CALCIUM RELATED PROPERTIES OF PLASMA MEMBRANES FROM GUINEA PIG PLACENTA

by

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in the Department

of

PHYSIOLOGY

We accept this thesis as conforming to the

required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September, 1974

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ABSTRACT

Calcium transport across the placenta is asymmetrical and is believed to be an active transport. An essential step in such a transport is translocation of the ion across a single plasma membrane. The objective of this thesis was to study the Ca^{2+}-related properties of the placental plasma membranes and to gain some knowledge of their role in Ca^{2+}-transport.

Three Ca^{2+}-related properties were studied:

1. Ca^{2+}-binding to the placental plasma membranes;
2. The membrane bound enzyme Ca^{2+}-ATPase; and
3. Ca^{2+}-uptake by the placental plasma membrane vesicles.

Ca^{2+}-binding properties of the membrane preparation were studied by the use of a new method, the flow dialysis system. Two types of sites for Ca^{2+} were found: 1) high affinity, low capacity sites, and 2) low affinity, high capacity sites. The high affinity sites had 10-fold higher affinity for Ca^{2+} than for Mg^{2+}.

A calcium-stimulated, membrane-bound enzyme, namely Ca^{2+}-ATPase, was located in the placental plasma membranes. This enzyme is distinct from the Na^{+}, K^{+}-ATPase and alkaline phosphatase. The enzyme can be activated by Mg^{2+} but with lower efficiency. Both Ca^{2+} and Mg^{2+} activate the enzyme at the same site. A formula was derived, enabling one to predict very precisely the velocity of the enzyme incubated under any combination of Ca^{2+} and Mg^{2+}; this relationship is presented in a three dimensional model. The formula can be used for other enzymes or other substrates, as was demonstrated with ATP and ADP.

The placental plasma membrane vesicles are capable of accumulating Ca^{2+}. Ca^{2+}-uptake was defined as the amount of Ca^{2+} which is not available for rapid exchange and cannot be displaced by a high concentration of competitor in the presence of ATP. This definition is different from and more accurate than the one which is widely used and cited in the literature. An intravesicular Ca^{2+} concentration of 190 mM was recorded, which was 24-fold higher than the external Ca^{2+} concentration (8 mM). Ca^{2+}-uptake was dependent on ATP hydrolysis by the placental Ca^{2+}-ATPase. This process was independent of Mg^{2+}.

It is suggested that while the substrate for Ca^{2+}-ATPase is Ca-ATP, the substrate for Ca^{2+}-uptake is Ca^{2+}.

The overall Ca^{2+}-related properties of the placental plasma membranes are independent of Mg^{2+} and the entire process from binding to membrane through activation of the enzyme and finally Ca^{2+}-uptake is dependent on Ca^{2+} alone. This situation is unique to the placental plasma membranes.

It is tempting to speculate that the link between the maternal and the fetal circulation is achieved by forming vesicles loaded with Ca^{2+} on the maternal side and unloading them through fusion with the basal plasma membrane on the fetal side.

The Ca^{2+}-related properties of placental plasma membranes described in this thesis, provide many answers regarding the first step in the asymmetrical transplacental Ca^{2+}-transport. Further investigation is required before a full understanding of the entire process is achieved.

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ACKNOWLEDGMENT

The assistance of a number of people contributed to the accomplishment of this thesis. First, I would like to thank my research supervisor, Dr. D. H. Copp, for the interest he showed in the subject and for the continuous encouragement which made the conclusion of this study possible. The help of Dr. Harold Messer in editing this thesis, and the helpful advice he provided me throughout the entire period, is greatly appreciated. I thank Dr. C. F. Cramer for his responsiveness while acting as the chairman of my research committee.

The just criticism of Dr. V. Palaty, which was done in the traditional manner of a good teacher, contributed to reaching a higher scientific level.

I am particularly indebted to Dr. I. C. Radde for introducing me to the subject and guiding me in the early stages.

The good and encouraging atmosphere which was provided by all the people in the laboratory was greatly appreciated.

Special thanks are due to Kurt Henze and Ralph Assina for the preparation of the thesis illustrations.

Finally, I wish to thank my wife, Chava, for her help, understanding and love, for without these I could not have made it.

GENERAL INTRODUCTION

Calcium ion has important roles associated with cellular function. These include:

1. Coupling between excitation - secretion at nerve endings (Eccles, 1964).
2. Coupling in excitation - contraction in muscle (Ebashi and Endo, 1968; Bianchi, 1969).
3. Maintenance of membrane integrity (Poste and Allison, 1973).
4. Membrane fusion. Thus all cellular events which include a stage of fusion of membrane are calcium dependent (Poste and Allison, 1973). These include cell fusion, endocytosis, exocrine (Hagen, 1959) and endocrine secretion (Hales and Milner, 1968; Curry et al., 1968) (exocytosis).
5. Regulation of enzyme activity. Ca^{2+} acts as an inhibitor of a large number of intracellular enzymes while it serves as an activator for only a few of them (Bianchi, 1968).
6. Control of hormone secretion (Copp, 1970).

Calcium ion concentration in the cytosol is estimated to be in the range of 10^{-5}M-10^{-8}M (Hodgkin and Keynes, 1957; Nanninga, 1961), in contrast to 10^{-3}M in extracellular fluid. The necessity for low Ca^{2+} concentration in the cytosol was explained some years ago as follows: high Ca^{2+} in the cytosol may react with the high (as then believed)

inorganic phosphate in the cytosol to form a calcium phosphate precipitate, which will impair cell function (Manery, 1969). This argument lost much of its basis with the finding that inorganic phosphate concentration in the cytosol is very low (Seraydarian et al., 1961). Some calcium phosphate precipitates can be found in cell organelles which are involved in maintaining low Ca^{2+} concentration in the cytosol (Martin and Matthews, 1970; Borle, 1973). By examining the roles for Ca^{2+} in cellular function, one can justifiably conclude that calcium ion is a powerful regulator of cell function, and for this reason its intracellular concentration is controlled very closely at a low level. The extracellular fluid is the most obvious "sink" for disposal of the excess intracellular calcium. The best available description of such a regulatory mechanism is that of the red blood cell (Vincenzi, 1971). According to this model, a membrane bound enzyme, namely a Ca^{2+}-Mg^{2+}-ATPase, is responsible for the active extrusion of calcium from the red blood cell. Since red blood cells in circulation contain no organelles, the plasma membrane "calcium pumps" is the only effective mechanism regulating intracellular Ca^{2+} concentration. Though Ca^{2+}-ATPase is found in plasma membranes of several other tissues (Martin et al., 1969; Parkinson and Radde, 1971; Ma et al., 1974), its involvement in Ca^{2+} extrusion in those tissues remains speculative at this stage.

Unlike the red blood cell, all other cells are equipped with organelles which can respond effectively to changes in cytosol Ca^{2+} concentration, accumulating or releasing Ca^{2+} as needed. The most efficient cell organelle in controlling cytosol Ca^{2+} is the sarcoplasmic

reticulum (Martonosi and Feretos, 1964). Once again Ca^{2+}-ATPase is implicated. However, sarcoplasmic reticulum is unique to muscle tissue. The less specialized endoplasmic reticulum (microsomal fraction) is also capable of accumulating Ca^{2+} (Alonso and Walser, 1968). This Ca^{2+} accumulation involves ATP hydrolysis. The mitochondrion is another cellular organelle which accumulates Ca^{2+} (Reynafarje and Lehninger, 1969).

The fate of the accumulated Ca^{2+} within the cellular organelles and the way it is finally extruded from the cell is not clear. One can speculate that at least the microsomes can fuse with the plasma membrane and secrete their contents into the extracellular fluids. Alternatively Ca^{2+} uptake by the cellular organelles may become important only when the plasma membrane calcium pump is unable temporarily to handle a large influx of Ca^{2+}. Thus the cellular organelles act as a "buffer" mechanism. Borle (1973) proposed a model of cellular calcium regulation in which Ca^{2+} influx is passive and the mitochondria act as the main regulator of cytoplasmic calcium activity and of calcium transport. He estimated that the efficiency of the plasma membrane calcium pump is about the same of that of the mitochondria. However, since the surface area of the mitochondria is so much larger than that of the plasma membrane, the contribution of the mitochondria to cellular Ca^{2+} regulation is 97% where the plasma membrane contributes only 3%. Thus in this model there are three major parameters: passive Ca^{2+} influx, active uptake by the mitochondria which acts as an ion buffer, and active extrusion of Ca^{2+} by the plasma membrane calcium pump. Hormones affecting calcium metabolism

(PTH, Calcitonin and Vitamin D Metabolite 1-25 DHCC) can act by modifying the three parameters that regulate the exchange between the cytosol, the mitochondria and the extracellular fluid.

The importance of maintaining intracellular Ca^{2+} at a low level creates special problems in transport of Ca^{2+} from one body compartment to another across a cellular barrier, since this must occur without increasing significantly the Ca^{2+} concentration in the cytosol of the cells involved. Such transport, which involves a net transfer of Ca^{2+} in one direction (asymmetric), occurs in the gut, bone, kidney, and placenta.

The placenta provides a suitable system for studying calcium transport from one body compartment to another. It was chosen as the subject of this study for the following reasons:

1) By the 60th day of gestation the placental barrier consists of fetal endothelial and trophoblastic layers. It may be assumed that the latter is involved in active transport of calcium.

2) It is easy to isolate significant quantities of plasma membranes primarily of the trophoblast.

3) In many mammalian species transfer of Ca^{2+} across the placenta is asymmetrical (net transfer from dam to fetus) and occurs against a concentration difference (Papadopoulos et al., 1967; Macdonald et al., 1965; Twardock and Austin, 1970).

4) This concentration difference can be easily measured.

5) Because of the large amounts of Ca^{2+} required by the fetus (Comar, 1956), it is possible that the transport system is operating at high velocity.

6) In vivo studies (Papadopoulos et al., 1967; Macdonald et al., 1965; Twardock and Austin, 1970) have suggested the presence of an active Ca^{2+} transport across the placenta. Comar (1956) estimated the net hourly transfer of Ca^{2+} from dam to fetus to be 7% of the total maternal plasma calcium in the human, 50% in the cow, 100% in the rabbit and 400% in the guinea pig.

In these four species the stress placed on calcium homeostatic mechanisms by pregnancy is far the greatest in guinea pig and the least in man. This fact and the similarity in the structure of the guinea pig placenta to that of man (both are classified as haemochorial), made the guinea pig placenta very attractive for use as a model.

The structure of the guinea pig placenta as shown by electron microscopy (Björkman, 1970) is presented in Plates 1 and 2. They show that the maternal and the fetal circulation are separated by a continuous layer of syncytiotrophoblast. This would appear to be the logical site for active Ca^{2+} transport across the placenta. However, the obvious limitations of in vivo studies preclude answers concerning the transport of Ca^{2+} at the cellular level.

Since Ca^{2+} transport occurs across the trophoblastic layer, it is assumed that the plasma membranes should demonstrate some calcium-related properties which could assist in Ca^{2+} transport.

The subject of this thesis is to characterize the Ca^{2+}-related properties of the placental plasma membranes and to try to utilize them in understanding Ca^{2+} transport, in the same way that Ca^{2+}-related properties of the sarcoplasmic reticulum (Martonosi and Feretos, 1964),

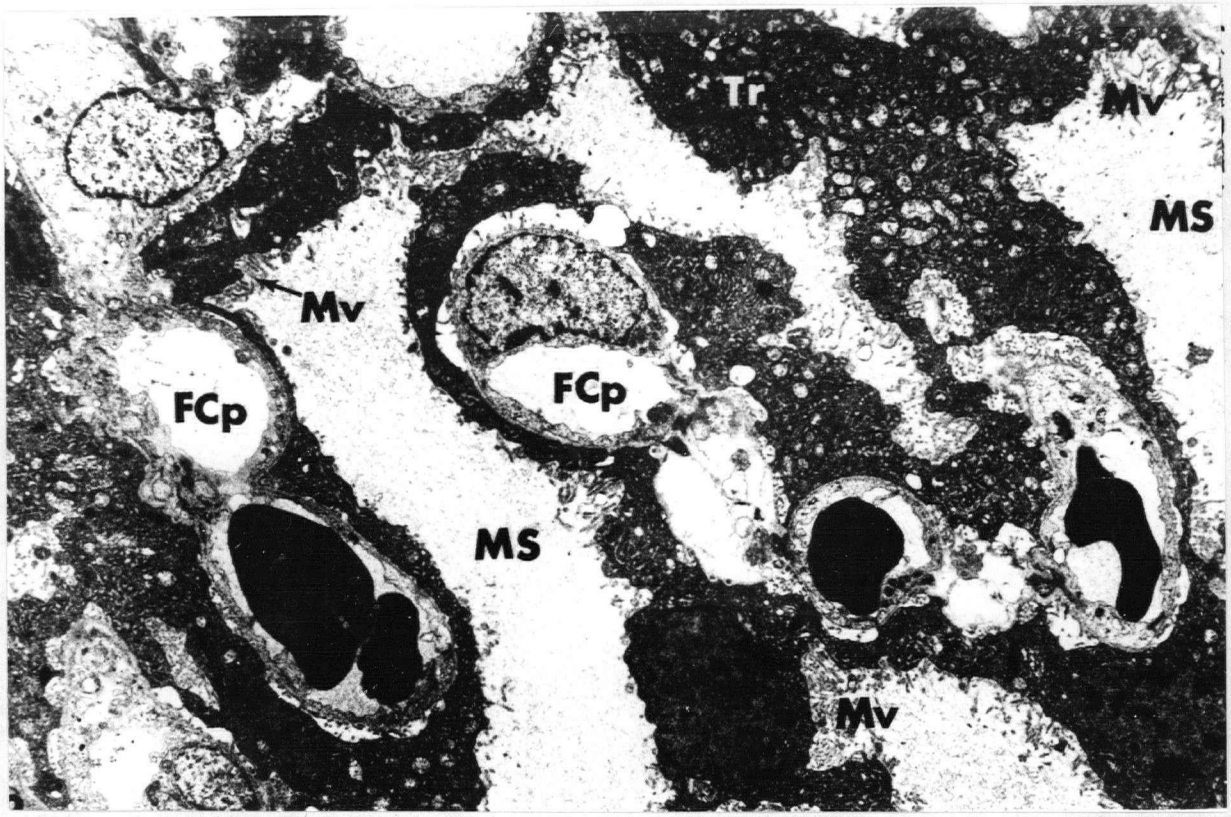


Plate 1. Survey picture showing the great variation in thickness of the trophoblastic layer (Tr) and its relation to the foetal capillaries (FCp). Note the microvilli (Mv) projecting into the maternal blood spaces (MS). Em. x 3600.

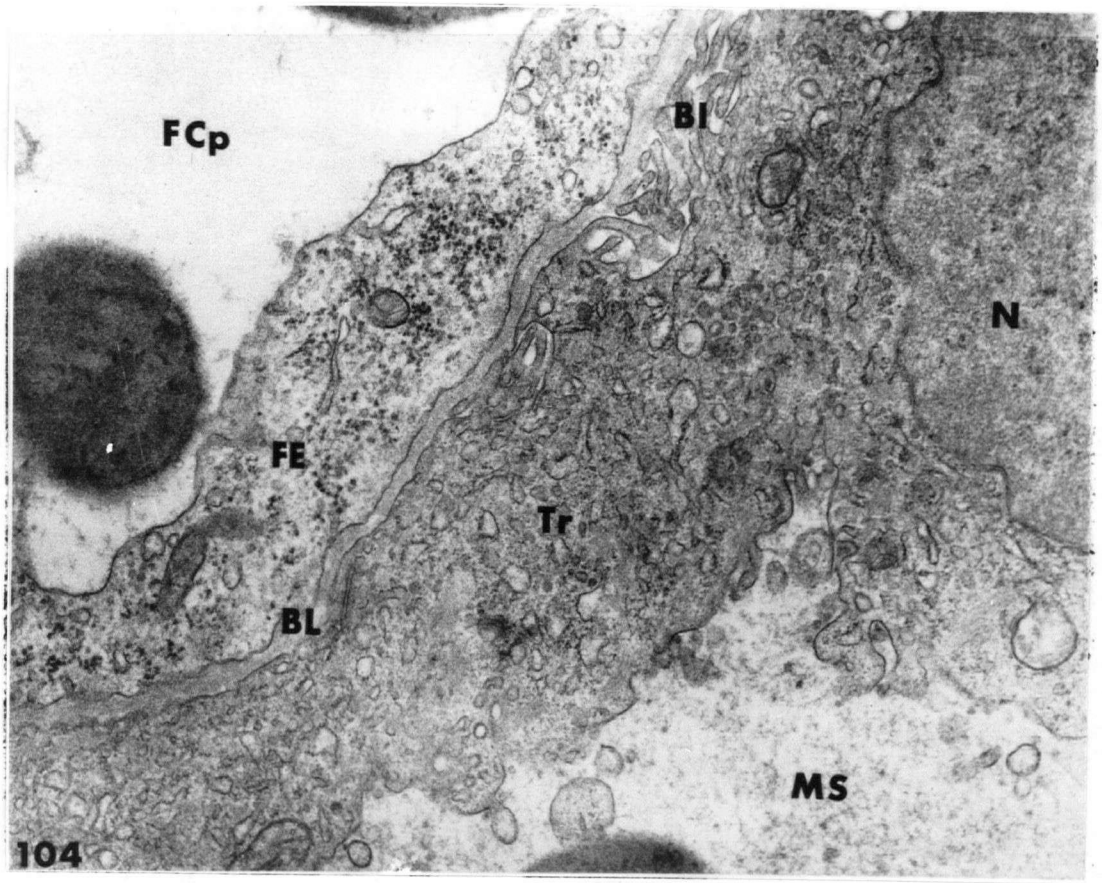


Plate 2. Detail of placental membrane between a maternal blood space (MS) and a foetal capillary (FCp). The intervening layers comprise a simple layer of trophoblast (Tr), a basal lamina (BL), and foetal endothelium (FE). The trophoblast appears to be highly differentiated with a well developed rough surfaced endoplasmic reticulum and infoldings of the basal plasma membrane (BI). Em. x 23,300.

mitochondria (Reynafarje and Lehninger, 1969), and red blood cell (Schatzmann and Rossi, 1971) membranes contributed to understanding of intracellular Ca^{2+} regulation. Since the sarcoplasmic reticulum has been most intensively studied with respect to its Ca^{2+}-related properties, it often serves as a reference in this thesis. However, one should keep in mind that unlike the placental Ca^{2+} transport, the sarcoplasmic Ca^{2+} transport is symmetric and is exposed to large changes in velocity in a very short time as part of its role in contraction-relaxation of muscle.

Three Ca^{2+}-related properties of the placental plasma membranes were chosen for study: Ca^{2+} binding to the membranes, membrane bound Ca^{2+} activated ATPase, and Ca^{2+} uptake by the placental plasma membrane vesicles.

This thesis is divided into three divisions, and the studies on each of the three Ca^{2+}-related properties are described and discussed in separate divisions. The reasons for choosing these properties are given in the introduction to each division.

To date there has been no full description of these three Ca^{2+}-related properties of any plasma membranes involved in asymmetrical Ca^{2+} transport. Therefore the experiments were designed to fill this gap. An attempt to relate these properties to Ca^{2+}-transport is made in the general conclusion of the thesis.

DIVISION I

CALCIUM BINDING TO THE PLACENTAL PLASMA MEMBRANES

CHAPTER I

CALCIUM BINDING TO THE PLACENTAL PLASMA MEMBRANES

INTRODUCTION

The present concepts of the mechanism of active transport require that a preliminary and essential step for calcium transport is the binding of the ion to the membrane involved. This binding is assumed to be passive. A knowledge of the number of calcium-binding sites, their specificity, relative affinities, and capacity is essential to an understanding of this process (binding to the membrane) and its relationship to active transport.

Perhaps the most important property is the specificity of the site for the ion to be transported. The ability of the membrane to transport ions selectively is dependent on the specificity of the site. The best described selectivity in biological membranes, is that between Na^+ and K^+. The "Sodium Pump" system has specific sites for Na^+ on the inner surface of the cell membrane and specific sites for K^+ on the outer surface of the cell membrane. The apparent affinities of each site are at least 50 times higher for the one ion than for the other (Garay and Garrahan, 1973). If we exclude the monovalent cations as competitors for the Ca^{2+} site, the only divalent cation whose physiological concentration enables it to act as a competitor for Ca^{2+} is Mg^{2+}.

The driving force underlying specificity, is the difference between the free energy of cation-site electrostatic interaction and the free energy of hydration of the cation. When Ca^{2+} and Mg^{2+} are present in the medium in equimolar concentration, the distribution of the occupancy of the sites by Ca^{2+} and Mg^{2+} is dependent on the ratio between the $-\Delta G$ (free energy) for the corresponding reactions. The free energies of the reactions can be estimated from the following equations. For Ca^{2+}:

$$\Delta G_{Ca} = \Delta G_{ICa} - \Delta G_{HCa}$$

where ΔG_{ICa} = the change in free energy of the system after the interaction of Ca^{2+} with the site (x)

$$Ca^{2+} + X \rightleftharpoons Ca\text{-}X$$

and ΔG_{HCa} = the change in the free energy of the system when Ca^{2+} is hydrated

$$Ca^{2+} + n\ H_2O \rightleftharpoons Ca^{2+}\ (H_2O)\ n$$

Since Ca^{2+} in aqueous solution is mainly hydrated, it must first be dehydrated ($+\Delta G_H$) before it can interact with the site. If the ΔG of the overall reaction is negative, it will proceed spontaneously. (ΔG Mg^{2+} can be calculated in the same way as for Ca^{2+}.) In the presence of both Mg^{2+} and Ca^{2+} in equimolar concentrations, the final ratio of calcium-occupied sites (Ca - X) to magnesium-occupied sites (Mg - X) will be as follows:

$$\frac{Ca - X}{Mg - X} = \frac{-\Delta G_{Ca^{2+}}}{-\Delta G_{Mg^{2+}}}$$

Since the diameter of Ca^{2+} (0.99 A°) is larger than that of Mg^{2+} (0.65 A°) its dehydration energy will be smaller. Since the dehydration energies are constant under physiological conditions, the only way for the cell to increase its selectivity is by construction of a specific site. Since the charge density of Ca^{2+} is smaller than that of the Mg^{2+}, it will react selectively with sites with low field. Strong field site (weak acid) will react favorably with Mg^{2+} (Diamond and Wright, 1969).

In this chapter, the Ca^{2+}-binding properties of the placental plasma membrane are described. Their possible relationship to Ca^{2+} transport is dealt with in the general conclusion.

The most popular technique for measurement of Ca^{2+}-binding is ultrafiltration. However, in the present study some uncontrolled non-specific binding to the filter was found. For example, it was observed that the binding of Ca^{2+} to the filter was time dependent. Thus with increasing membrane concentration of the filtered sample, the filtration time increased as did the uncontrolled non-specific Ca^{2+}-binding to the filter. Because of these difficulties, a method based on the measurement of the rate of diffusion in a flow dialysis system was used (Colowick and Womack, 1969). This method has not previously been used for measuring Ca^{2+}-binding, so it had to be modified as described in the methods section of this chapter. The advantages of the flow dialysis method are given in the discussion.

