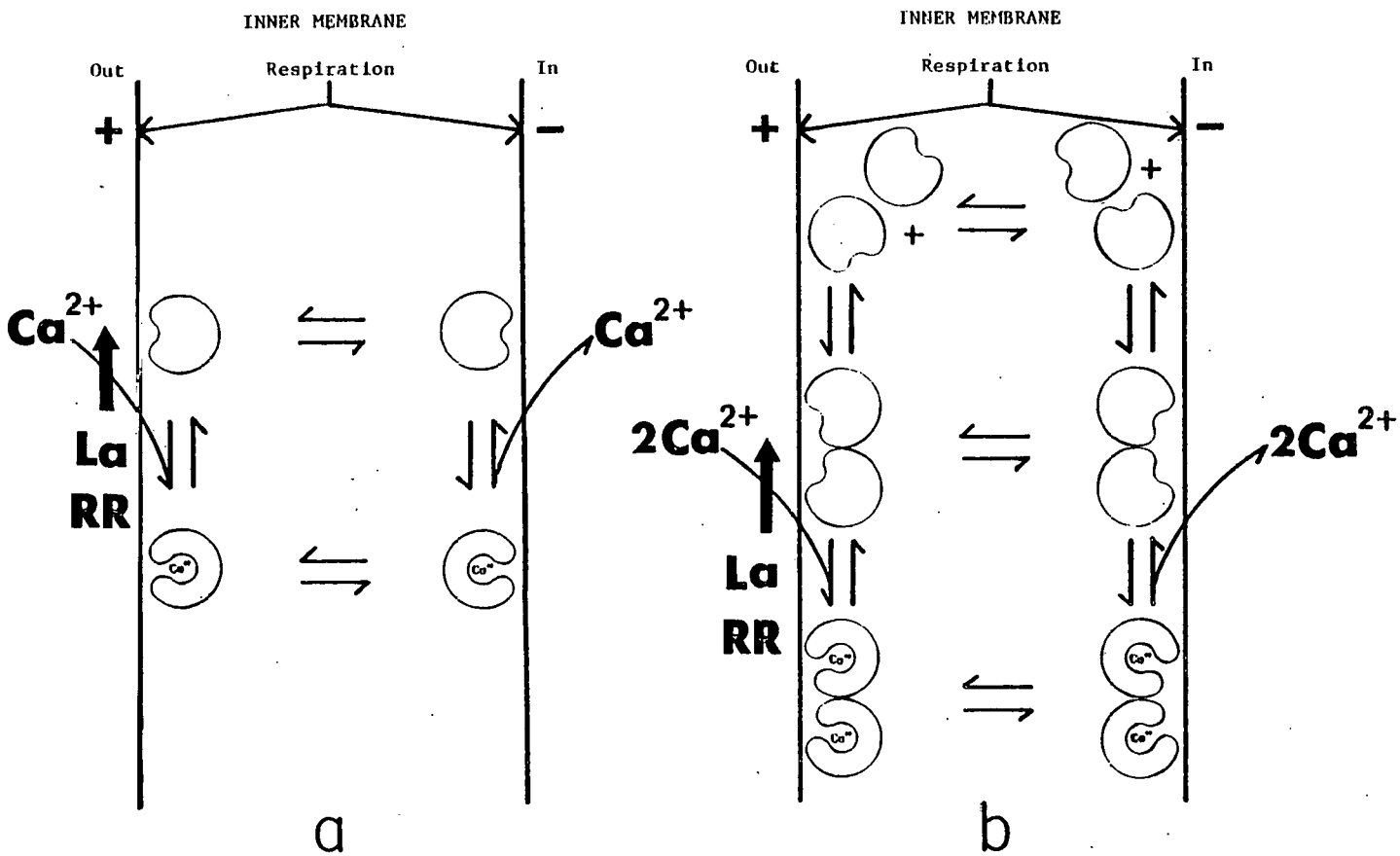

Calciphorin bound/(Claciphorin free)², M⁻¹
10⁸
10⁷
10⁶
10⁵
10⁴
10⁻⁶
10⁻⁵
10⁻⁴
[Ca],M