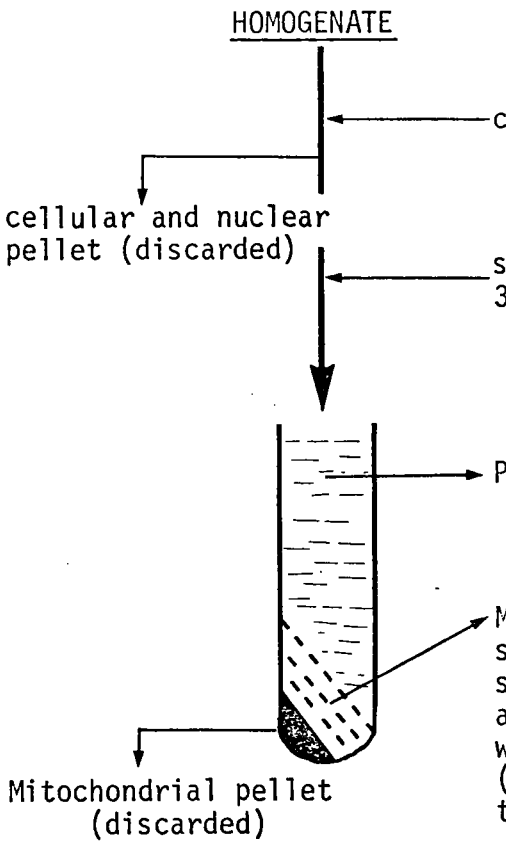
MATERIALS AND METHODS

Isolation of Placental Plasma Membranes

The method of Post and Sen (1967) for the isolation of renal plasma membranes was adapted for this study of placental plasma membranes. The purity of the final preparation was checked by electron microscopy and by enzyme markers to ensure that the method was suitable for isolation of the placental plasma membranes.

Pregnant guinea pigs around the 60th day of gestation were anaesthetized with Na pentobarbital (100 mg/kg). The abdomen was opened and the amniotic sacs with the fetuses were removed. Each placenta was carefully freed from membranes, gross blood vessels and uterine tissue and placed in ice-cold 0.9% NaCl solution. Using a Thomas tissue grinder, size C with Teflon pestle, for 10 strokes at 1500 rpm, the placentas were homogenized in 25 ml of a solution containing 87 g sucrose, 1.169 g NaCl, 1.860 g Na_2H_2EDTA, 0.2 g $MgCl_2 6\ H_2O$, and 0.68 g imidazole per litre. The tissues were then processed in a manner similar to that described by Post and Sen (1967) for the isolation of renal plasma membranes, omitting the urea stage. The procedure consists of a series of centrifugations at 35,000 xg for 30 min in different solutions, and is given in detail in the accompanying flow chart. The isolated membranes were finally suspended in 5×10^{-4} M imidazole-histidine, 5 mM Tris-HCl buffer (pH 7.6) and stored at +4°C until assay. This procedure for isolating the placental plasma membranes was used throughout the study except in certain cases, when it was modified to serve different purposes.

Isolation of Placental Plasma Membranes



Composition of Solutions Used in Isolation of the Plasma Membranes:

(1)	(2)	(3)	(4)
0.25 M Sucrose	0.25 M Sucrose	15 mM NaCl	10 mM Imidazole
0.02 M NaCl	2 mM Na_2EDTA	1 mM Na_2EDTA	0.1 mM Na_2EDTA
5 mM Na_2EDTA	0.1 mM $MgCl_2$	3 mM Imidazole	
1 mM $MgCl_2$	4 mM Imidazole		
10 mM Imidazole	0.02% (W/V) Na-Heparin		

Determination of Membrane Concentration

The protein concentration of the final preparation was taken as an indicator of membrane content in the medium. The total protein was measured by the procedure of Lowry et al. (1951) using bovine albumin standards. All the results are expressed per mg of membrane protein.

Enzyme Marker Assays

The supernatants from the 35,000 x g centrifugations were pooled, as were the pellets. These fractions and the final preparations were assayed for alkaline phosphatase, glucose-6-phosphatase and succinate dehydrogenase.

Alkaline Phosphatase (EC 3.1.3.1)

Alkaline phosphatase as a marker for plasma membranes (Dixon and Webb, 1964a) was assayed by the method of Bessey et al. (1946) as modified by Kelly and Hamilton (1970). Aliquots (50 µl) from the different fractions were incubated for 30 min at 30°C in 2-amino-2-methyl-1-propanol buffer (Eastman Organic Chemical) 0.75 M pH 10.0 using 5 mM disodium p-nitrophenyl phosphate (Sigma) as substrate. The final volume was 0.55 ml. The reaction was terminated by adding 5 ml of 0.05 N NaOH, and p-nitrophenol release was determined spectrophotometrically by reading absorbence at 410 nm, compared to a standard p-nitrophenol curve. Blank samples (in the absence of enzyme) were incubated under the same conditions.

Glucose-6-Phosphatase (EC 3.1.3.9)

Glucose-6-phosphatase is a predominantly microsomal enzyme (Ginsburg and Hers, 1970) and was used in this study as a marker for the assessment of the purity of the subcellular fractions. The enzyme was tested as described by Hübscher and West (1965). The incubation mixture contained: 43 mM maleate buffer (pH 6.0), 2 mM KF, 4 mM EDTA, 28.5 mM glucose-6-phosphate as substrate and approximately 1 mg protein from the different fractions. The final volume was 0.7 ml and it was incubated for 15 min at 37°C. The reaction was terminated with 1.3 ml 10% (W/V) TCA and the amount of inorganic phosphate liberated was measured by the GOMORI method (Gomori, 1942) using the auto analyzer (Technicon). The Pi released in the absence of protein was subtracted before calculating the specific activity.

Succinate Dehydrogenase (EC 1.3.99.1)

Succinate dehydrogenase is suitable as a marker for mitochondria (Dixon and Webb, 1964a). The activity was measured as described by Pennington (1961) using sodium succinate (50 mM) as substrate, INT 0.1% [2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-2H-tetrazolium chloride] (Eastman) as hydrogen acceptor in 50 mM potassium phosphate buffer pH 7.4 in the presence or absence of aliquots (0.1 ml) from the different fractions. The samples (final volume 1 ml) were incubated for 15 min at 37°C and the reaction terminated with 1 ml 10% (W/V) TCA. The formazan was extracted with 4 ml ethyl acetate and its absorbence measured at 490 nm. Non-enzymatic reduction of INT was measured and subtracted from the total activity.

Electron Microscopic Examination of the Final Preparation

An aliquot from the final preparation was centrifuged at 90,000 x g for 10 min. The pellet was fixed in phosphate-buffered osmium tetraoxide (1%), dehydrated, and embedded in Epon. The sections were stained with uranyl acetate and lead citrate.

Evaluation of the Method

Marker Enzymes

Table I summarizes the distribution of marker enzymes. The specific activity of all the enzymes in the supernatant was low. Glucose-6-phosphatase and alkaline phosphatase were distributed in the same ratio of 1.1 between the "final preparation" and the pellet, indicating that the final preparation is truly a microsomal fraction. The high specific activity of the two phosphatases in the pellet is due to incomplete recovery of the heavier plasma membranes fragments and microsomes. However, the distribution of succinate dehydrogenase showed a ratio of 0.1, indicating that the "final preparation" contains very few mitochondria. No attempt was made to separate the plasma membranes from the microsomes. The electron microscopic picture of the trophoblastic layer (plates 1, 2) showed a continuous network of endoplasmic reticulum with the plasma membranes which project into the maternal circulation as micro-villi. Thus, following homogenization, both form fragments with the same density and size, which together comprise the microsomal fraction. This fraction was used for studying the Ca^{2+}-related properties of these placental components.

TABLE I. Specific activities of marker enzymes in different fractions, and ratio between the specific activity found in the final preparation (F.P.) over the specific activity found in the pellet.

	Alkaline Phosphatase*	Glucose-6-Phosphatase*	Succinate Dehydrogenase**
Supernatant	0.22	0.015	0
F.P.	2.35	0.688	0.398
Pellet	2.05	0.602	3.76
Ratio F.P./Pellet	1.14	1.14	0.105

*Activity of Phosphatases (μmole Pi/mg protein per 30 min)

**O.D./mg protein/ 15 min.

Electron Microscopic Examination

The electron microscopic examination of the final preparation revealed no contamination with mitochondria confirming the results with the enzyme markers. The preparation consisted mostly of plasma membranes in the form of vesicles and fragments (Plates 3 and 4).

Calcium binding assay

The calcium-binding properties were investigated in a flow dialysis system based on that described by Colowick and Womack (1969). The method is based on the principle that the rate of diffusion is proportional to the concentration of the free diffusible molecule; this rate will be constant when equilibrium is achieved with the macromolecule in the upper chamber of the dialysis cell. Equilibrium will be reached within seconds, and a constant diffusion rate will occur within 1.5-2 minutes when the effluent volume pumped through the lower chamber is about 5 times its volume (Colowick and Womack, 1969).

The dialysis cell (Figure 1) was prepared as described by Colowick and Womack (1969) using a standard cellophane dialysis membrane (Fisher Scientific Company). The placental plasma membranes and ^{45}Ca (New England Nuclear) were premixed, and the pH adjusted to 8.0 if necessary, before addition to the upper chamber. All experiments were conducted at room temperature (24°C). Initial $^{45}Ca^{2+}$ concentration was 1.5×10^{-5}M (approximately 0.04mCi/ml in final reaction mixture) unless indicated differently.

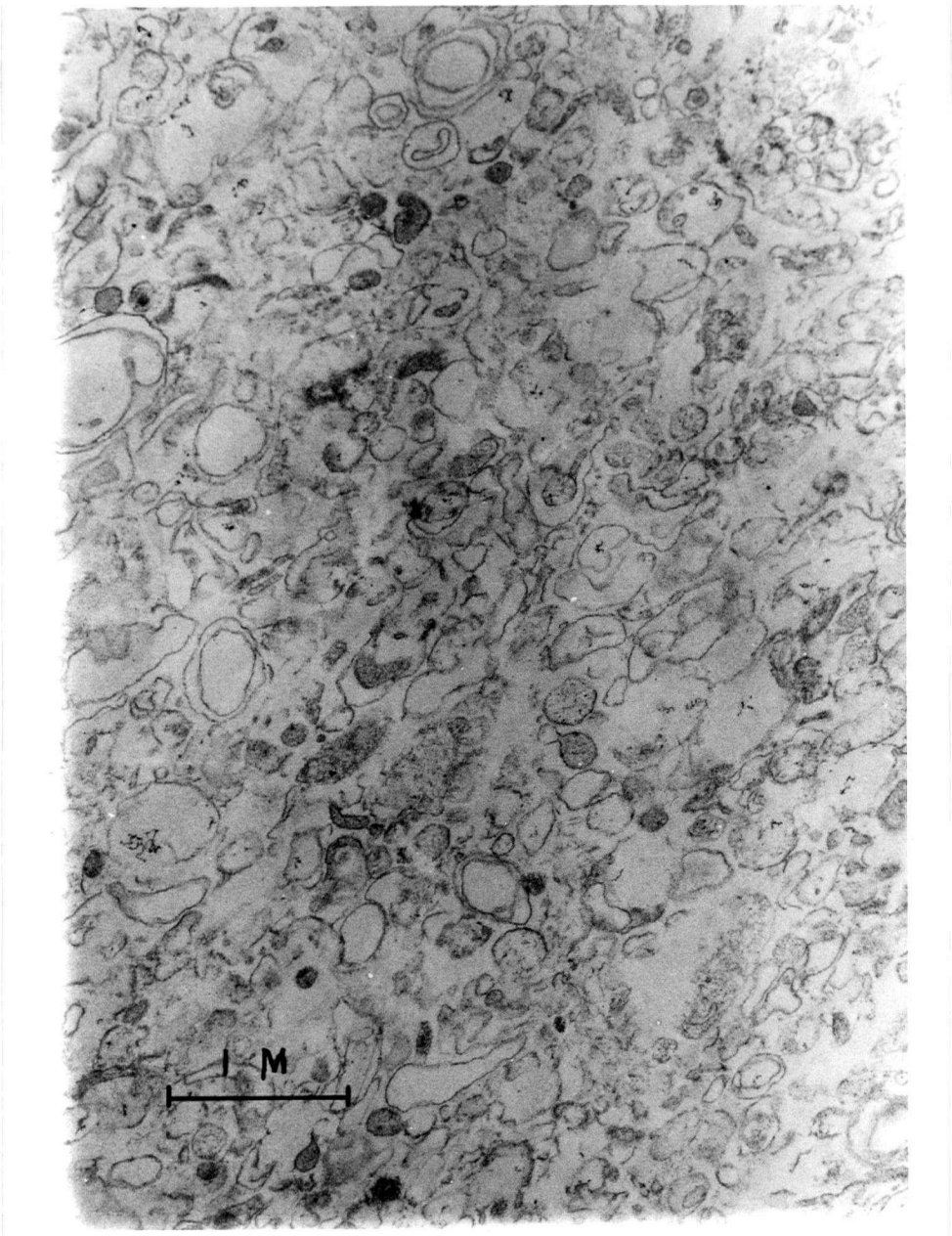


Plate 3. The final membrane preparation (x 24,282).

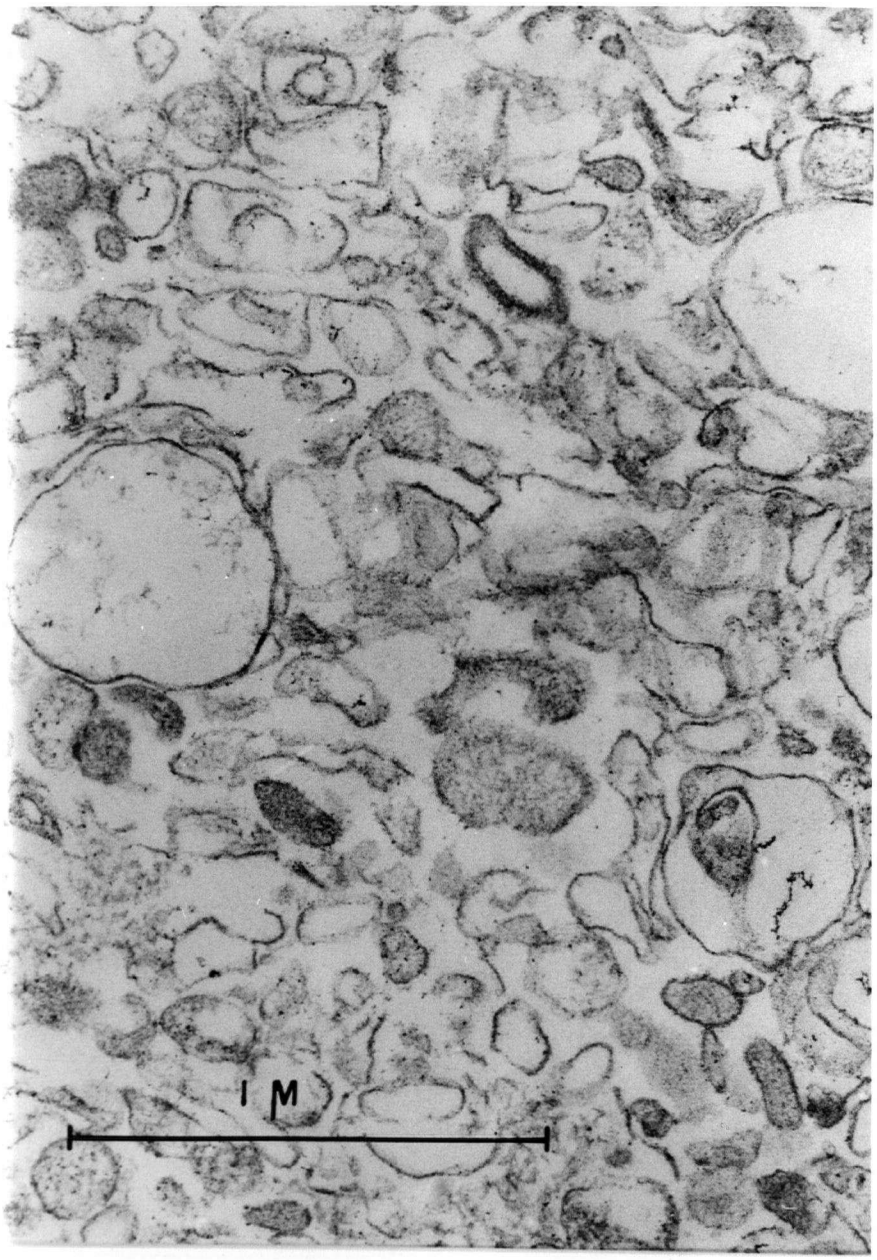


Plate 4. The final membrane preparation (x 61,560).

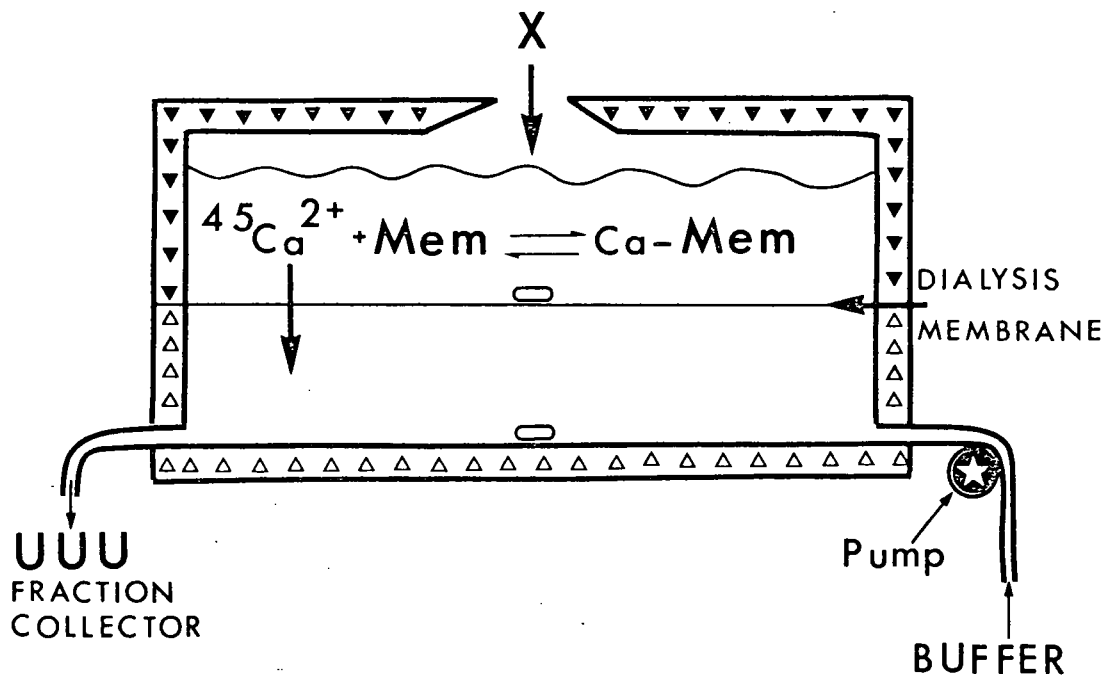


Figure 1. Diagram of the apparatus used for measuring calcium binding by rate of dialysis.

The dialysis buffer contained 20 mM Tris-HCl (pH 8.0) and 100 mM NaCl. The NaCl concentration was raised to 100 mM (as suggested by Reed (1973)) in order to prevent an excessive non-specific binding to the dialysis membrane. The flow rate was maintained at 8 ml/min and the effluent was collected in 2 ml aliquots with a fraction collector. One ml samples were added to 10 ml scintillation fluid (aquasol-New England Nuclear) and were counted in a Beckman model LS-233 liquid scintillation counter. Protein was determined by the procedure of Lowry et al. (1951).

Calculation of calcium binding

The difference between the control diffusion rate without plasma membranes and the diffusion rate observed in the presence of plasma membranes represents the fraction of bound Ca^{2+} in the medium. However, the control diffusion rate is not constant because dilution occurs with each addition to the upper chamber, and because of cpm ($^{45}Ca^{2+}$) loss in the effluent during the experiment. For these reasons the initial control diffusion rate must be corrected for dilution and cpm loss for each step. The dilution effect can be duplicated in the control simply by adding the same volumes to the upper chamber without plasma membranes present. The cpm loss cannot be duplicated because differences in the diffusion rates in the presence and absence of plasma membranes produce different rates of loss from the upper chamber.

For each experiment, the loss of cpm for each step was calculated from the cpm found in the effluent collected in each step. These figures were then subtracted from the initial cpm in the upper chamber

to give actual cpm concentration for each step. The total loss of cpm during experiments of 25-30 min, with a flow rate of 8 ml/min was up to 10%. The control diffusion rate for each step was calculated by multiplying the experimental initial control diffusion rate by the dilution factor and by the cpm loss factor.

C.D.R. = I.C.D.R. x D.F. x Cpm.L.F.

Where: C.D.R. = Control Diffusion Rate,

I.C.D.R. = Initial Control Diffusion Rate at time zero in the absence of plasma membranes,

D.F. = Dilution Factor (due to volume increase with each addition to the upper chamber),

and Cpm L.F. = Cpm Loss Factor (due to loss of Cpm from the upper chamber to the effluent).

Thus: $\%\ Ca^{2+}\ \text{bound} = \frac{C.D.R. - O.D.R.}{C.D.R.} \times 100$

Where O.D.R. = Observed Diffusion Rate in the presence of plasma membranes.

Calcium Binding by Bovine Albumin

For comparison, Ca^{2+}-binding by Bovine Albumin (Fraction V, Armour Pharm. Comp.) was also studied, under the same conditions as described for the placental plasma membranes. It is assumed that Bovine Albumin is representative of a class of proteins which do not bind Ca^{2+} specifically.

RESULTS

Evaluation of the Method

The suitability of the flow dialysis method for Ca^{2+}-binding studies is demonstrated in Figure 2. The addition of $^{45}Ca^{2+}$ to the upper chamber in the absence of plasma membranes (Figure 2, upper curve) caused a linear increase in diffusion rate (Figure 3), suggesting that only a small fraction of $^{45}Ca^{2+}$ was bound. The lower curve in Figure 2 represents the results obtained under the same conditions but in the presence of plasma membranes in the upper chamber. The difference in the diffusion rates in the absence and the presence of plasma membranes is due to $^{45}Ca^{2+}$ binding by the placental plasma membranes.

The addition of a large excess of $^{40}Ca^{2+}$ (10^{-2}M) in the absence of plasma membranes produced a sharp spike in the diffusion rate as a result of $^{45}Ca^{2+}$ displacement from the dialysis membrane by $^{40}Ca^{2+}$. However the amount bound was negligible (0.1% of the total $^{45}Ca^{2+}$ present) and after a few samples the diffusion rate returned to the same level as before the addition of $^{40}Ca^{2+}$. In the presence of plasma membranes, excess $^{40}Ca^{2+}$ (10^{-2}M) increased the diffusion rate to the level of the control, indicating complete displacement of $^{45}Ca^{2+}$ from the plasma membranes. The absence of a spike in the presence of plasma membranes is due to a reduced binding of $^{45}Ca^{2+}$ to the dialysis membrane because of the higher affinity of the plasma membranes for the available $^{45}Ca^{2+}$.

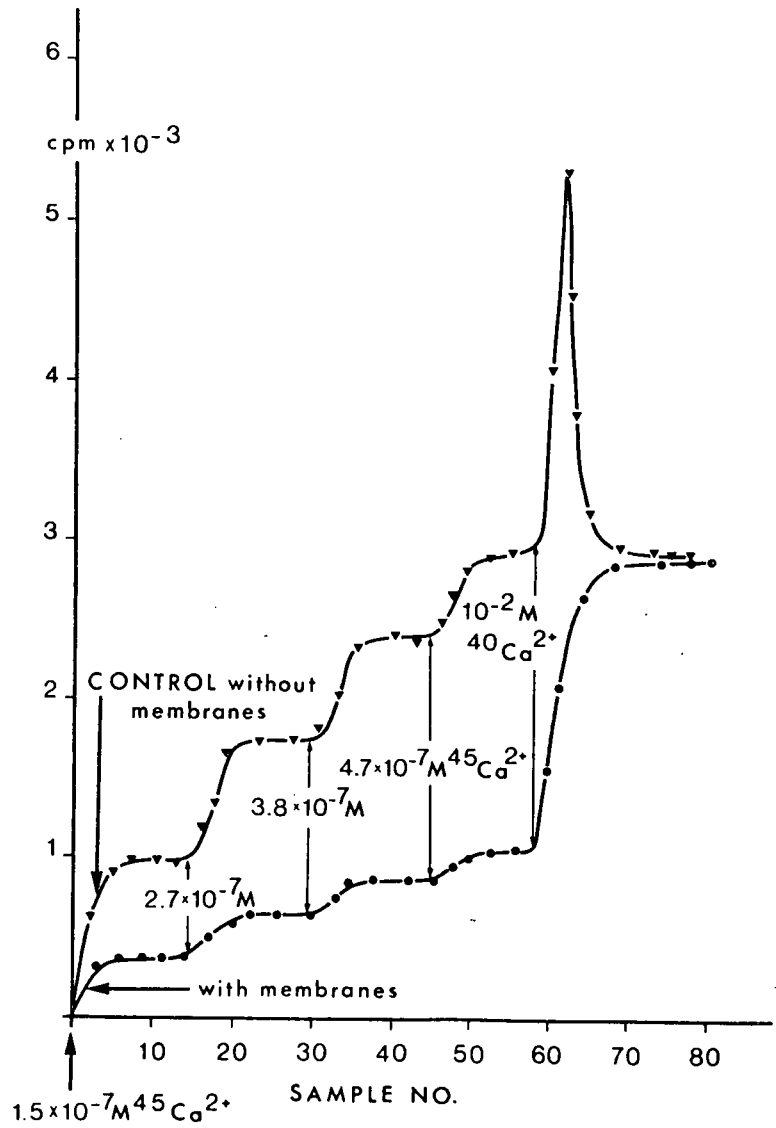


Figure 2. Measurement of $^{45}Ca^{2+}$ diffusion rate at various $^{45}Ca^{2+}$ concentrations ▼--------▼ without plasma membranes ●--------● with plasma membranes. The medium and dialysis buffer contained 20 mM Tris-HCl (pH 8.0), 100 mM NaCl. Protein concentration was 2.52 mg/ml, the effluent was collected in 2 ml fractions of which 1 ml was counted for ^{45}Ca. Each step was allowed 15 samples before increasing $^{45}Ca^{2+}$ concentration. The results are expressed in cpm/ml effluent.

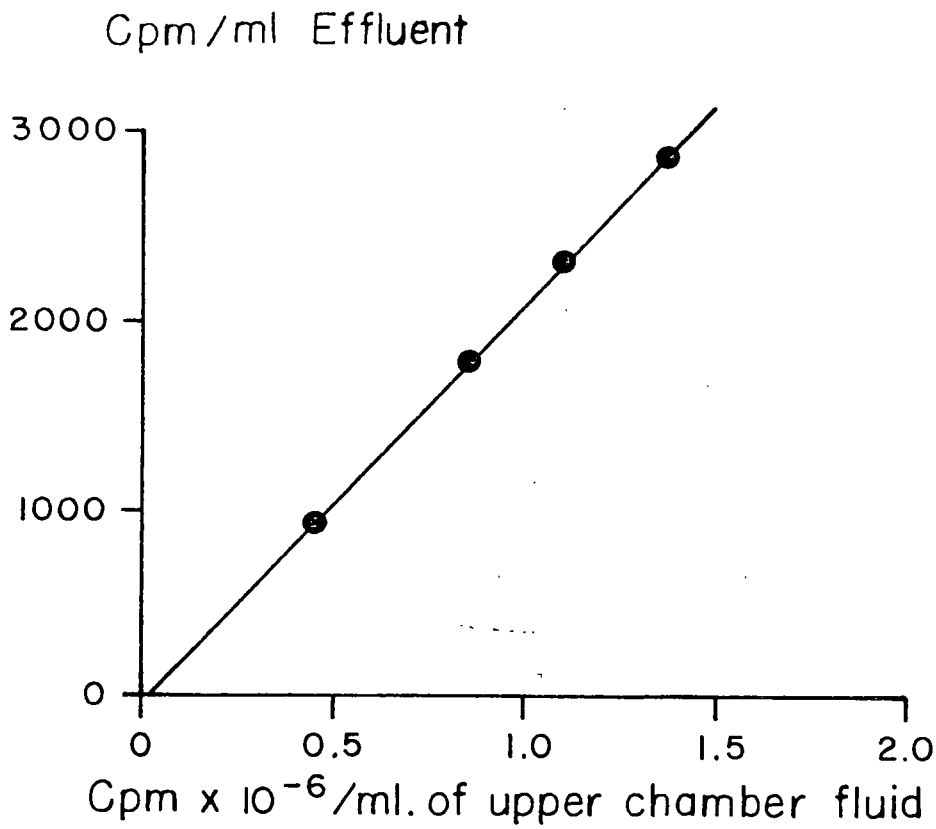


Figure 3. Measurement of $^{45}Ca^{2+}$ diffusion rate as a function of cpm concentration in the upper chamber. The data presented here were obtained from Figure 2 (upper curve).

A typical $^{45}Ca^{2+}$ diffusion rate profile is presented in Figure 4. The control diffusion rate after the first step (samples 1-15) was corrected for dilution and loss of $^{45}Ca^{2+}$, as described in the Methods section. For all experiments, the data were first plotted in this manner, to allow calculation of the extent of Ca^{2+}-binding for each step. The Ca^{2+}-binding was then replotted against the different parameters used in subsequent experiments.

Calcium Binding by Placental Plasma Membranes

At low Ca^{2+} concentrations (10^{-7}-5 x 10^{-7}M) calcium binding per mg protein was linear (Figure 5A: data derived from Figure 2), indicating a constant percentage bound in this concentration range. In a double reciprocal plot the line passed through the origin, suggesting non-saturable kinetics.

At higher Ca^{2+} concentration (10^{-6}-10^{-2}M) the amount of Ca^{2+} bound per mg protein increased sigmoidally (Figure 5B). A Scatchard plot (Figure 6) of Ca^{2+}-binding revealed two types of sites: high affinity sites accommodating 26 $\pm$ 2 (mean $\pm$ S.E.) nmoles per mg protein and dissociation constant 3.1 $\pm$ 0.4 x 10^{-5}M; and low affinity sites accommodating 266 $\pm$ 27 nmoles per mg protein and dissociation constant 1.1 $\pm$ 0.1 x 10^{-3}M (Table II).

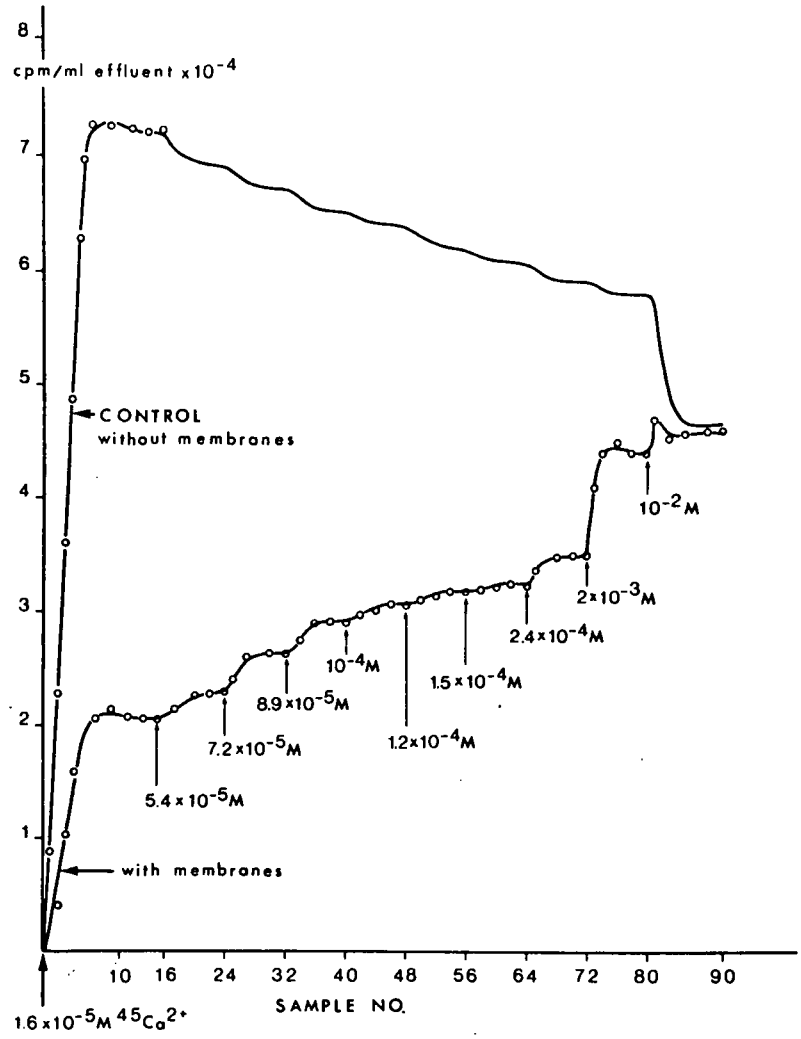


Figure 4: Flow dialysis profiles of calcium binding at various calcium concentrations: The medium and dialysis buffer contained 20 mM Tris-HCl (pH 8.0), 100 mM NaCl. At time zero 1.6 x 10^{-5}M $^{45}Ca^{2+}$ was added to the medium in the presence or absence of plasma membranes. The lower curve represents the diffusion rate profile of $^{45}Ca^{2+}$ in the presence of plasma membranes (protein concentration 2.4 mg/ml) at various $^{40}Ca^{2+}$ concentrations, as indicated under the arrows. The upper curve is a control curve in the absence of plasma membranes. Up to sample No. 16 the control curve represents experimental values. The remainder is a corrected control curve compensated for dilution and cpm loss for each step. The flow rate was 8 ml/min, each step was allowed 8 samples and results are expressed as cpm per ml effluent.

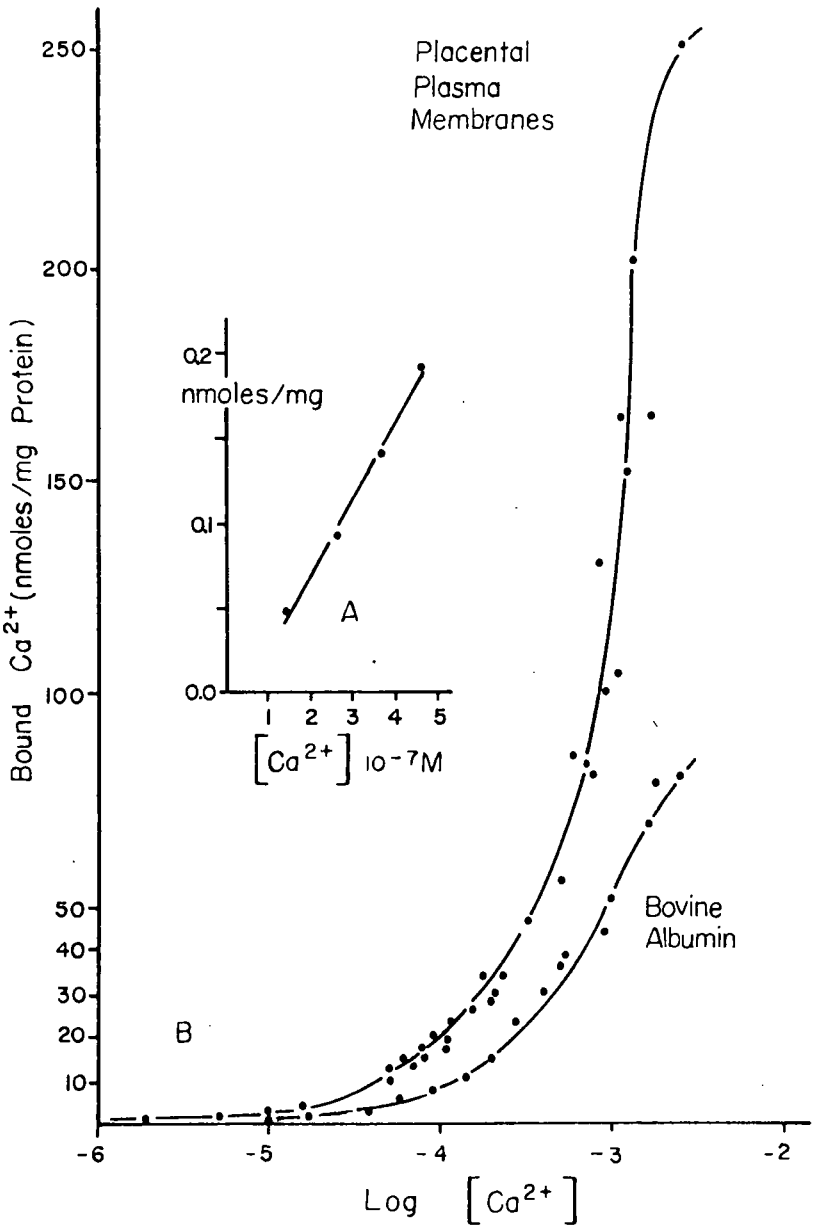


Figure 5. Ca^{2+} concentration effect on Ca^{2+}-binding levels by placental plasma membranes and Bovine Albumin. (A)Ca^{2+}-binding at low Ca^{2+} concentration ($1 \cdot 10^{-7}$-$5 \cdot 10^{-7}$M). This curve was derived from Figure 1. The results are expressed in nmoles Ca^{2+} bound per mg protein. (B)Ca^{2+} binding at higher Ca^{2+} concentrations ($1 \cdot 10^{-6}$-$1 \cdot 10^{-2}$M). This curve was derived from four different experiments carried out under the same conditions as described in Figure 2. The Ca^{2+} concentration was varied slightly from one experiment to another to cover the wide range. The bound calcium is expressed in nmoles Ca^{2+} per mg protein.

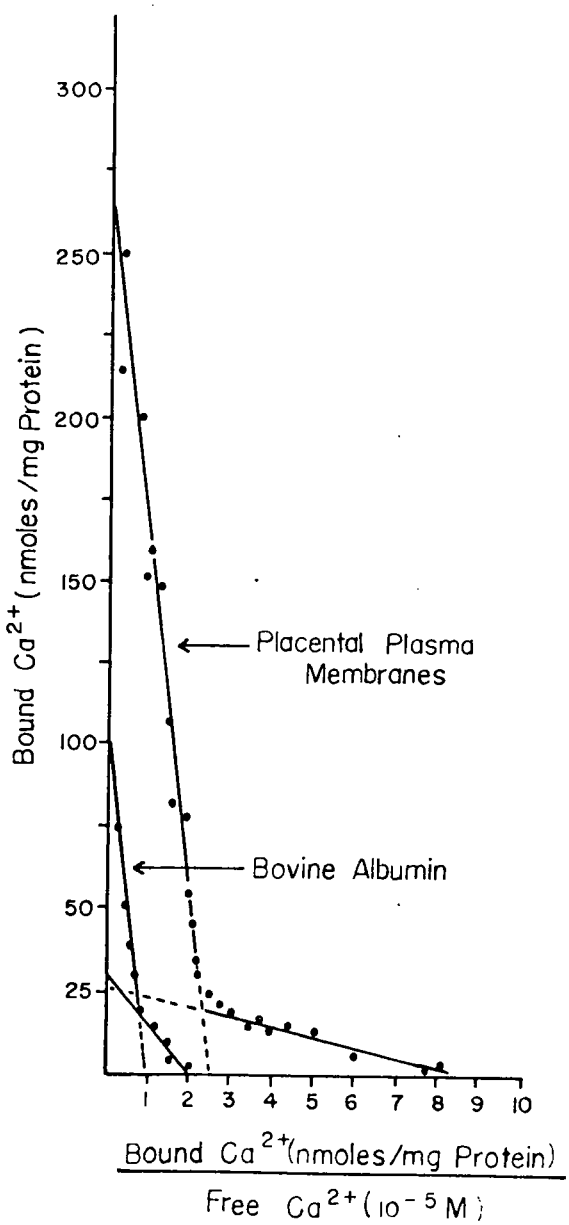


Figure 6. Scatchard plot of Ca^{2+}-binding by placental plasma membranes and Bovine Albumin (derived from the steady values which were used to construct Figure 5B).

Table II. Affinity and capacity of placental membranes and Bovine Albumin for Ca^{2+}

	K_{s1}*	n_1**	K_{s2}*	n_2**
Placental Plasma Membranes	$3.1 \pm 0.4 \times 10^{-5}$M	26±2	$1.1 \pm 0.1 \times 10^{-3}$M	266±27
Bovine Albumin	$1.4 \pm 0.06 \times 10^{-4}$M	28±1	$9.1 \pm 0.6 \times 10^{-4}$M	99±5

All the results are expressed as mean ± S.E.

*K_s = dissociation constant

**nmoles Ca^{2+} per mg protein

Calcium Binding by Bovine Albumin

The results are plotted in Figures 5-6 and summarized in Table II. Two types of sites for Ca^{2+} were revealed. The high affinity sites have the same capacity as the high affinity sites of the plasma membranes but the affinity is almost five-fold lower. The low affinity sites have the same affinity with only 40% of the capacity of the low affinity sites of the plasma membranes.

Time, Temperature, Protein Concentration and pH Effects

The maximal diffusion rate was achieved after 1.5 min, and when no further additions or treatments were performed, the diffusion rate was constant (after compensating for cpm loss) for at least 30 min. Thus calcium binding by placental plasma membranes reached a maximum within 1.5 min, and thereafter was independent of incubation time.

Reducing the temperature from 24°C to 4°C did not change significantly the extent of Ca^{2+}-binding.

The binding of Ca^{2+} increased linearly with protein concentration over the range 0.25-2.7 mg protein/ml when $[Ca^{2+}]$ was 1.5×10^{-5}M (Figure 7).

Calcium binding was dependent on pH, and practically no binding was detected at pH 4.0. The binding increased with increasing pH up to pH 11.0, and leveled off (Figure 8). Since the membranes were subjected to each pH for no more than 2.5 min, denaturation effects of extreme pH's were minimized; the pH effect was reversible over the entire pH range (3.0-11.8).

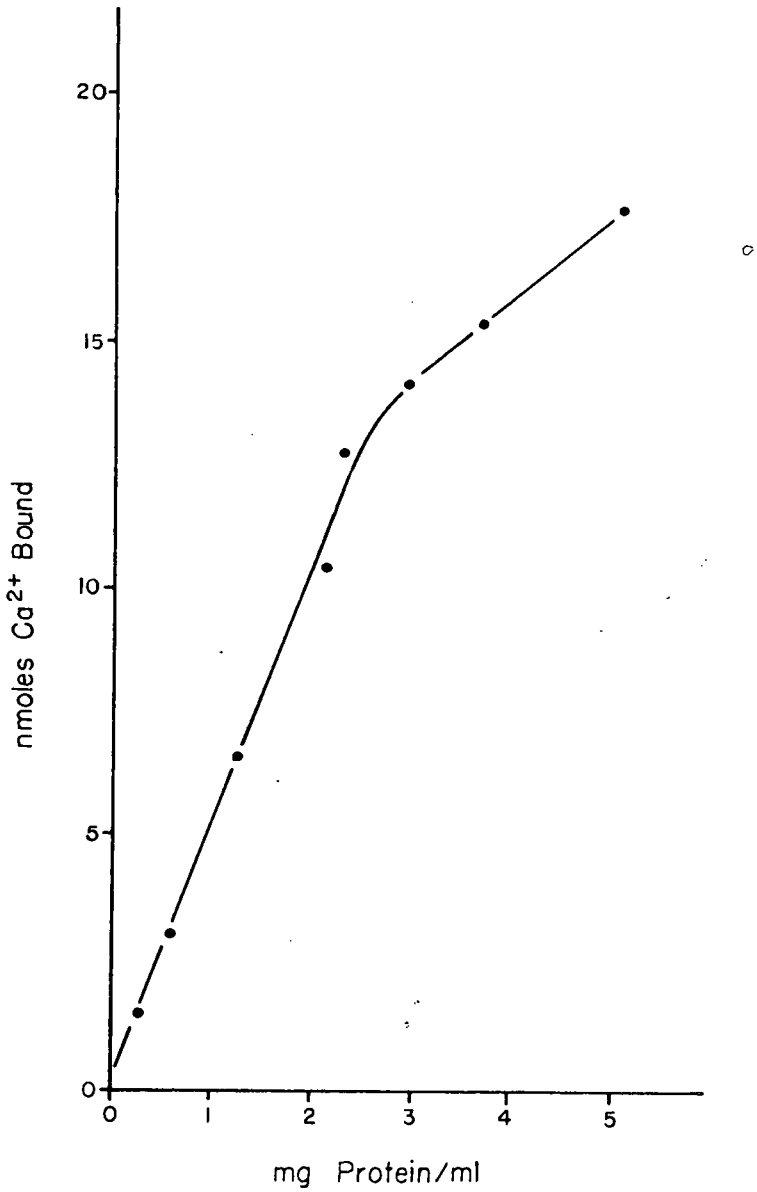


Figure 7. Ca^{2+} binding as a function of placental plasma membrane protein concentration. The medium contained 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and $1.5 \cdot 10^{-5}$M $^{45}Ca^{2+}$. The plasma membranes were added to the upper chamber to give the indicated concentration. The results are expressed as total nmoles Ca^{2+} bound.

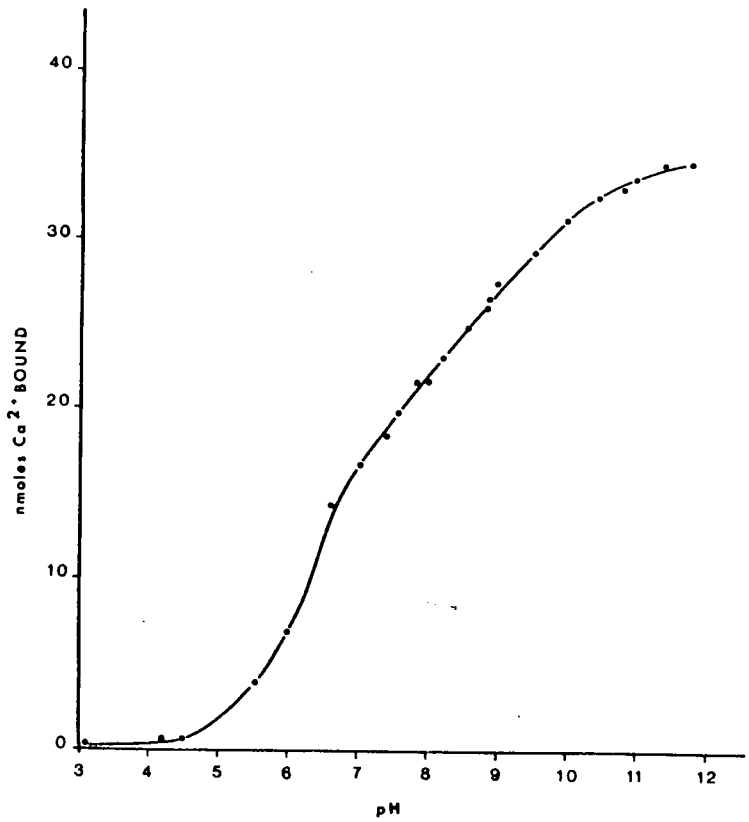


Figure 8. pH effect on Ca^{2+} binding. The pH in the upper chamber was measured with a pH electrode, and controlled by addition of HCl or NaOH (0.1 M). The plasma membranes were exposed to each pH for not more than 2.5 min. The medium contained 20 mM Tris-HCl, 100 mM NaCl $2.2 \cdot 10^{-3}$M $^{45}Ca^{2+}$ and protein concentration was 2.5 mg/ml. The results are expressed as total nmoles Ca^{2+} bound.