transport are depicted in Fig. 14. Fig. 14(a) shows the possibility that the transport unit of calciphorin is a monomer. Ca^{2+} binds to the Ca^{2+}-binding site of calciphorin at the external surface of the inner mitochondrial membrane. This process can be blocked by either ruthenium red or La^{3+}. According to Table 1, calciphorin becomes more hydrophobic in the presence of Ca^{2+} at the physiological pH. The binding of Ca^{2+} to calciphorin may induce a conformational change of the calciphorin molecule such that Ca^{2+} and its ligands reside in the interior of the complex leaving the exterior more hydrophobic. This Ca^{2+}-calciphorin complex can respond to a negative-inside membrane potential generated by respiration or ATP hydrolysis. Bound Ca^{2+} is then dissociated into the matrix compartment either in its free form or bound to the negatively charged groups (Gunter and Puskin, 1972). Afterwards, free calciphorin diffuses back to the external surface of the inner mitochondrial membrane and recycles. Fig. 14 (b) shows another possible mechanism of calciphorin-mediated Ca^{2+}-transport, where functional calciphorin is assumed to be in a dimeric form. In the presence of Ca^{2+}, calciphorin is dimerized. Two molecules of calciphorin bind two Ca^{2+} (Fig. 13). This binding process can be blocked by ruthenium red or La^{3+}. The two Ca^{2+}-binding sites of the two calciphorin molecules may be nearby so that only one molecule of ruthenium red or La^{3+} is required to inhibit two Ca^{2+} extracted by a calciphorin dimer (Fig. 6). Again, calciphorin may undergo a conformational change, becoming more hydrophobic, when Ca^{2+} is bound. The dimerized Ca^{2+}-calciphorin

Figure 14. Possible mechanisms of calciphorin-mediated Ca^{2+} transport in mitochondria. The C-shaped molecule stands for calciphorin. The invagination of the "C" represents the Ca^{2+}-binding site.

(a) The transport unit of calciphorin is a monomer.

(b) The transport unit of calciphorin is a dimer.


INNER MEMBRANE
Out
Respiration
In
+
−
Ca2+
La
RR
Ca2+
a
INNER MEMBRANE
Out
Respiration
In
+
−
2Ca2+
La
RR
2Ca2+
b

complex can move to the inside of the inner mitochondrial membrane in response to a negative-inside membrane potential. Ca^{2+} is then dissociated and calciphorin diffuses back to the external phase either in monomeric or dimeric form.

In the bulk methylene chloride phase, there are probably three different complexes in the dimer hypothesis: monomers in equilibrium with dimers with no Ca^{2+} bound (C_2^0), dimers with 1 Ca^{2+} bound (C_2^1), and dimers with 2 Ca^{2+} bound (C_2^2). In the organic solvent extraction experiments, calciphorin is shown to have a pK_a around 8.0-8.1 (Fig.4). Probably C_2^2 dominates at higher pH and C_2^1 at low pH. Assuming there is 20% C_2^0 in the organic phase, it is expected to have 80% C_2^1 at pH 6.5. Therefore, a stoichiometry of 0.4 mole Ca^{2+} bound to each mole of calciphorin is expected (Fig. 4). At pH 8.5, the ratio of C_2^2 to C_2^1 calculated is 70:30, assuming the pK_a is 8.1. Therefore ,in the organic phase the relative abundance of C_2^0:C_2^1:C_2^2 is 20%:25%:55% at pH 8.5. The stoichiometry is then: 25% x 0.5 + 55% x 1 = 0.68 (Fig 4). C_2^1 is expected to exist prior to the formation of C_2^2. At pH 8.5, Ca^{2+}-binding sites of C_2^1 probably have higher affinity and the number of binding sites is expected to be 80% x 0.5 = 0.4 mole Ca^{2+} per mole calciphorin or 133 nmoles Ca^{2+} per mg calciphorin (compared with 129 nmoles per mg calciphorin shown in Table 3). The second Ca^{2+} bound in C_2^2 probably has lower affinity and the number of binding sites is expected to be 55% x 0.5 = 0.28 mole Ca^{2+} per mole calciphorin or 93 nmoles Ca^{2+} per mg calciphorin (compared with 96 nmoles Ca^{2+}

per mg calciphorin shown in Table 3). At pH 6.5, where the relative abundance of C_2^0: C_2^1: C_2^2 is assumed to be 20%:80%:0%, it is expected there is only one affinity constant observed in the Ca^{2+}-binding studies by organic solvent extraction experiments. Although the Scatchard plots shown in Fig. 5 at pH 6.5 are not monophasic, the difference in the two dissociation constants obtained at pH 6.5 do not differ as drastically as that shown at pH 8.5. Probably the addition of Ca^{2+} shifts the equilibrium favoring formation of dimer from monomer and renders more Ca^{2+} bound.

Evidence presented here suggests that calciphorin may be a strong candidate for the Ca^{2+} carrier responsible for Ca^{2+} influx in mitochondria. However, whether the functional calciphorin is a monomer or a dimer is not certain. The dimer hypothesis can explain most of the experimental results presented here and also fits the kinetics data, where 2 Ca^{2+} are required to bind to the carrier in order to have Ca^{2+} transported at significant rates (Vinogradov and Scarpa, 1973).

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