Ca^{2+} Displacement from Its Sites by Mg^{2+} and Sr^{2+}

Figure 9A shows the ^{45}Ca displacement from its sites by $^{40}Ca^{2+}$, Mg^{2+}, and Sr^{2+}. The percent $^{45}Ca^{2+}$ bound at the end of the first step was regarded as zero percent displaced. The ability of Mg^{2+} and Sr^{2+} to displace $^{45}Ca^{2+}$ from its sites was much lower than that of $^{40}Ca^{2+}$ (Figure 9A).

The double reciprocal plots for displacement of $^{45}Ca^{2+}$ from the plasma membranes by $^{40}Ca^{2+}$, Mg^{2+}, and Sr^{2+} (Figure 9B, C, D) revealed two apparent Km's for each ion. These values appear in Table III. The affinity of Mg^{2+} and Sr^{2+} for the high affinity Ca^{2+} sites was approximately 10-fold lower than that of $^{40}Ca^{2+}$. The affinity of Mg^{2+} for the high affinity Ca^{2+} sites was higher than that of Sr^{2+}. The wide gap of affinities was narrower for the low affinity sites (4-fold lower for Mg^{2+} and 8-fold lower for Sr^{2+}). These results indicate high specificity of these sites for calcium, with greater specificity in the high affinity sites.

DISCUSSION

The flow dialysis method was found in the present study to be a very useful tool for studying Ca^{2+}-binding. The process of binding has met the requirements for the use of this method as defined by Colowick and Womack (1969): 1) chemical equilibrium was achieved within a few seconds; 2) a constant diffusion rate was established after 1.5 min as predicted; 3) the dissociation constants of this binding reaction were

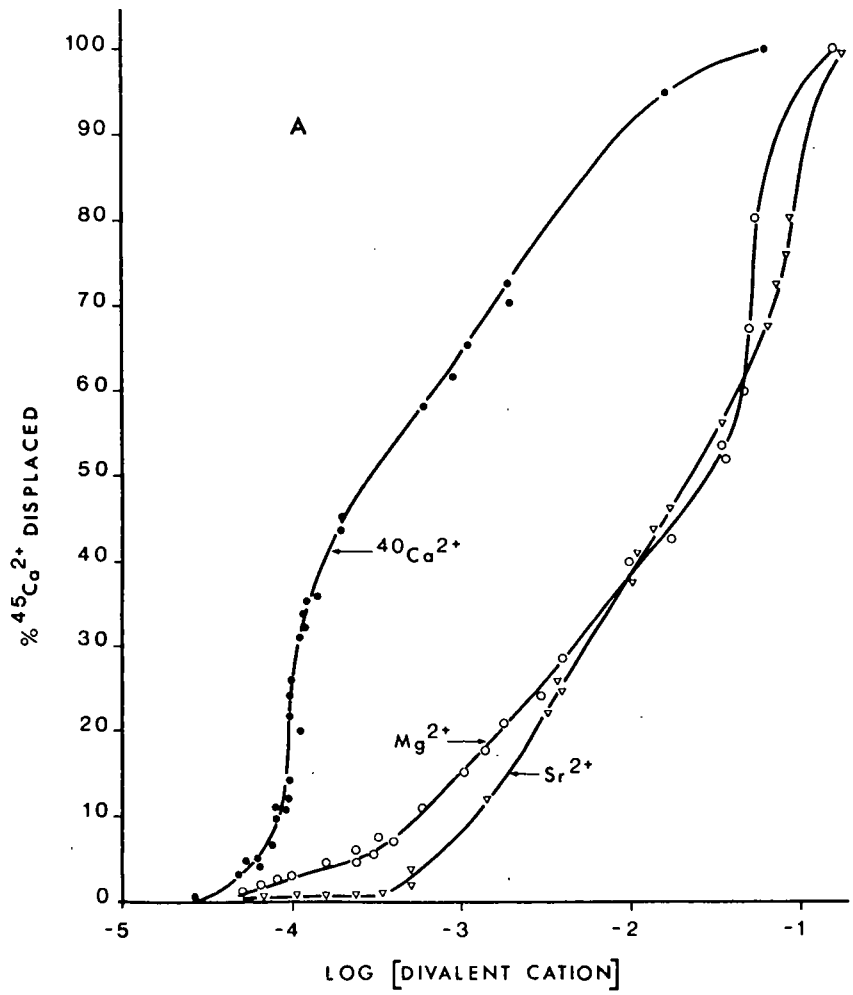


Figure 9A. $^{45}Ca^{2+}$ displacement by divalent cations. (A) The experiments were carried out as described for Figure 2. $^{40}Ca^{2+}$, Mg^{2+}, and Sr^{2+} were added in increasing concentrations to the upper chamber as indicated. The results are expressed as a percentage of $^{45}Ca^{2+}$ displaced from the plasma membranes. Zero displacement is defined as the amount of $^{45}Ca^{2+}$ bound at the end of the first step.

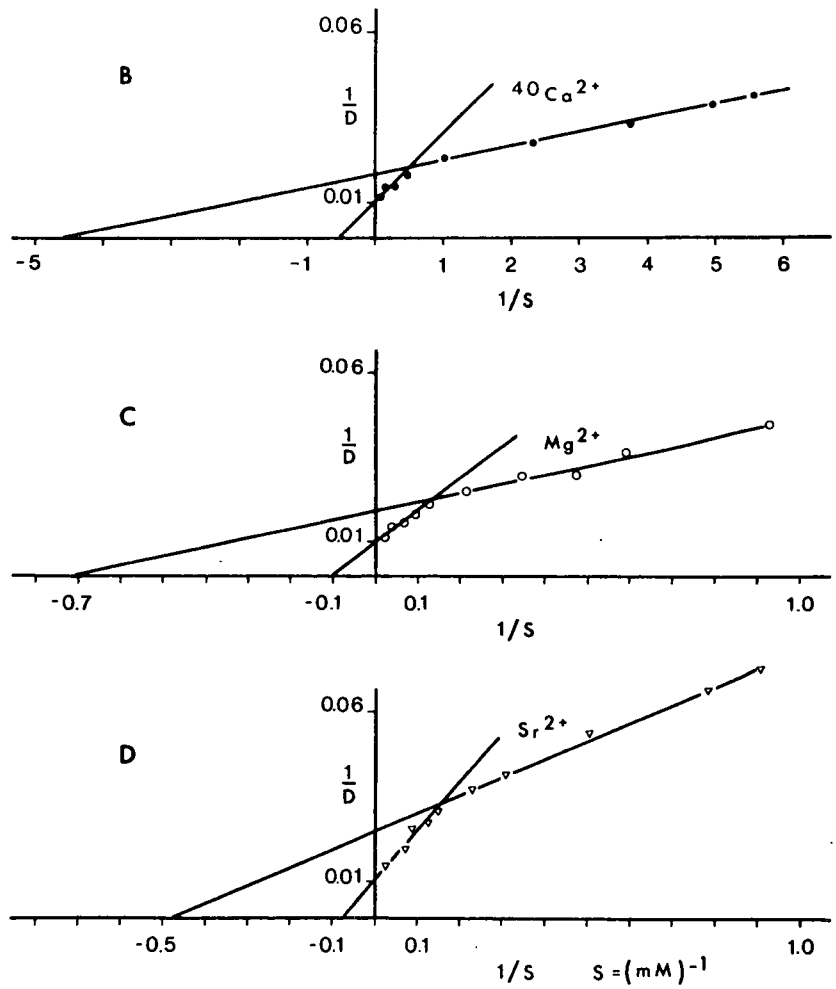


Figure 9B, C and D. Double reciprocal plots for $^{45}Ca^{2+}$ displacement by $^{40}Ca^{2+}$, Mg^{2+}, and Sr^{2+}, respectively. D is expressed as percent $^{45}Ca^{2+}$ displaced and [S] as millimolar divalent cation. These plots were derived from data used in Figure 9A.

TABLE III. Apparent Km's for $^{45}Ca^{2+}$ displacement by $^{40}Ca^{2+}$, Mg^{2+}, Sr^{2+}. The Km's were calculated from the double reciprocal plots presented in Figure 9B-D.

ION	Km_1 (M ± S.E.)	Km_2 (M ± S.E.
$^{40}Ca^{2+}$	$(2.2 \pm 0.3) \times 10^{-4}$M	$(2 \pm 0.2) \times 10^{-3}$M
Mg^{2+}	$(1.4 \pm 0.15) \times 10^{-3}$M	$(9 \pm 1) \times 10^{-3}$M
Sr^{2+}	$(2.2 \pm 0.25) \times 10^{-3}$M	$(1.5 \pm 0.1) \times 10^{-2}$M

within the effective range of the method (10^{-3}-10^{-6}M). The use of $^{45}Ca^{2+}$ as the diffusible molecule, which has a relatively high diffusion rate through the dialysis membrane, made this system sensitive to changes in free $^{45}Ca^{2+}$ in the upper chamber.

The flow dialysis system offers several advantages for Ca^{2+}-binding measurements over equilibrium dialysis and ultrafiltration. It is faster than the equilibrium dialysis method, since each step can be completed within 2 minutes. Washing the membrane, which is necessary in the ultrafiltration method, is avoided here; this eliminates a possible displacement of ^{45}Ca, particularly from the low affinity sites. The continuous monitoring of the $^{45}Ca^{2+}$ diffusion rate gives a clearer picture of the kinetic behavior of the investigated reaction. In studying pH effects, the membranes are exposed to each pH for not more than 2.5 min. Thus denaturation effects by extreme pH's can be minimized. Using the other methods, the exposure to each pH is much longer and denaturation effects cannot be excluded. However in calculating Ca^{2+}-binding using the flow dialysis method two effects must be taken into account: the dilution effect and cpm loss during the experiments. These two factors should be calculated for each step to give the actual control diffusion rate for each step. An excessive nonspecific Ca^{2+}-binding to the dialysis membrane was successfully prevented by raising NaCl concentration up to 100 mM, as suggested by Reed (1973). In contrast to the ultrafiltration method the flow dialysis method (without modfication) is not sensitive enough to measure calcium uptake. Because uptake is a relatively slow process, the effect on the diffusion rate will be small,

and difficult to detect. Another limitation of this method is that it requires high protein concentrations (2-3 mg/ml).

Two types of Ca^{2+}-binding sites can be distinguished on the placental plasma membranes: high affinity sites with a capacity of 26 nmoles per mg protein and $K_s = 3.1 \times 10^{-5}M$, and low affinity sites with a capacity of 266 nmoles per mg protein and $K_s = 1.1 \times 10^{-3}M$. Bovine Albumin which was used as a reference in this study, was also shown to possess two types of Ca^{2+}-binding sites. While the capacity of the high affinity sites for albumin was equal to that of the membranes, the affinity was five-fold lower ($K_{s1} = 1.4 \times 10^{-4}M$). It is tempting to speculate that the higher affinity for Ca^{2+} of the placental plasma membranes may be related to a function in Ca^{2+} transport.

Membranes from several sources show more than one class of calcium-binding sites. The sarcoplasmic reticulum has been most intensively studied with respect to its Ca^{2+}-binding properties, but even with this one type of membrane, different methods have yielded conflicting results. Cohen and Selinger (1969) reported two classes of sites with the same dissociation constant ($K_s = 4 \times 10^{-5}M$), while a more recent study by Chevallier and Butow (1971) revealed three classes of sites with the following dissociation constants: $1.3 \times 10^{-6}M$; $3.2 \times 10^{-5}M$; $3.2 \times 10^{-4}M$.

Despite the difficulties one faces trying to compare Ca^{2+}-binding properties of preparations obtained by the various methods, the existence of high affinity sites is typical of membranes that are involved in the active regulation of intracellular calcium. In addition to the sarcoplasmic reticulum, high affinity sites for calcium are found in the

mitochondria (Reynafarje and Lehninger, 1969) and in cardiac microsomes (Repke and Katz, 1972). Lower affinity sites are found in rat liver plasma membranes (Schlatz and Marinetti, 1972) (K_s = 2.5 x 10^{-4}M and 3.1 x 10^{-3}M), and in red blood cell membranes (Gent et al., 1964) (K_s = 2.8 x 10^{-4}M). The placental plasma membranes, in respect to Ca^{2+}-binding affinity (K_s = 3.1 x 10^{-5}M), ranks with the sarcoplasmic reticulum, mitochondria and cardiac microsomes. It is interesting to note that liver plasma membranes (Schlatz and Marinetti, 1972) have binding sites with a 10-fold higher dissociation constant and a correspondingly lower affinity for calcium than those sites in the placental plasma membranes. It is tempting to attribute this difference in affinity for Ca^{2+} to the fact that the liver plasma membranes, unlike the placental plasma membranes, are not involved in active Ca^{2+}-transport between body compartments.

The pH-dependent Ca^{2+} binding profile by the placental plasma membranes shows a pH optimum of 11.0, and at pH 4.0 there is practically no binding. Up to pH 7.0 the pH profile agrees with that already described for liver plasma membranes (Schlatz and Marinetti, 1972), sarcoplasmic reticulum (Cohen and Selinger, 1969), and muscle microsomes (Carvalho, 1966). Above pH 7-8 a decrease was reported (Cohen and Selinger, 1969; Gent et al, 1964). In the present study no such decrease was observed even above pH 11.0. These differences can be at least partly accounted for by the different methods used. The time of exposure to each pH varied from 40 min (10 min incubation and 30 min centrigugation) (Cohen and Selinger, 1969) up to 42 hr (Shlatz and Marinetti, 1972) using the equilibrium dialysis method. The use of flow dialysis

reduced the exposure time to 2.5 min, minimizing long term pH effects. In addition, no attempt to reverse the pH effect was mentioned in these studies (Cohen and Selinger, 1969; Shlatz and Marinetti, 1972) so that long term effects cannot be excluded.

The affinity of Mg^{2+} and Sr^{2+} for the Ca^{2+} high affinity sites was found to be 10-fold lower than that of Ca^{2+}. The binding of Mg^{2+} and Sr^{2+} was not studied, so that the possibility of specific sites for these ions cannot be excluded. The effect of Na^{+} on Ca^{2+} binding could not be studied in detail since it was necessary to maintain a high Na^{+} concentration to prevent nonspecific Ca^{2+}-binding to the dialysis membrane. However, in several short experiments when NaCl (100 mM) was added after the first step (before measurable amounts of $^{45}Ca^{2+}$ were bound to the dialysis membrane, which is time dependent) (Reed, 1973) no significant changes in $^{45}Ca^{2+}$ diffusion rate could be detected. The more efficient displacement of $^{45}Ca^{2+}$ by Mg^{2+} and Sr^{2+} from the low affinity sites indicates a reduced specificity for Ca^{2+}.

The specificity of Ca^{2+}-binding sites of different membranes shows wide variations. The sites on the liver mitochondria (Reynafarje and Lehninger, 1969) are insensitive to Mg^{2+}, but Sr^{2+} displaces Ca^{2+} quite effectively. The sarcoplasmic reticulum (Cohen and Selinger, 1969) sites are unaffected by Mg^{2+} up to 10^{-2}M, while Sr^{2+} displaces at a level close to that found in this study. The Ca^{2+}-binding sites of another plasma membrane (derived from liver; Sclatz and Marinetti, 1972) are insensitive to K^{+} and Na^{+} while Mg^{2+} reduced Ca^{2+}-binding to the low affinity sites only. The degree of specificity observed in the present

study enables us to conclude that at physiological concentrations of Mg^{2+}, Ca^{2+} and normal levels of Sr^{2+}, practically no binding other than Ca^{2+} to these sites will be detected.

The concept behind this study is that a passive binding of Ca^{2+} to the membrane is an essential first step in the process of an active Ca^{2+} transport. The placental plasma membranes were found to contain sites for Ca^{2+} with capacity, specificity and affinity within the range reported for other membranes involved in active transport of Ca^{2+}. Unlike the sarcoplasmic reticulum and mitochondria, which are exposed to only two different Ca^{2+} concentrations, the placental plasma membranes are exposed to three different Ca^{2+} concentrations. The outer plasma membranes on the maternal side are exposed to a Ca^{2+} concentration of 2.9 mequiv/L, and on the fetal side to 3.8 mequiv/L (Papadopoulos et al, 1967). The inner surface of the membranes is exposed to a low Ca^{2+} concentration, estimated to be around 10^{-5}-10^{-6}M. It is premature to assign the distribution of the two classes of sites to the different surfaces of the placental plasma membranes. However, it should be noticed that the dissociation constants obtained are close to the physiological concentrations of Ca^{2+} to which the membranes are exposed. Although high affinity specific Ca^{2+} sites are present in placental plasma membranes, the direct involvement of these sites in active transport of Ca^{2+} remains speculative at this stage.

SUMMARY

The Ca-binding properties of placental plasma membranes were studied using a flow dialysis system.

Ca-binding was not detectable at pH 4.0, but increased at higher pH's to a maximum binding at pH 11.0.

Two types of Ca-binding sites were identified: high affinity sites with dissociation constant $K_s = 3.1 \times 10^{-5}$M and a capacity of 26 nmoles per mg protein; low affinity sites with $K_s = 1.1 \times 10^{-3}$M and a capacity of 266 nmoles per mg protein.

The affinities of Mg^{2+} and Sr^{2+} for the high affinity sites were 10-fold lower than that of Ca^{2+}, and for the low affinity sites were 4- and 8-fold lower respectively.

The placental plasma membranes contain sites for Ca^{2+} with capacity, specificity and affinity within the range reported for other membranes involved in an active transport of Ca^{2+} (mitochondria, sarcoplasmic reticulum, cardiac microsomes).

DIVISION II

Ca^{2+}-STIMULATED ATPase OF THE GUINEA PIG PLACENTAL PLASMA MEMBRANES

CHAPTER II

CHARACTERIZATION OF CALCIUM-STIMULATED ATPase

INTRODUCTION

It has been proposed that calcium transport across the placenta is an active transport (Papadopoulos et al., 1967). Most energy-utilizing processes are driven by the breakdown of ATP (Dixon and Webb, 1964b). To be effective the hydrolysis of ATP must be enzymatic and coupled with the energy-utilizing process, in this case, calcium transport. The coupling between the two events must take place within the membrane, so that the enzyme hydrolyzing the ATP is fixed in space; this makes possible the coupling between the chemical reaction (ATP hydrolysis) and translocation of the molecule (Curie-Prigogine principle) (Katchalski and Curran, 1967).

Skou (1965) has established the relationship between Na^+ and K^+ fluxes and ATP hydrolysis by the membrane-bound (Na^+, K^+)-activated Mg^{2+}-dependent ATPase (EC 3.6.1.3). Other membrane-bound ATPases activated by Ca^{2+} and Mg^{2+}, and involved in Ca^{2+}-transport, have been described in the red blood cell (Schatzmann and Rossi, 1971) and the sarcoplasmic reticulum (Martonosi and Feretos, 1964).

In this chapter the properties of such a (Ca^{2+}, Mg^{2+})-activated ATPase located in the placental plasma membranes are described, and compared with the properties of other (Ca^{2+}, Mg^{2+}) ATPases.

MATERIALS AND METHODS

Histochemical Localization of Ca^{2+}-ATPase in the Placenta

The placenta, after removal, was frozen (to conserve enzymatic activity) and sectioned (20μ). The sections were incubated for an hour in 20 mM Tris-HCl buffer pH 8.5, 30 mM $CaCl_2$ and 12 mM ATP. The sections were stained as described by McManus et al (1960). The staining method is based on precipitating the released Pi as calcium phosphate, replacing the calcium with cobalt by washing with 2% cobalt acetate and replacing the Pi with sulfide by placing the sections for one minute in 2% aqueous yellow ammonium sulfide. The result is that the ATPase sites are stained brown-black by the precipitate of cobalt-sulfide. Though this method is not specific for Ca^{2+}-ATPase, the presence of high Ca^{2+} concentration (30 mM) and 12 mM ATP in the incubation medium make the contributions of other phosphatases to ATP hydrolysis negligible.

ATP Hydrolysis by the Placental Plasma Membranes

To test the various enzymatic properties, 0.1 ml of membrane suspension containing 10-20 μg of protein, was incubated with 1.0 ml of solutions containing calcium or magnesium in appropriate amounts (0.1 to 10 mM); 20 mM Tris-HCl buffer (pH 8.2) and 70 mM Na^+ (as NaCl); Na_2ATP (Sigma) was added to make 5 mM. Blank specimens did not contain

bivalent cations. The samples were incubated for 30 min in a Dubnoff shaking water bath at 37°C. The reaction was terminated by plunging the tubes into an ice-water bath and adding 1.0 ml 10% (W/V) trichloroacetic acid. The rate of ATP hydrolysis was determined by measuring the amount of Pi released from the samples. Using the AutoAnalyzer inorganic phosphate was measured by GOMORI (1942) method, total protein by the Lowry et al. (1951) procedure. Results are expressed as μmole Pi released per mg of protein in 30 min. Every value of the various experiments was the mean of triplicate samples and every whole experiment was repeated at least three times.

The following compounds were obtained from the sources indicated:

1. Na_2ATP (Sigma No. A-3127);
2. Tris-ATP [Di-Tris (hydroxymethyl)-amino-metane salt];
3. ITP-Inosine 5'-triphosphate sodium salt (Sigma No. I-5000);
4. GTP-Guanosine 5'-triphosphate sodium salt (Sigma No. G-8752);
5. ADP-Adenosine 5' diphosphate disodium salt (Sigma No. A-0127);
6. AMP-Adenosine 5'-monophosphoric acid sodium salt (Sigma No. A-1877);
7. EDTA-disodium ethylenediamine-tetraacetate (Fisher S-311);
8. EGTA - ethyleneglycol-bis-(-aminoethyl ether)n, n'-tetraacetic acid (Sigma No. E-3251);
9. Mersalyl acid (Sigma No. M-8125);
10. Oligomycin (Sigma No. O-1295);
11. Ruthenium Red (K and K Laboratories No. 2603-A); and
12. Maleic anhydride (Fisher A-168).

RESULTS

Histochemical Localization of ATPase Activity in the Placenta

Plate 5 shows clearly that the ATPase activity (black-brown staining) is localized in the trophoblastic layer between the maternal and the fetal circulations.

Distribution of Ca^{2+}-ATPase Activity

The Ca^{2+}-ATPase activity of the different fractions (see Chapter I for definition of the fractions) was assayed in the presence of 5 mM Ca^{2+} under standard conditions as described in the Methods section. The distribution was as follows: supernatant, 1.3; final preparation, 16.8; and pellet, 15.0 (μmoles Pi/mg protein per 30 min). This follows the distribution of alkaline phosphatase and glucose-6-phosphatase (Table I), with the same ratio (1:1) between the activity in the final preparation and the pellet. These results indicate that the three phosphatases are located in the membranes of the microsomal fraction.

Activation of the Enzyme by Divalent Cations

Activation of the enzyme by calcium ions was assessed by varying the calcium concentration in the incubation medium from 0.02 to 10 mM. Figure 10 depicts the rate of Pi production at the various calcium concentrations. The apparent K_m for Ca^{2+} of placentas from 5 different guinea pigs was 0.26 $\pm$ 0.01 mM (m$\pm$SEm) (Figure 11). The rate of Pi release at peak activity ranged from 15.0 to 22.0 μmole Pi per mg protein in 30 min.

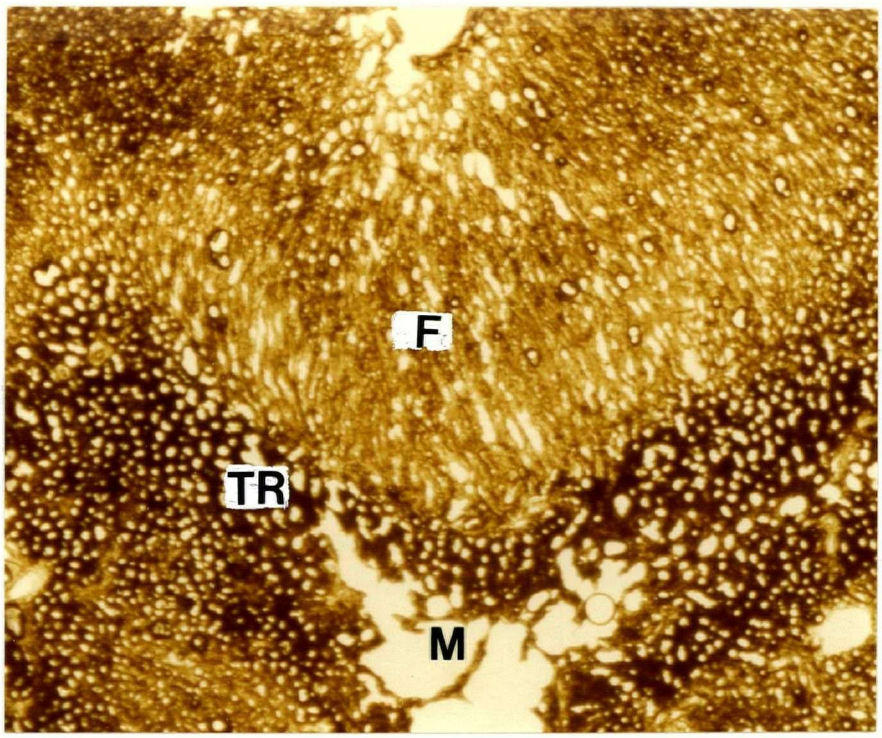


Plate 5. Histochemical localization of ATPase activity in the guinea pig placenta. F = fetal circulation, M = maternal circulation, and TR = trophoblast. The brown staining indicates ATPase activity.

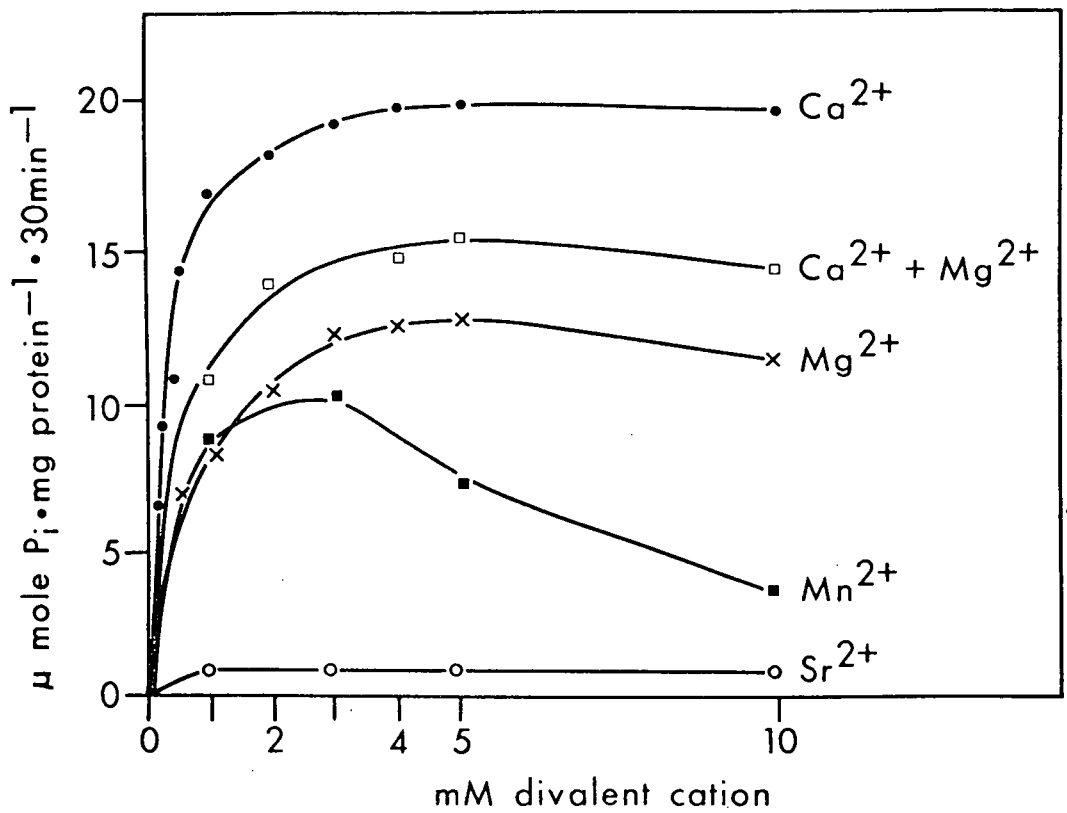


Figure 10. Stimulation of ATP hydrolysis by divalent cations. Unless indicated incubation fluids contained 70 mM Na^+ (as NaCl), 20 mM Tris-HCl (pH 8.2) and 5 mM Na_2ATP. ●——● activation by Ca^{2+}; □——□ $Ca^{2+} + Mg^{2+}$ (in equimolar concentrations); X —— X Mg^{2+}; ■——■ Mn^{2+}; O —— O Sr^{2+}.

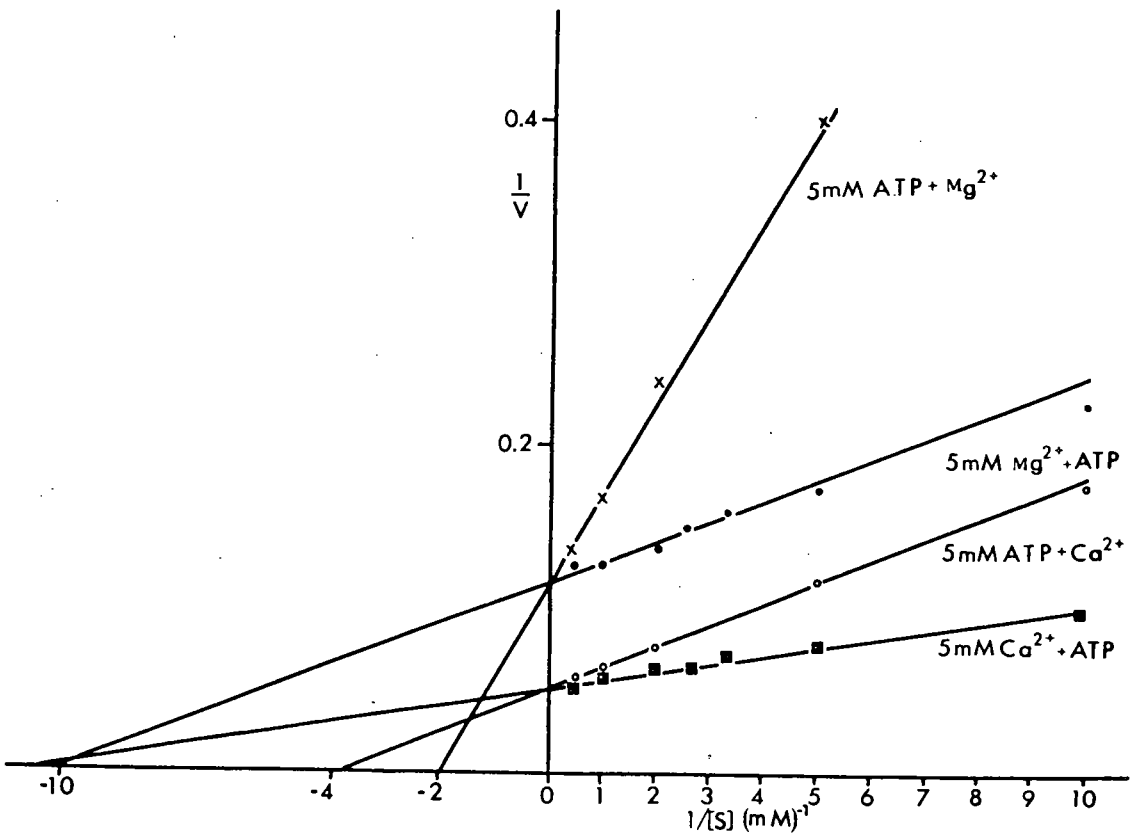


Figure 11. Variations in Lineweaver-Burk plots of ATPase activity at various concentrations of ATP, Ca^{2+} and Mg^{2+}. V in μmole Pi formed per mg protein in 30 min [S] in mM. The incubation mixture contained 20 mM Tris HCl (pH 8.2), 70 mM Na^{+}.

X —— X 5 mM ATP + Mg^{2+}

O —— O 5 mM ATP + Ca^{2+}

● —— ● 5 mM Mg^{2+} + ATP

■ —— ■ 5 mM Ca^{2+} + ATP

In the absence of calcium, magnesium ion also activated the enzyme but always less effectively than calcium (Figure 10). The apparent K_m for magnesium was 0.56 $\pm$ 0.03 mM (m$\pm$SEm) (Figure 11). When instead of the single cation a 1:1 combination of Ca^{2+} + Mg^{2+} was used, the resultant curve lay between the two curves obtained from incubation with Ca^{2+} or Mg^{2+} alone (Figure 10).

The effect of other divalent cations (Mn^{2+}, Sr^{2+}) on enzyme activation was also tested. Figure 10 shows that manganese activated the enzyme but strontium did not. Maximal activation of the enzyme with Mn^{2+} occurred at 2 mM, higher concentrations producing inhibition.

Activation of enzyme by Ca^{2+} and Mg^{2+} was further tested by using a constant Ca^{2+} concentration (5 mM) and adding increasing amounts of Mg^{2+}, as well as by using a constant Mg^{2+} concentration (5 mM) and adding increasing amounts of Ca^{2+} (up to 8 mM). The addition of Ca^{2+} to 5 mM Mg^{2+} led to increased stimulation, whereas the addition of Mg^{2+} to 5 mM Ca^{2+} led to inhibition of the enzyme. Figure 12 gives the curve so obtained. The point of intersection of the two curves was at 5 mM Ca^{2+} + 5 mM Mg^{2+}.

The Effect of Incubation Time on the Concentration of H^+ and Pi in the Medium

Frequent recording of pH and Pi release (Figure 13), revealed that during 4 hrs of incubation the pH dropped only 0.1 pH unit, and Pi release was linear. During the first 30 min the drop in pH was very small indicating that the selection of Tris as buffer was appropriate.

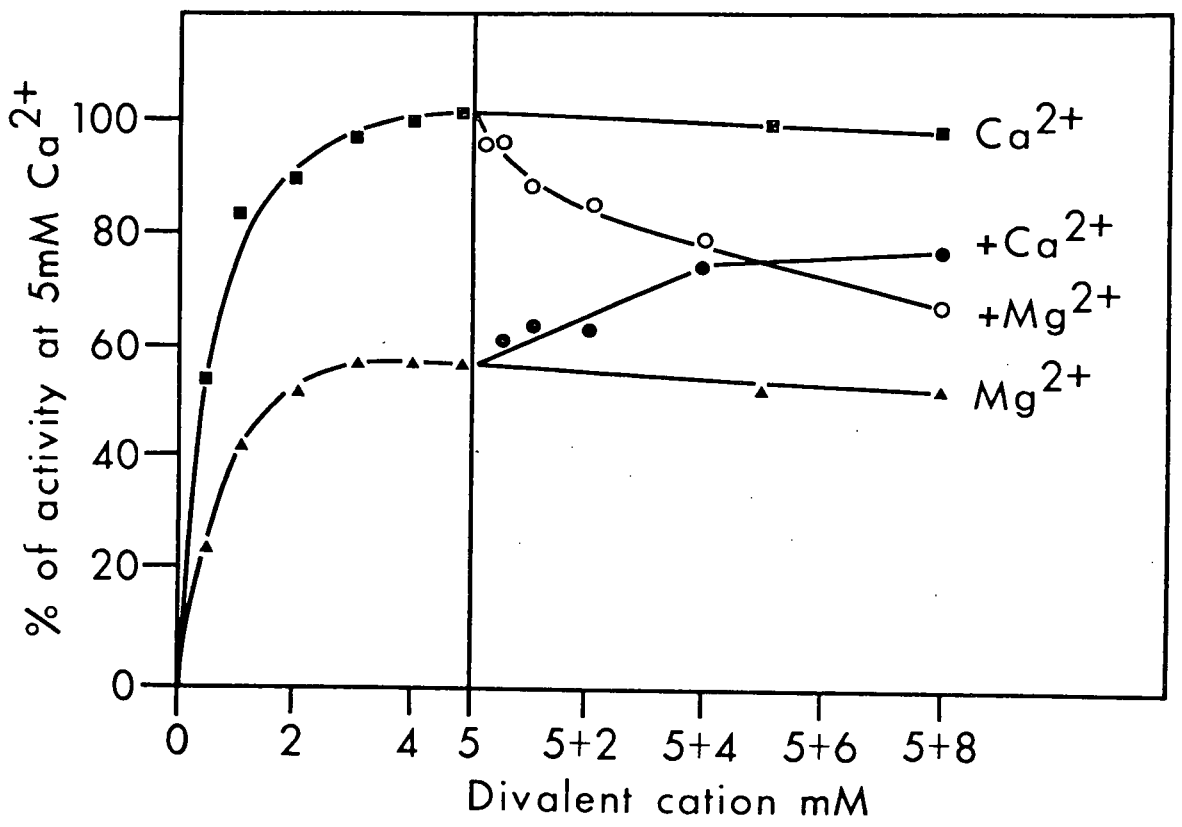


Figure 12. Enzyme activation by Ca^{2+} and Mg^{2+}.

○——○ Constant Ca^{2+} (5 mM) + varying Mg^{2+} concentrations.

●——● Constant Mg^{2+} (5 mM) + varying Ca^{2+} concentrations.

■——■ Ca^{2+} alone

▼——▼ Mg^{2+} alone

Incubation fluids contained 20 mM Tris HCl (pH 8.2) 70 mM Na^{+} (as NaCl) and 5 mM Na_2ATP.

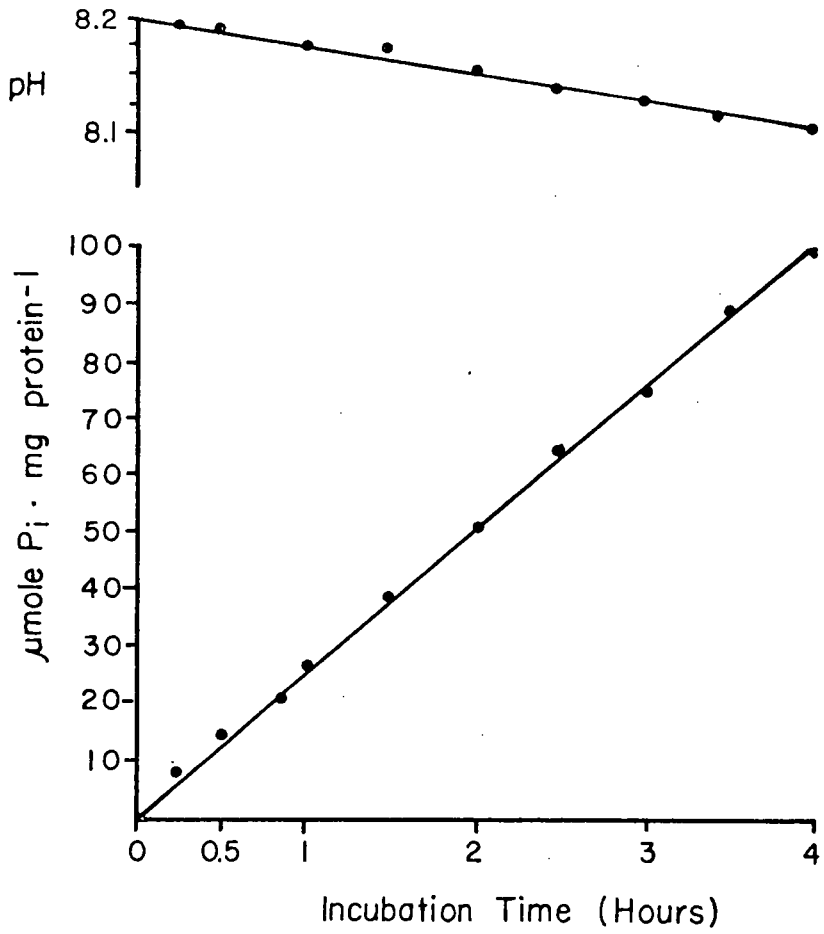


Figure 13. The effect of incubation time on Pi release and pH of the incubation medium. The incubation fluid contained 5 mM Ca^{2+}, 70 mM NaCl, 5 mM Na_2ATP and 20 mM Tris-HCl (pH 8.2).

Protein Concentration Effect on Pi Release

Inorganic phosphate release was linear within the protein concentration range tested 5-60μg/ml incubation fluid (Figure 14).

Sodium Independence of the Enzyme

The requirement of sodium ions for enzyme activation was tested by incubating the enzyme in 0 or 70 mM Na and adding Tris-ATP instead of Na_2ATP. Specimens incubated without sodium showed slightly higher activation than those incubated with sodium (Figure 15).

The Effect of pH on ATP hydrolysis

The calcium and magnesium activation curves of the enzyme were obtained by incubating the enzyme preparation with 0 or 5 mM Ca^{2+} or Mg^{2+} at a pH ranging from 6.5 to 10.7. In each instance the pH was adjusted both in the incubation fluid and Na_2ATP solutions and determined before and after incubation. Figure 16 shows the curves obtained. The pH optimum of Ca^{2+}ATPase was between 8.2 and 8.5. At pH 7.1 and 9.6 the enzyme was stimulated by calcium ions to only 50% of peak activity. The pH optimum of Mg^{2+}ATPase was between 8.2 and 9.3 with approximately 60% of the activation by Ca^{2+}. Below pH 7.0 and above pH 9.7, Mg^{2+} produced higher activation than Ca^{2+}.

Inhibitors

The effects of different inhibitors on calcium activation of the enzyme were tested. Ouabain (1 mM) was added to the solutions

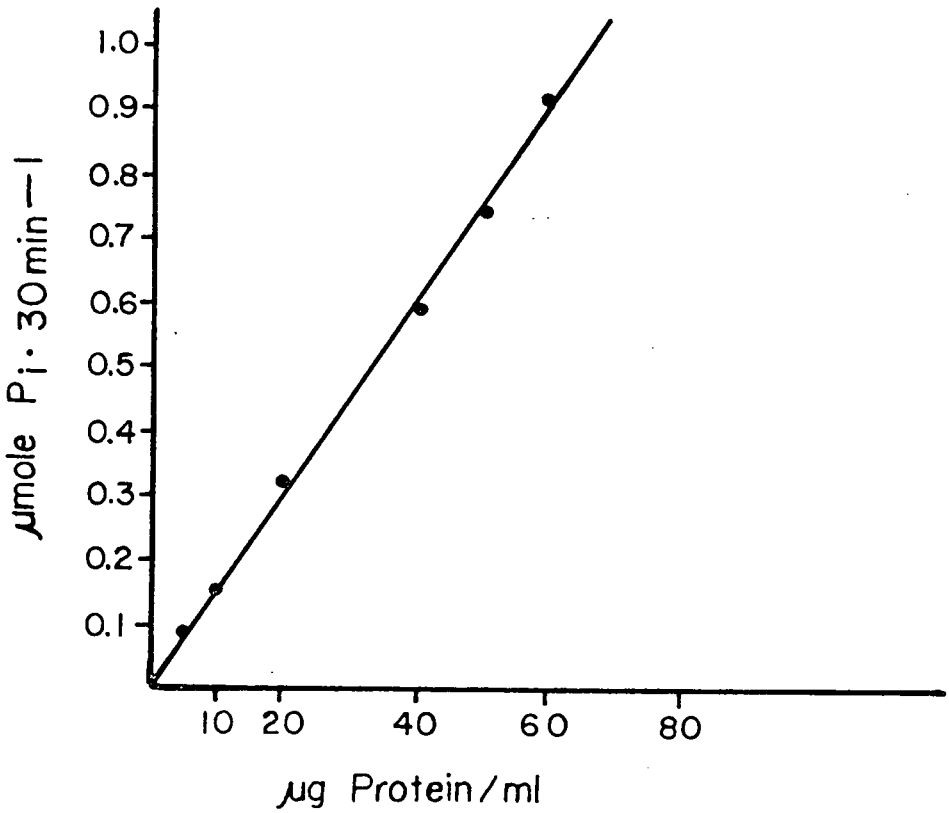


Figure 14. Protein concentration effect on Pi release. The incubation medium contained 5 mM ATP, 20 mM Tris-HCl (pH 8.2) and membrane protein at the concentrations as indicated.

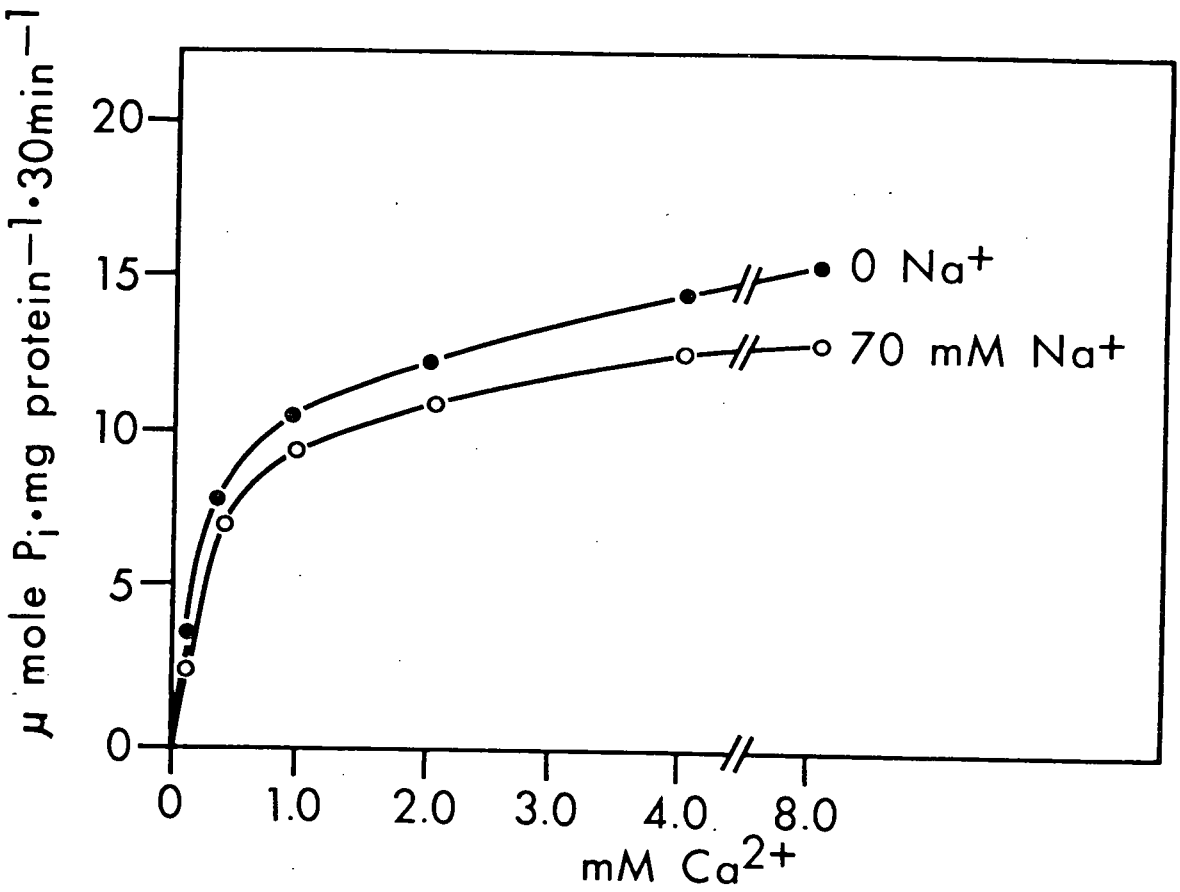


Figure 15. Effect of Na^+ on enzyme activation by Ca^{2+}. ●——● without Na^+, ○——○ with 70 mM Na^+. Incubation medium contained 20 mM Tris-HCl (pH 8.2), and 5 mM Tris-ATP.

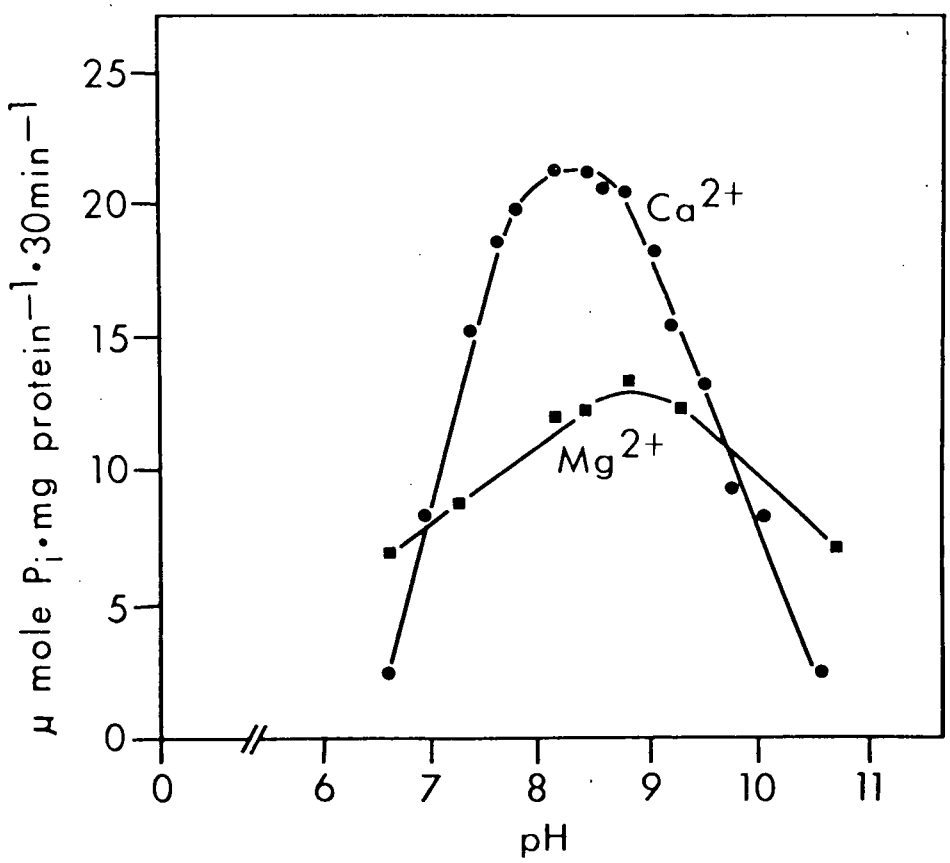


Figure 16. Effect of pH on enzyme activity. Incubation solutions contained 5 mM Ca^{2+}, 20 mM Tris HCl, 70 mM Na^{+} and 5 mM Na_2ATP.

●——● pH effect with 5 mM Ca^{2+}

■——■ pH effect with 5 mM Mg^{2+}

containing 0 or 5 mM calcium, 20 mM Tris HCl buffer (pH 8.2), 70 mM Na^+ and 5 mM Na_2ATP. Figure 17 shows that ouabain did not inhibit the enzyme.

Ethacrynic acid, added to the samples to produce concentrations between 0.15 and 5.0 mM, inhibited enzyme activity by Ca^{2+} (Figure 17); 5.0 mM ethacrynic acid producing 55% inhibition.

When the enzyme was preincubated at 37° with 1 mM mersalyl, time-dependent inhibition was shown (Fig. 18A-a). The inhibitory effect of mersalyl was then tested with a concentration range of 10^{-5}M to 10^{-2}M. The resulting inhibition curve (Figure 18A-b) shows three stages of inhibition with a fall-off in activity between 10^{-3}M and 10^{-2}M. The effect of 1 mM mersalyl on Ca^{2+} activation of the enzyme (Figure 18B) showed the V_{max} to be 80% of the control and K_m remaining unchanged (Figure 18C).

When Na_2H_2EDTA was added in increasing amounts to the incubation solutions containing 5 mM Ca^{2+}, the Ca^{2+}-sensitive ATPase activity decreased as shown in Figure 19. The increase in degree of chelation of calcium ions led to decrease in enzyme activity; the enzyme activation by Ca^{2+} could be inhibited completely by the addition of 5 mM Na_2H_2EDTA. However, the inhibition obtained with EDTA was greater than expected from the non-chelated Ca^{2+} activation curve.

EGTA, a specific chelator of Ca^{2+}, was used to inhibit the enzyme. The inhibition curve was a symmetrical mirror image of the Ca^{2+} activation curve of the enzyme (Figure 20A). When the incubation medium contained equimolar concentrations of Ca^{2+}, Mg^{2+}, and EGTA, EGTA led to a decrease in the activity to a level similar to that obtained by Mg^{2+} alone (Figure 20B). Increasing the EGTA concentration to 10 mM did not cause further inhibition and the Mg^{2+} activation of the enzyme was not affected.

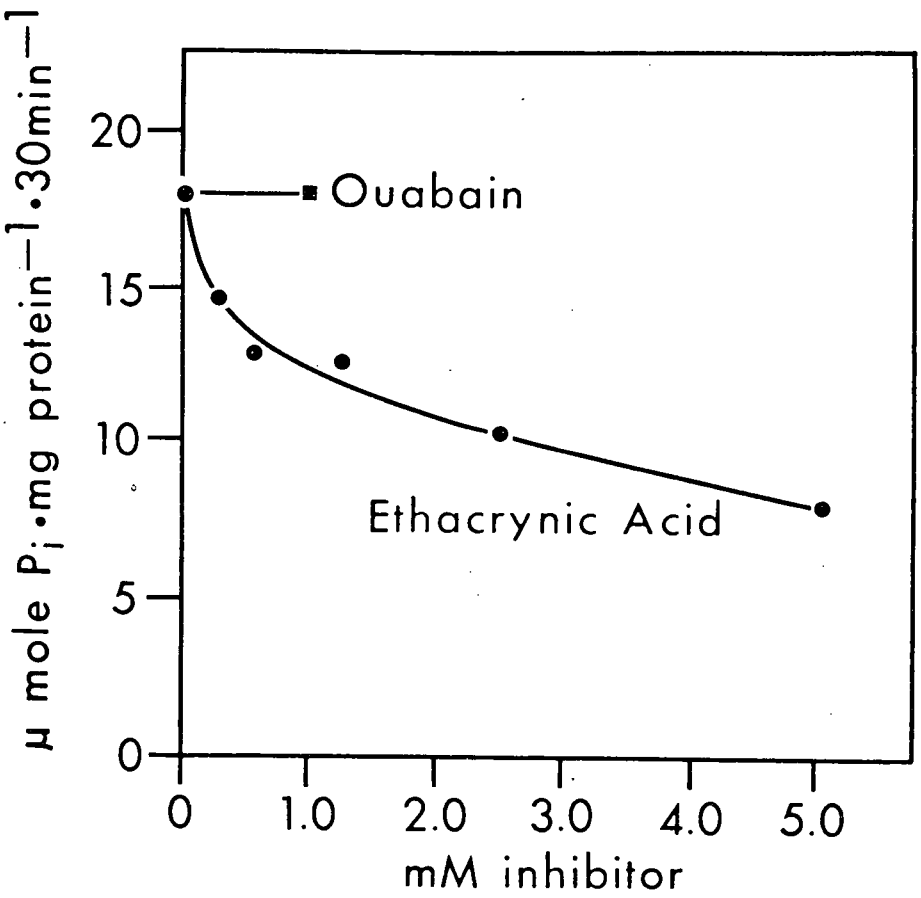


Figure 17. Effect of inhibitors. Incubation contained 5 mM Ca^{2+}, 70 mM Na^+, 20 mM Tris HCl (pH 8.2) and 5 mM Na_2ATP. ■——■ Ouabain, ●——● Ethacrynic acid.

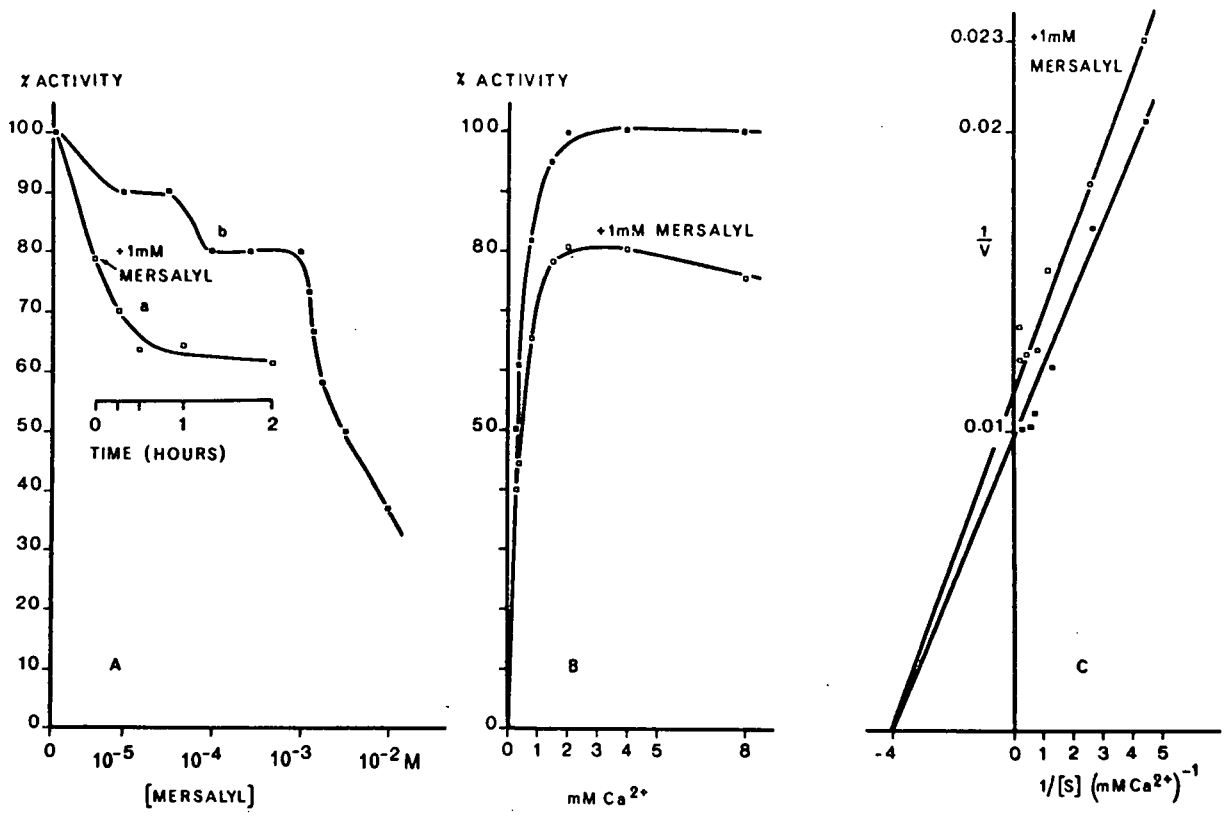


Figure 18. Effect of mersalyl on Ca^{2+} activation of the enzyme. Incubation media contained 70 mM NaCl, 20 mM Tris-HCL (pH 8.2), 5 mM Na_2ATP and Ca^{2+} and mersalyl as indicated. Activity is expressed as % of Pi production with 5 mM Ca^{2+}.

A-a: □——□ effect of preincubating the enzyme with 1 mM mersalyl on the V_{max} obtained with 5 mM Ca^{2+}. The Na_2ATP was added at the time indicated; ATP hydrolysis was determined after 30 min of further incubation.

A-b: ■——■ effect of increasing mersalyl concentrations on the V_{max} of the enzyme incubated with 5 mM Ca^{2+}.

B: Effect of 1 mM mersalyl on Ca^{2+} activation of the enzyme, ■——■ control, □——□ + mersalyl.

C: Lineweaver-Burk plot of the effect of 1 mM mersalyl on Ca^{2+} activation of the enzyme. ■——■ control, □——□ with 1 mM mersalyl. V is expressed as percent Pi production with 5 mM Ca^{2+}.

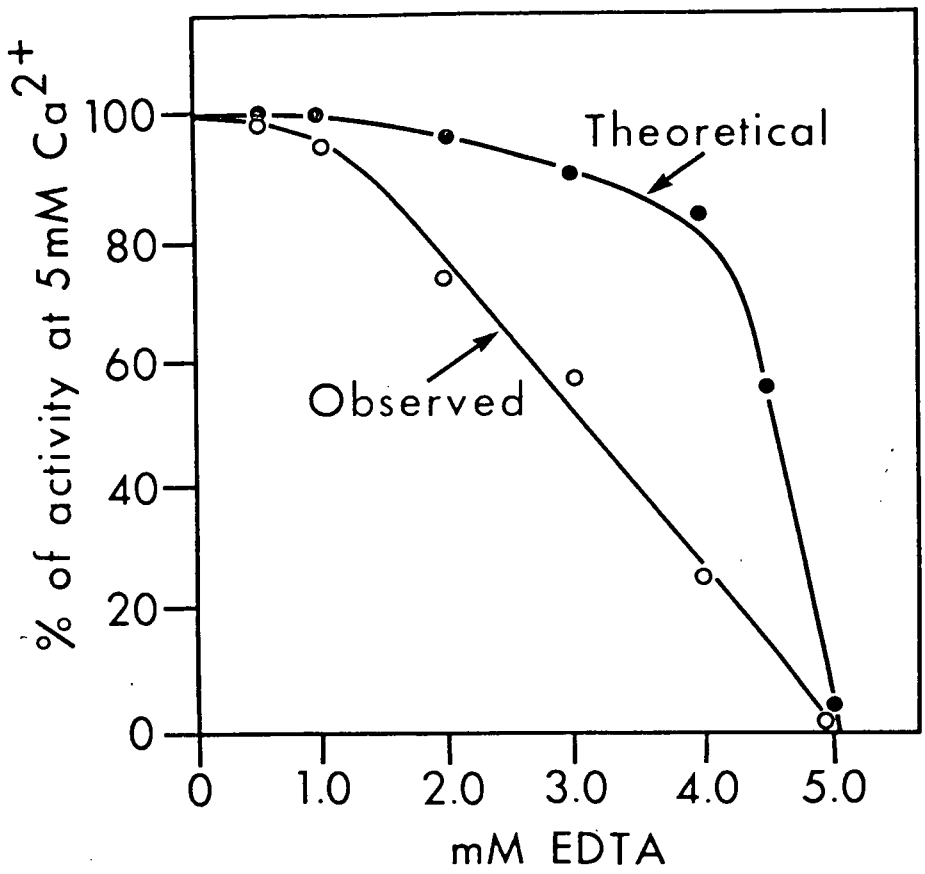


Figure 19. Effect of EDTA on enzyme activation by Ca^{2+}, O —— O "Observed" curve obtained from incubation with 5 mM Ca^{2+}, 20 mM Tris-HCL (pH 8.2), 70 mM Na^{+} and 5 mM Na_2ATP. ●——● "Theoretical" curve obtained with Ca^{2+} concentrations in the incubation fluid corresponding to the non-chelated Ca^{2+} of the "observed" curve.

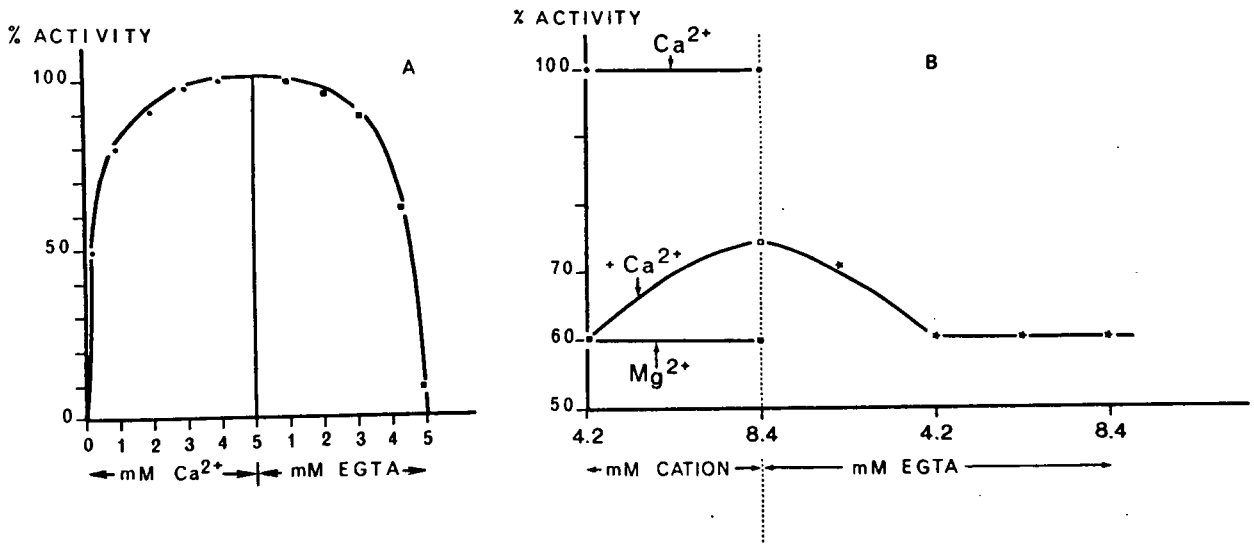


Figure 20. EGTA effect on Ca^{2+} and Mg^{2+} activation of the enzyme.

A: ●——● activation of the enzyme by Ca^{2+}, ■——■ activity obtained when the incubation medium contained 5 mM Ca^{2+} plus increasing amounts of EGTA.

B: ●——● activation by Ca^{2+}; ■——■ activation by Mg^{2+} alone; □——□ activation by 4.2 mM Ca^{2+} + 4.2 mM Mg^{2+}; ✱——✱ activation by (4.2 mM Ca^{2+} + 4.2 mM Mg^{2+}) + EGTA.

Complete modification of amino groups on the enzyme with 25 mg maleic anhydride per mg of protein was carried out as described by Freedman et al. (1968). This modification led to complete inactivation of the enzyme (Figure 21A). When the enzyme was treated with 1 mg maleic anhydride per mg protein the V_{max} was 70% of the control (Figure 21A) but the K_m was unchanged (Figure 21B).

Ruthenium red (prepared by the method of Luft (1971)) in a concentration range of 10^{-6}M to 10^{-4}M did not inhibit the Ca^{2+} or the Mg^{2+} activation of the enzyme.

Oligomycin in a final concentration of 1 µg/ml also did not inhibit the enzyme.

Substrate Specificity

The substrate specificity of the enzyme was tested by adding increasing amounts (up to 5 mM) of Na_2GTP, Na_2ITP, Na_2ADP or AMP to the incubation fluid plus enzyme and comparing the Pi release to that produced from Na_2ATP. When the Na_2ATP concentration was varied between 0.1 and 5 mM, maximal calcium activation occurred at 5 mM (Figure 22) with an apparent K_m of 0.08 mM-0.10 mM. The two other triphosphates and ADP also served as substrates, but GTP and ADP gave a greater K_m (0.38 and 0.15, respectively), whereas the apparent K_m of ITP (0.08 mM) was the same as that for ATP. No change in the K_m for Na_2ATP (0.08-0.1 mM) was obtained when 5 mM Ca^{2+} was replaced by 5 mM Mg^{2+} (Figure 11) but the V_{max} with ATP was higher. AMP was not hydrolyzed at all.

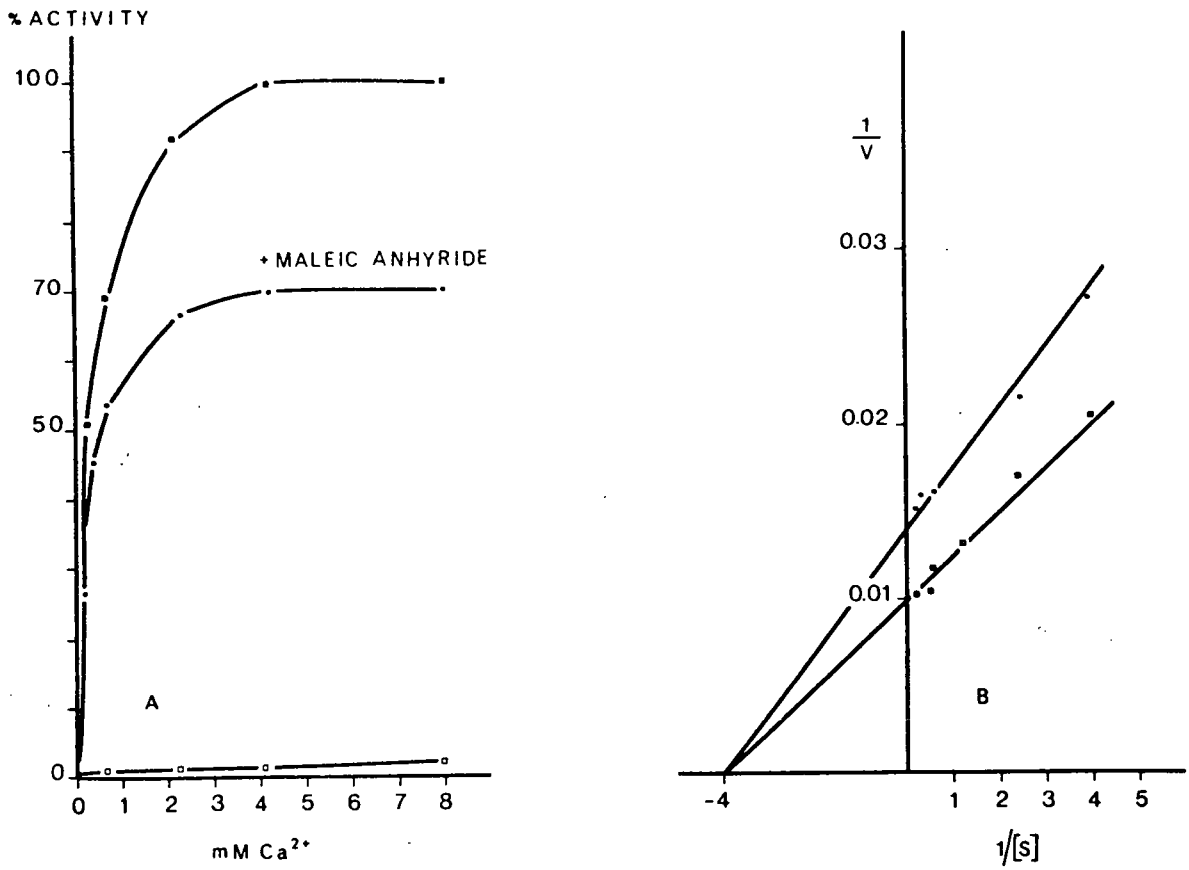


Figure 21. The effect of blocking NH_3^+ groups with maleic anhydride. Incubation media contained 70 mM NaCl, 20 mM Tris-HCl (pH 8.2), 5 mM Na_2 ATP, 5 mM $CaCl_2$ and maleic anhydride as indicated.

A: ■——■ activation by Ca^{2+}; ●——● effect of 1 mg maleic anhydride per mg protein on Ca^{2+} activation of the enzyme; □——□ 25 mg maleic anhydride per mg protein.

B: Lineweaver-Burk plot of the effect of 1 mg maleic anhydride per mg protein on Ca^{2+} activation of the enzyme. ■——■ control, ●——● with maleic anhydride; 1 µg/mg protein.

V is expressed as percent of Pi production with 5 mM Ca^{2+}; and S in mM Ca^{2+}.

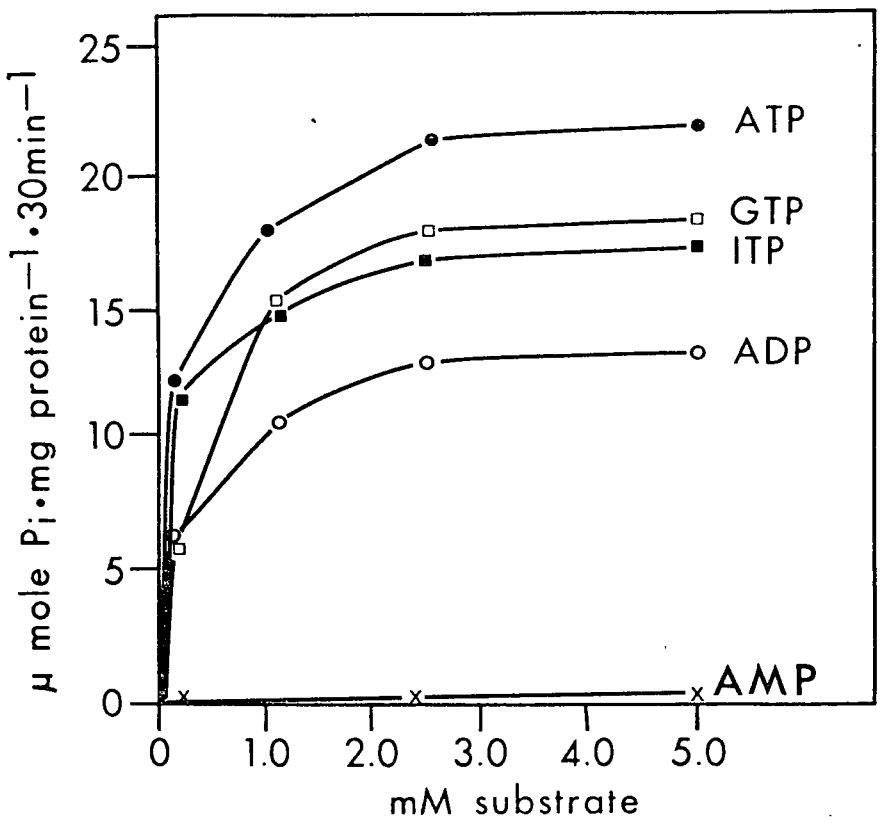


Figure 22. Substrate specificity: Hydrolysis of ATP compared to that of other high energy tri- and diphosphate nucleotides.

●——● ATP: □——□ GTP:

■——■ ITP: 0 —— 0 ADP: X——X AMP.

Incubation fluid contained 5 mM Ca^{2+}, 70 mM Na^{+}, 20 mM Tris-HCl (pH 8.2).

Temperature Effect on ATP Hydrolysis

Samples were incubated at temperatures in the range 1°C to 80°C in 5 mM Ca^{2+}, 20 mM Tris-HCl (pH 8.2), and 5 mM Na_2ATP. ATP hydrolysis was stimulated at temperatures up to 50°C, levelled between 50°C and 70°C, and dropped sharply between 70°C and 80°C (Figure 21A). The Q_{10} values (calculated according to the method of Giese (1968) decreased from 1.9 for the range 1°C-11°C to 1.3 for the 27-37°C range and became 0.15 above 70°C (Figure 23B). The arrhenius plot (Figure 23B) revealed that the activation energy of ATP hydrolysis was 6.31 KCal/mole between 1°C-45°C.

Stability of the Enzyme

A decrease of only 10% in enzyme activity was noted after two months of storage at 4°C. Freezing the samples resulted in rapid loss of activity.

DISCUSSION

A Ca^{2+}-stimulated ATPase is found in several tissues in which active transport of calcium is thought to occur, such as the intestinal mucosa (Martin et al., 1969) and renal tubules (Parkinson and Radde, 1971). Calcium-sensitive ATPases have also been described in other tissues in which calcium ions are needed for specific functions, such as sarcoplasmic reticulum (MacLennan, 1970), brain and nerve tissue (Berl and Puszkin, 1970; Nakamaru et al., 1967). Another Ca^{2+}-sensitive ATPase has been characterized in the red cell (Schatzmann and Vincenzi, 1969; Cha et al., 1971).

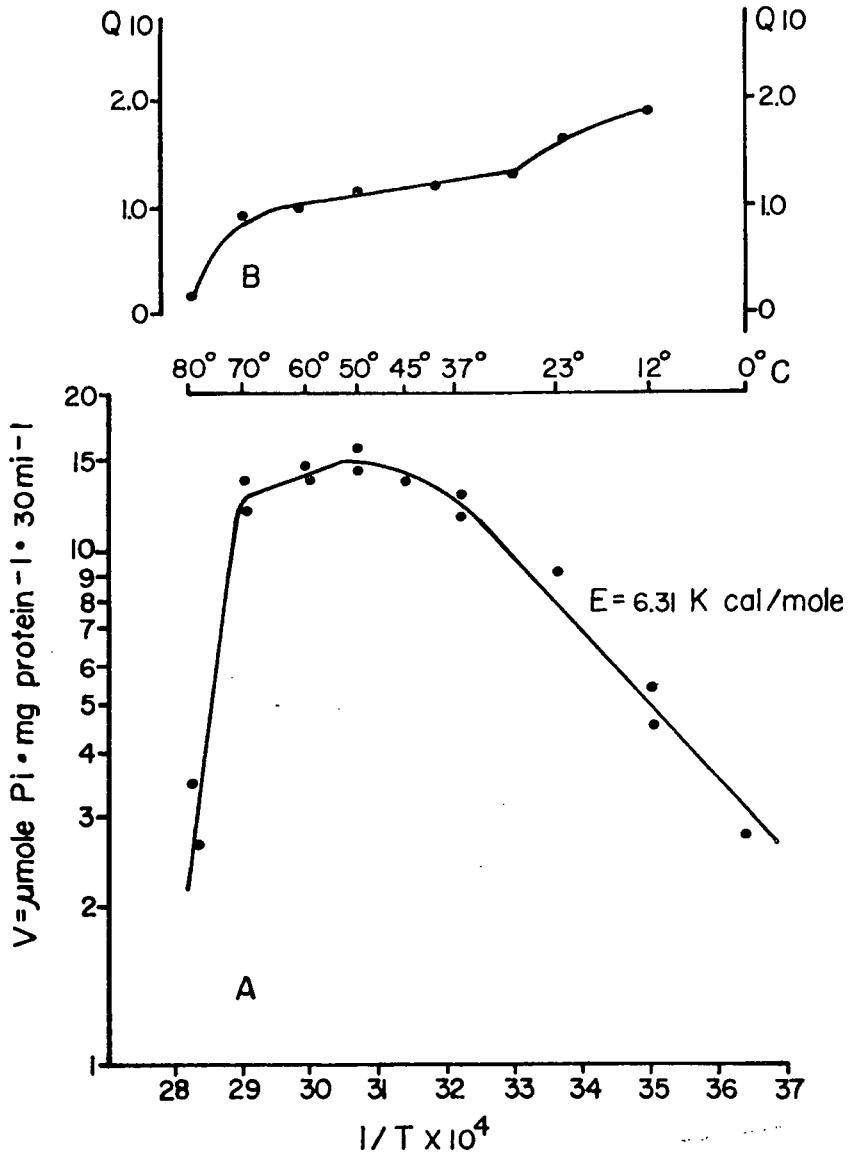


Figure 23. Temperature effect on enzyme activity.

A - Arrhenius plot for ATPase activity

B - Q_{10} at different temperatures.

This chapter describes such an enzyme isolated from the guinea pig placenta. Histochemically the placental Ca^{2+}-ATPase was localized in the trophoblastic layer, and more specifically in the microsomal fraction, as concluded from the distribution of enzyme markers. The enzyme appears to be membrane bound as indicated by the low activity in the 35,000 x g supernatant.

The common properties of the various Ca^{2+} Mg^{2+} ATPases are that they do not require Na^{+} or K^{+} for activation (MacLennan, 1970; Berl and Puszkin, 1970), and that ouabain does not inhibit their activity. This is also true for the placental Ca^{2+}-ATPase. However, the cation requirements differ for each ATPase. In sarcoplasmic reticulum, whereas Mn^{2+} can substitute for both Mg^{2+} and Ca^{2+}, Sr^{2+} can substitute for Ca^{2+} but not for Mg^{2+} (MacLennan, 1970). In the erythrocyte (Schatzmann and Vincenzi, 1969; Rosenthal et al., 1970) the enzyme is stimulated by Ca^{2+} and inhibited by Mg^{2+}. In the kidney (Parkinson and Radde, 1971) and intestinal mucosa (Martin et al, 1969), Mg^{2+} always stimulates the enzyme more than Ca^{2+} but either ion could be substituted for the other. Brain Ca^{2+} ATPase (Berl and Puszkin, 1970; Nakamaru et al., 1967) can be stimulated equally well by Ca^{2+} and Mg^{2+}.

Calcium ions are the preferential cations for stimulation of the placental Ca^{2+}-ATPase. Only 60% of maximal activity produced by Ca^{2+} ions can be achieved by substituting 5 mM Mg^{2+} for 5 mM Ca^{2+}. However, there is no essential requirement by the enzyme for either, and both produce stimulation. Maximal activation produced by either ion can be modified by the other ion. Thus, an inhibition was produded by adding increasing concentrations of Mg^{2+} to 5 mM Ca^{2+}, and stimulation

by adding increasing concentrations of Ca^{2+} to 5 mM Mg^{2+}. It seems therefore, that there is competition between Ca^{2+} and Mg^{2+} for the active sites, and since the affinity and the V_{max} of Ca^{2+} is higher than that of Mg^{2+}, any combination of these two ions will give lower activity than Ca^{2+} alone, but higher than with Mg^{2+} alone.

The role of the divalent cations in this enzyme system is believed to be the production of a divalent ion - ATP complex (Melancon and Deluca, 1970; Hyde and Rimai, 1971) which serves as a substrate for the enzyme. Although the same K_m for Na_2ATP was obtained by replacing 5 mM Ca^{2+} with 5 mM Mg^{2+}, the V_{max} for Mg^{2+} ATPase was only 60% of the V_{max} for Ca^{2+} ATPase. This finding indicates that the Ca^{2+} ATP-complex is the preferential substrate. The kinetics of the competition between Ca^{2+} and Mg^{2+} will be described in the next chapter.

The optimal pH for the Ca^{2+}-activated ATPase of the placenta also differs from that for similar enzymes in other tissues. For example, it ranges from 7.5 in sarcoplasmic reticulum (Martonosi and Feretos, 1964) to pH 9.0 in brain (Nakamaru et al, 1967). Although the enzyme was not isolated, the narrow pH curve suggests that only a few or perhaps a single enzyme is active. Since the optimal pH of this enzyme is 8.2, it is unlikely that an alkaline phosphatase is activated, whose optimum is at 10.3 in guinea pig placenta (Manning et al, 1970). A more detailed comparison with alkaline phosphatase will be given later in the thesis.

There are contradictory reports in the literature about substrate specificity for (Na^+, K^+) ATPase. Some claim that ATP is the only substrate, while others showed hydrolysis of GTP and ITP as well. These

results have been reviewed by Whittam and Wheeler (1970). A more recent study by Watson et al. (1971a) on the red blood cell (Ca^{2+} + Mg^{2+})-ATPase suggests that other nucleotides cannot serve as substrate.

In this study ATP was the preferential substrate, but GTP, ITP, and ADP were also hydrolyzed to a great extent. Similar results were obtained for other Ca^{2+}-ATPases from plasma membranes of bacteria (Davies and Bragg, 1972; Mirsky and Barlow, 1971), kidney (Parkinson and Radde, 1971), gills (Ma et al., 1974), and to a certain extent in the sarcoplasmic reticulum (MacLennan, 1970).

The absence of substrate specificity makes it likely that this enzyme is a general triphosphatase with ATP as the preferential substrate, and this is the main justification for calling the enzyme a Ca^{2+}-ATPase.

The search for a specific inhibitor of Ca^{2+}-ATPase has thus far been unsuccessful. Ouabain (Berl and Puszkin, 1970) and oligomycin (Dunham and Glynn, 1961), specific inhibitors of (Na^{+} + K^{+})-ATPase, have no effect on Ca^{2+}-ATPases, and so it is in this study. Ruthenium red, an inorganic dye, has been shown to inhibit selectively Ca^{2+}-ATPase of the red blood cell (Watson et al., 1971b); it had no effect on the placental Ca^{2+}-ATPase. Some non-specific inhibitors of enzyme activity such as ethacrynic acid (Vincenzi, 1968) and mersalyl (Schatzmann and Vincenzi, 1969) also inhibit Ca^{2+}-ATPase.

The inhibition obtained with ethacrynic acid suggests that there is an SH group in or near the active center of the enzyme, since ethacrynic acid is believed to block SH groups (Davis, 1970). This is similar to its action in the kidney where it inhibits $Na^{+}K^{+}$-ATPase to cause

diuresis (Davis, 1970; Duggan and Noll, 1965). The essentiality of SH-groups for Ca^{2+}-ATPase and Ca^{2+}-transport has been demonstrated in sarcoplasmic reticulum (Hasselbach, 1966a) by using other SH-blockers. In the red blood cell both Ca^{2+}-ATPase and the Ca^{2+} pump are inhibited by ethacrynic acid (Vincenzi, 1968). Mersalyl is also believed to be an SH-blocker (Slater, 1967). Therefore inhibition of Ca^{2+}-ATPase activity caused by this compound is an additional indication that free SH-groups are essential for full expression of the enzyme activation. The time-dependent as well as the three-stage inhibition of the enzyme by mersalyl suggest that there are several types of SH-groups, presumably located in different layers of the plasma membrane. If the inhibitor has to penetrate the membrane to different depths to interact with SH-groups, this will produce time-dependency. Thus low concentrations of mersalyl (10^{-5}M to 5 x 10^{-5}M) would saturate only external SH-groups, while higher concentration (5 x 10^{-5}M to 10^{-3}M) may block deeper groups and concentrations above 10^{-3} appear to give the greatest inhibition. The innermost SH-groups are presumably the most essential ones because the greatest degree of inhibition is obtained by saturating them. The existence of three different types of -SH groups was demonstrated in sarcoplasmic membranes (Hasselbach, 1966b), although not all of them are involved in ATPase activity. Since the K_m for Ca^{2+} was not affected by mersalyl, the inhibition is non-competitive.

The positive charge on the free amino groups probably plays a major role in the binding of the metal-ATP complex to the enzyme. Treating the enzyme with maleic anhydride replaced the positive charge

donated by the free amino group with a negative charge (Freedman et al., 1968), and led to a complete loss of activity. Wolf (1972) in his model of the $Ca^{2+}Mg^{2+}$-ATPase in the erythrocyte described the role of the NH_3^+ in the splitting site of the active center. Since the K_m for Ca^{2+} was unchanged with the lower concentration of maleic anhydride (1.0 μg/mg protein), while the V_{max} was reduced to 70%, it seems that treatment of the enzyme with maleic anhydride reduced the number of sites available for ATP hydrolysis.

The placental Ca^{2+}-ATPase was found to be relatively heat resistant and a marked fall in velocity was noticed only between 70°C-80°C. The activation energy for the hydrolysis of ATP by the placental Ca^{2+}-ATPase was 6.31 Kcal/mole. A higher activation energy for hydrolysis of ATP was reported for the acto-heavy meromyosin ATPase (25-30 Kcal/mole) (Barouch and Moos, 1971). A discontinuity in the Arrhenius plot for the hydrolysis of ATP by (Na^+, K^+) ATPase was found in a few cases. Gruner and Avi-Dor (1966) obtained 29.5 Kcal/mole activation energy for the 0°C-6°C range and 7.8 Kcal/mole for the 20°C-37°C range. Similar results were reported by Bowler and Duncan (1968). A possible discontinuity in Arrhenius plot around 23°C can be detected in this study (Figure 23). However, even if two lines are drawn instead of one, the difference in the activation energies is not impressive (8.7 Kcal/mole for the 1°C-23°C range and 3.5 Kcal/mole for the 23°C-45°C range).

The transition temperature of brain phospholipid dispersed in water is 23°C (Luzzati and Husson, 1962). Thus the possibility that the activation energy of ATP hydrolysis by the placental CA^{2+}-ATPase

is affected to a certain degree by the state of the phospholipidis in the membrane is not excluded.

SUMMARY

Guinea pig placental homeogenate was found to contain a Ca^{2+}-ATPase. Further purification and tests for marker enzyme showed that the Ca^{2+}-ATPase is located in the plasma membranes.

This enzyme is preferentially activated by calcium ions, in the presence of 5 mM ATP, maximal enzyme activity being obtained at 3 to 5 mM Ca^{2+}. The maximal rate of ATP hydrolysis varies between 15 and 22 μmole Pi/mg protein in 30 min.

Mg^{2+} also activates the enzyme, but always to a lesser degree than Ca^{2+}. Mn^{2+}, but not Sr^{2+}, activates the enzyme. At optimal calcium concentration, addition of magnesium is always inhibitory. Ca^{2+} and Mg^{2+} seem to act on the same site.

The enzyme does not require Na^{+} or K^{+} for activation by Ca^{2+}.

The optimal pH for Ca^{2+} activation of the enzyme lies between 8.2 and 8.5; at pH 7.1 and 9.5 only 50% of maximal activation occurs. Ruthenium red, in the concentration range of 10^{-6}M to 10^{-4}M, Ouabain (10^{-3}), and oligomycin (1.0 μg/ml) did not inhibit the enzyme.

Other triphosphates may serve as substrate; but the V_{max} for ATP is highest.

Addition of increasing amounts of Na_2H_2EDTA leads to a progressive decrease in activity, complete inhibition occurring at 5 mM when the incubation fluid contains 5 mM Ca^{2+}.

EGTA, a specific chelator of Ca^{2+}, symmetrically reversed the Ca^{2+} activation of the enzyme. The activation of the enzyme by Mg^{2+} was not affected.

The inhibition of the enzyme by mersalyl, an SH-blocker, was such that it suggested the presence of three types of SH-groups involved in the activation of the enzyme. Mersalyl acted as a non-competitive inhibitor of the enzyme.

Free amino groups are essential for ATP-hydrolysis. Complete modification of the amino groups with maleic anhydride led to total inactivation of the enzyme, whereas partial blocking of NH_3^+-groups reduced the V_{max} without affecting the K_m of Ca^{2+}.

In conclusion, the properties of the placental Ca^{2+}-ATPase are sufficiently similar to the properties of other Ca^{2+}-ATPases involved in Ca^{2+}-transport, to suggest that it may possibly have a similar role in translocation of Ca^{2+} in the placenta.

CHAPTER III

THE EFFECT OF Ca^{2+}/Mg^{2+} CONCENTRATION RATIO ON PLACENTAL (Ca^{2+}-Mg^{2+})-ATPase ACTIVITY

INTRODUCTION

The antagonism between Ca^{2+} and Mg^{2+} on enzyme activity is well documented. Dixon and Webb (1964c) and Williams (1959) give lists of antagonistic effects on different enzymes. Since Ca^{2+} is the major divalent cation in the extracellular fluid and Mg^{2+} is the major divalent cation found intracellularly, it has been suggested that Ca^{2+}/Mg^{2+} concentration ratio acts as regulator of enzyme activity (Bianchi, 1968).

In the previous chapter it was suggested that Ca^{2+} and Mg^{2+} compete for the same site on the placental (Ca^{2+}-Mg^{2+})-ATPase. This suggestion was based on the results that are presented in Figures 10 and 12. To prove this postulate, an equation expressing ATPase activity as a function of Ca^{2+}/Mg^{2+} concentration ratio has been derived. The following assumptions were made:

1. Calcium ions and magnesium ions compete for the available ATP to form divalent cation-ATP complexes, which serve as alternate substrates for the enzyme on the same site.

2. The association of Ca^{2+}, Mg^{2+}, and ATP directly with the enzyme is low compared with Ca-ATP and Mg-ATP association constants.

The postulated pathways are presented in Figure 24.

In accordance with the preceding assumptions Ca^{2+} reacts in the following way:

$$Ca^{2+} + ATP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Ca\ ATP \tag{1}$$

The stability constant of the Ca ATP complex will be:

$$K_1 = \frac{k_1}{k_2} = \frac{[Ca\ ATP]}{[Ca^{2+}]\ [ATP]} \tag{2}$$

$$Ca\ ATP + E \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\ Ca\ ATP \tag{3}$$

$$K_2 = \frac{k_4}{k_3} = \frac{[Ca\ ATP]\ [E]}{[E\ Ca\ ATP]} \tag{4}$$

$$E\ Ca\ ATP \xrightarrow{k_5} E + Products \tag{5}$$

$$V_1 = k_5\ [E\ Ca\ ATP] \tag{6}$$

Similarly for Mg^{2+} the following holds:

$$Mg^{2+} + ATP \underset{k_7}{\overset{k_6}{\rightleftharpoons}} Mg\ ATP \tag{7}$$

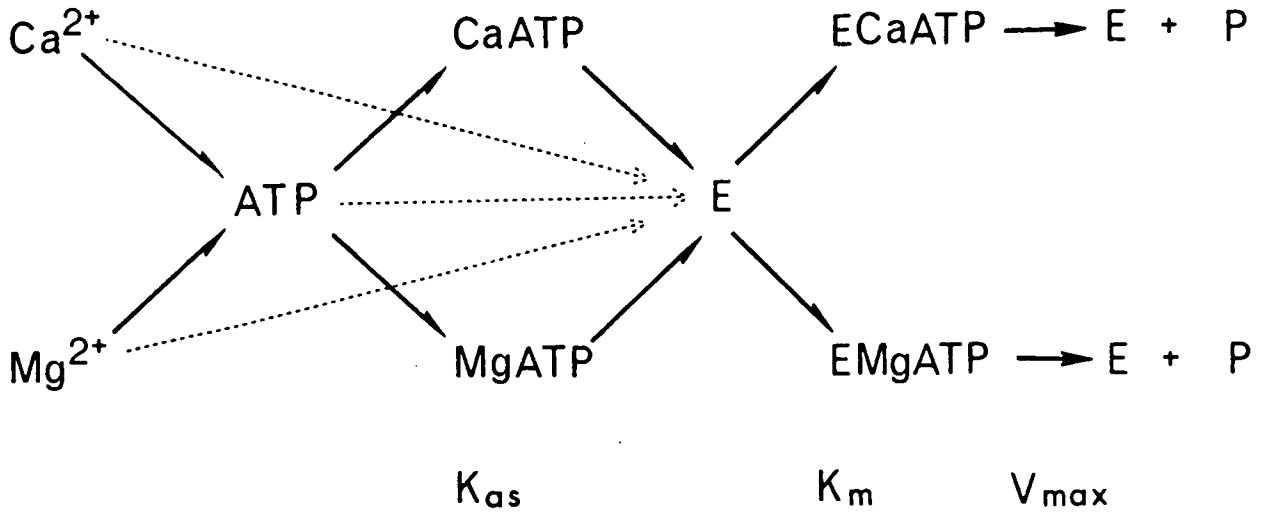


Figure 24. Postulated scheme for ATP hydrolysis in the presence of Ca^{2+} and Mg^{2+}.
Where: K_{as} = Association constant
E = Enzyme, P = Products and broken lines indicate minor interactions.

The stability constant of Mg ATP Complex will be:

$$K_3 = \frac{k_6}{k_7} = \frac{[Mg\ ATP]}{[Mg^{2+}]\ [ATP]} \tag{8}$$

$$E + Mg\ ATP \underset{k_9}{\overset{k_8}{\rightleftharpoons}} E\ Mg\ ATP \tag{9}$$

$$K_4 = \frac{k_9}{k_8} = \frac{[Mg\ ATP]\ [E]}{[E\ Mg\ ATP]} \tag{10}$$

$$E\ Mg\ ATP \xrightarrow{k_{10}} E + Products \tag{11}$$

$$V_2 = k_{10}\ [E\ Mg\ ATP] \tag{12}$$

Since there is competition between Ca^{2+} and Mg^{2+} for ATP in the first stage and again between Ca ATP and Mg ATP for the enzyme (E), the ratio [E Ca ATP] to [E Mg ATP] after introducing the terms obtained from equations 2, 4, 8, 10, will be:

$$\frac{[E\ Ca\ ATP]}{[E\ Mg\ ATP]} = \frac{K_1\ K_4\ [Ca^{2+}]}{K_3\ K_2\ [Mg^{2+}]} \tag{13}$$

Saturating the enzyme by using at least one of the ions at the optimal concentration, the total [E] will form complexes with both substrates in the following way:

$$\frac{[E\text{-}Mg\text{-}ATP]}{[E]} = \frac{100}{\frac{K_1 K_4 [Ca^{2+}]}{K_3 K_2 [Mg^{2+}]} + 1} \tag{14}$$

This is the fraction of the enzyme which is complexed with Mg ATP. Since the enzyme is saturated, the rest of the enzyme which is not complexed with Mg ATP, will complex with Ca ATP. Thus E Ca ATP complex in percent of total enzyme will be:

$$\frac{[E\ Ca\ ATP]}{[E]} = 100 - \frac{100}{\frac{K_1 K_4 [Ca^{2+}]}{K_3 K_2 [Mg^{2+}]} + 1} \tag{15}$$

Since the total activity is

$$A = V_1 + V_2 = k_5 [E\ Ca\ ATP] + K_{10} [E\ Mg\ ATP], \tag{16}$$

we can write:

$$A = \left(\frac{100}{\frac{K_1 K_4 [Ca^{2+}]}{K_3 K_2 [Mg^{2+}]} + 1} \right) k_{10} + \left(100 - \frac{100}{\frac{K_1 K_4 [Ca^{2+}]}{K_3 K_2 [Mg^{2+}]} + 1} \right) k_5 \tag{17}$$

Introducing the constants into equation 17 and substituting $Vmax_1$ for k_5, $Vmax_2$ for k_{10}, we get the total activity in percent by referring to the $Vmax_1$ for Ca^{2+} as 1.0.

$$A = \left(\frac{100}{\frac{K_1 K_4 [Ca^{2+}]}{K_3 K_2 [Mg^{2+}]} + 1} \right) Vmax_2 + \left(100 - \frac{100}{\frac{K_1 K_4 [Ca^{2+}]}{K_3 K_2 [Mg^{2+}]} + 1} \right) Vmax_1 \quad (18)$$

Since we assume that the last stages of the pathway for equations 5 and 11 are irreversible we can use Km_1 instead of K_2, and Km_2 instead of K_4. (K_2 and K_4 are the dissociation constants.) K_1 and K_3 are the stability constants of Ca ATP and Mg ATP. Thus the only variable is either $[Ca^{2+}]$ or $[Mg^{2+}]$ while the other ion is at optimal concentration.

Finally the formula will be:

$$A = \left(\frac{100}{\frac{K_1 Km_2 [Ca^{2+}]}{K_3 Km_1 [Mg^{2+}]} + 1} \right) Vmax_2 + \left(100 - \frac{100}{\frac{K_1 Km_2 [Ca^{2+}]}{K_3 Km_1 [Mg^{2+}]} + 1} \right) Vmax_1 \quad (19)$$

MATERIALS AND METHODS

Guinea pig placental plasma membranes were isolated according to the method of Post and Sen (1967). The experiment was carried out as described in the previous chapter. The amount of inorganic phosphate released was measured by the Gomori (1942) method and the protein in the sample by the Lowry et al. (1951) procedure.

RESULTS

The activation curves obtained by either of the two ions, Ca^{2+} and Mg^{2+}, are rectangular hyperbolas of typical substrate concentration curves (Figure 25).

Adding increasing amounts of Mg^{2+} to 5 mM Ca^{2+} resulted in inhibition of ATP hydrolysis; stimulation was produced by adding increasing amounts of Ca^{2+} to 5 mM Mg^{2+} or 10 mM Mg^{2+}. The results obtained are shown in Figure 25. The apparent K_m (calculated from a Lineweaver-Burk plot) was 0.24 mM for Ca^{2+} and 0.52 mM for Mg^{2+}. For Ca^{2+}, the $Vmax_1$ = 1, and for Mg^{2+}, $Vmax_2$ = 0.6. The stability constants for Ca ATP and Mg ATP (Hyde and Rimai, 1971) (K_1 and K_3) are log K_1 = 3.9 and log K_3 = 4.5. The curves I, II and III in Figure 25 are the theoretical curves obtained by using equation 19. Each experimental value was the mean of samples done in triplicate and the whole experiment was repeated four times. The goodness of fit between the theoretical curves and the experimental points was tested by using $\chi 2$ test, and the values were for each of four experiments: $0.99 > P_1 > 0.97$; $0.995 > P_2 > 0.99$; $P_3 = 0.97$; $0.9 > P_4 > 0.75$.

Since the concentration ratio is the only variable and all other values are constant, it is possible to plot A (activity) against $[Mg^{2+}]/[Ca^{2+}]$ while either Mg^{2+} or Ca^{2+} is at optimal concentration (5 mM). The calculated theoretical curve so obtained by using the above equation and the experimental results are shown in Figure 26A. It is also possible to calculate the change in the apparent Km for the total divalent cations

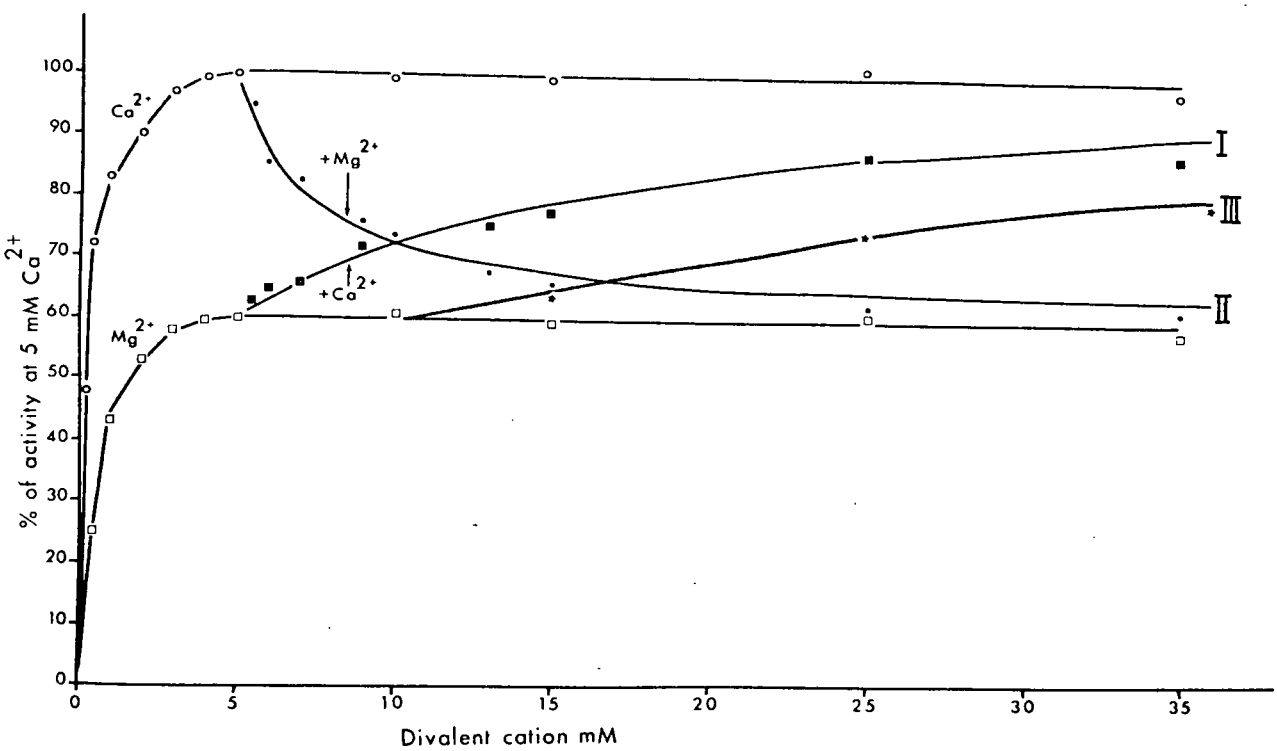


Figure 25. The effects of Ca^{2+} and Mg^{2+} on ATP hydrolysis. The incubation medium contained 70 mM NaCl, 20 mM Tris-HCl (pH 8.2), 5 mM Na_2ATP and Ca^{2+} or Mg^{2+} or Ca^{2+} + Mg^{2+} in concentration as indicated. Results expressed as percent of activity at 5 mM Ca^{2+} which ranged between 15 and 22 μmole/Pi per mg protein in 30 min at 37°. o——o activation by Ca^{2+}, □——□ activation by Mg^{2+}; I, II, III, theoretical curves obtained by using the equation. ○, ●, experimental values for 5 mM Ca^{2+} and increasing Mg^{2+} concentrations; □, ■, experimental values for 5 mM Mg^{2+} and increasing Ca^{2+} concentrations, *, experimental values for 10 mM Mg^{2+} and increasing Ca^{2+} concentration.

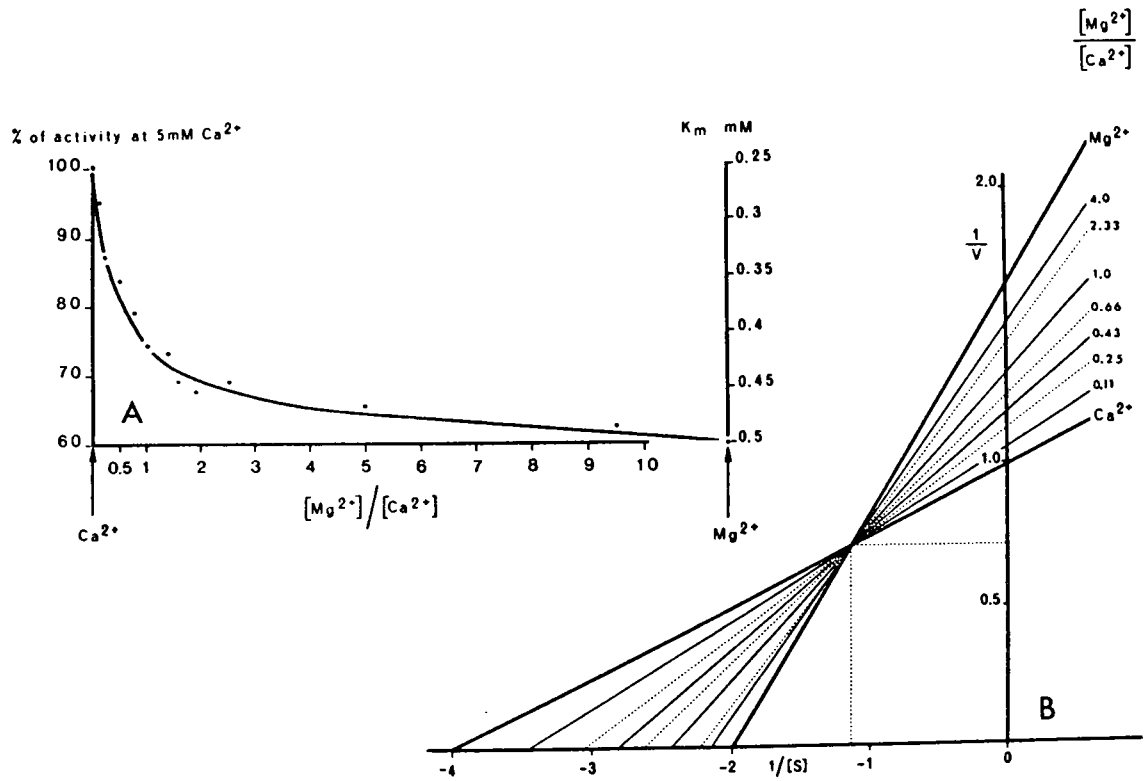


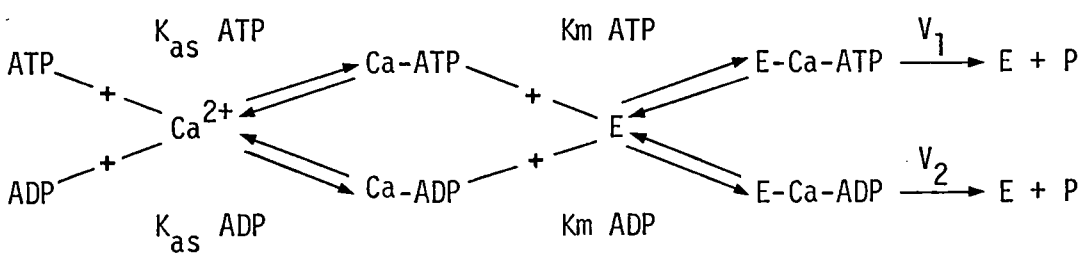
Figure 26. A - The effect of changing the concentration ratio of Mg^{2+} to Ca^{2+}, while at least one cation is at the optimal concentration of 5 mM. The solid line represents the theoretical, calculated curve and individual dots (● ●) are experimental values obtained when the $Vmax_2$ for Mg^{2+} was 60% of $Vmax_1$ for Ca^{2+}.

B - The change in Km as a function of Mg^{2+}/Ca^{2+}. V values for the various combinations obtained from Figure 26A.

as a function of Ca^{2+}/Mg^{2+} concentration ratio. The "Km" drops from 0.54 mM for Mg^{2+} to 0.25 mM for Ca^{2+} (Figure 26B).

Finally a three dimensional model describing the relationship between $[Mg^{2+}]/[Ca^{2+}]$ and the activity of the placental Ca^{2+}-ATPase was built. In this model the Y and the X axes are the concentration of Ca^{2+} and Mg^{2+} respectively, and the Z axis is the velocity of the reaction. The surface formed by the activation curves at different $[Mg^{2+}]/[Ca^{2+}]$ represent the velocity of the reaction under any possible combination up to the 10 mM Ca^{2+} + 10 mM Mg^{2+}. The model is shown in Figure 27.

To test further the validity of equation (19) for competition between substrates, the formula was used for predicting the velocity of the reaction as a function of ATP/ADP concentration ratio. ADP is a production of ATP hydrolysis by ATPase and can itself be hydrolyzed further by the enzyme. The hypothesis was that if ADP and ATP compete for the same site, Pi release will be inhibited. The following pathway was assumed:



The formation of the Ca-ADP and Ca-ATP complexes is dependent on the association constants. Log K for Ca-ATP = 3.6, and log K for Ca-ADP = 2.78 as quoted by Williams (1959). The Km for ATP is 0.1 mM (Figure 11)

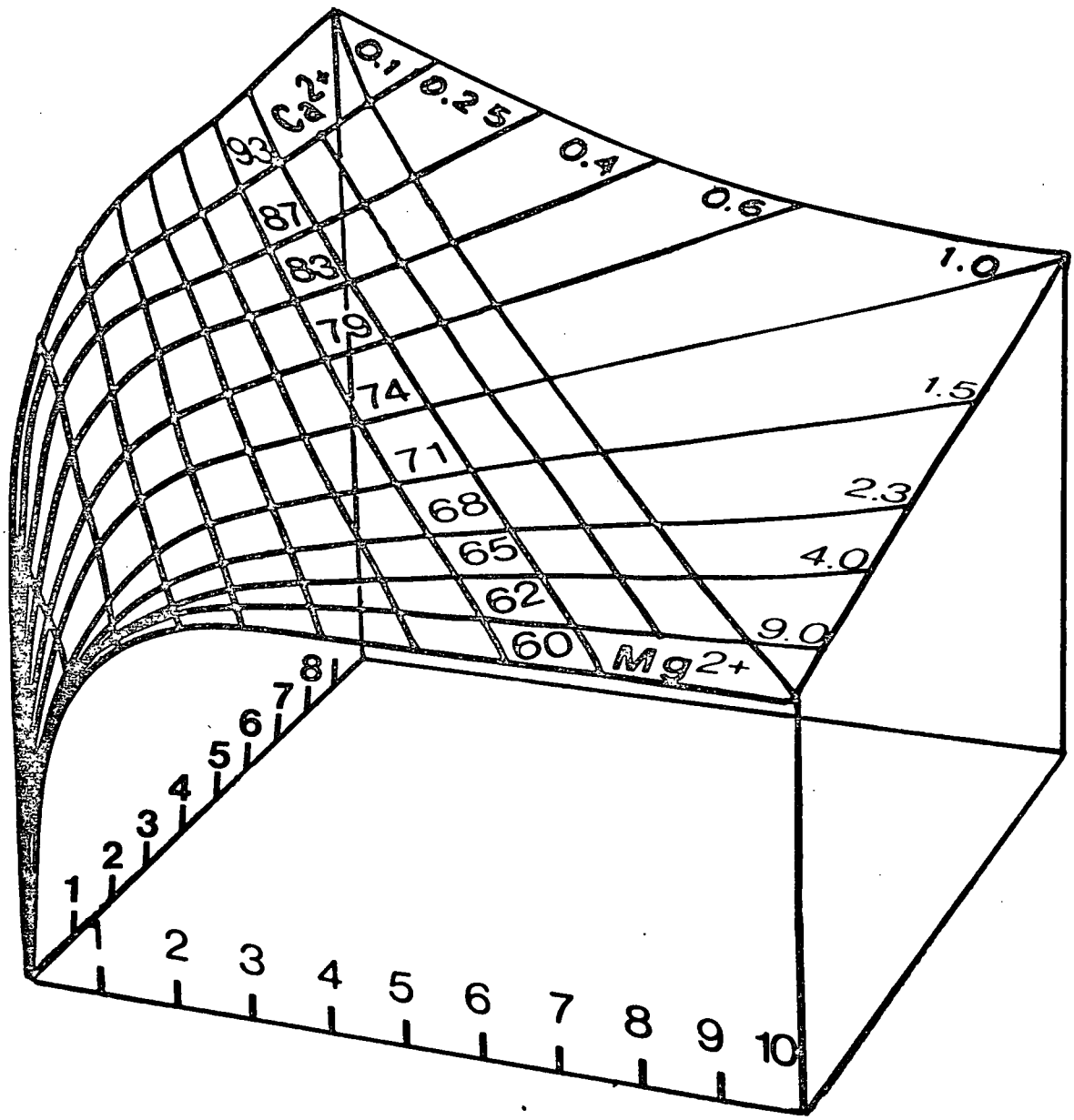


Figure 27. A three dimensional model describing the relationship between $[Mg^{2+}]/[Ca^{2+}]$ and the activity of the placental Ca^{2+}-ATPase. The Y and X axes are the concentration of Ca^{2+} and Mg^{2+} respectively, and the Z axis is the velocity of the reaction.

and for ADP Km = 0.15 mM (calculated from Figure 22). Samples were incubated with 5 mM ATP ADP and 2.5 mM ATP + 2.5 mM ADP. V_1 for ATP was considered as 1.0, and V_2 with 5 mM ADP was 0.69. Using all the constants mentioned, equation (19) will be as follows:

$$A = \left(\frac{100}{\frac{K_{as}ATP(K_mATP)[ATP]}{K_{as}ADP(K_mADP)[ADP]} + 1} \right) V_2ADP + \left(100 - \frac{100}{\frac{K_{as}ATP(K_mATP)[ATP]}{K_{as}ADP(K_mADP)[ADP]} + 1} \right) V_1ATP$$

$$A = \left(\frac{100}{\frac{7782\ (0.15)\ (2.5)}{892\ (0.10)\ (2.5)} + 1} \right) 0.69 + \left(100 - \frac{100}{\frac{7782\ (0.15)\ (2.5)}{892\ (0.10)\ (2.5)} + 1} \right) 1.0$$

$$A = 4.89 + 92.9 = 97.8\%$$

Thus the calculated velocity in the presence of 2.5 mM ATP + 2.5 mM ADP is 97.8% of that with 5 mM ATP alone. The actual experimental value was 96% for 1:1 [ATP]/[ADP] concentration ratio, which is in good agreement with the predicted value. Thus ATP and ADP appear to be hydrolyzed by the same site, and the inhibitory effect of ADP is very small. Even when [ATP]/[ADP] = 0.1, Pi release will decrease only 13% (calculated value).

DISCUSSION

The use of the formula presented in this chapter was very successful in predicting the velocity of the placental (Ca^{2+}-Mg^{2+})-ATPase incubated with numerous combinations of [Ca^{2+}]/[Mg^{2+}]. It is concluded

that Ca^{2+} and Mg^{2+} compete for the same site, or more precisely the corresponding complexes with ATP compete for the same site. It has been shown very clearly that the placental ATPase activity can be regulated by the Ca^{2+} to Mg^{2+} concentration ratio. The role of these two ions in regulating cellular enzymes has been the subject of several studies. Shikama (1971) calculated the standard free energy for ATP hydrolysis as a function of pMg and pCa. He found that the $[Ca^{2+}]/[Mg^{2+}]$ ratio plays an important role as a sensitive modifier of the thermodynamic potential of the ATP molecule. Thus the energy that can be derived from ATP hydrolysis by ATPase is regulated by at least two steps, both dependent on $[Ca^{2+}]/[Mg^{2+}]$. Step I, the rate of ATP hydrolysis; Step II, the change of free energy ($-\Delta G°$) for ATP hydrolysis.

The role of Ca^{2+} and Mg^{2+} in the ATPase of sarcoplasmic reticulum was studied by Panet et al. (1971). Their results suggest that phosphorylation of the enzyme (an intermediate step in ATP hydrolysis) is dependent on Ca^{2+}, while dephosphorylation is increased by Mg^{2+} and decreased by Ca^{2+}. In the placental (Ca^{2+}-Mg^{2+})-ATPase it is unlikely that this is true, since the hydrolysis of ATP proceeds with either ion alone. Though the possibility of formation of a phosphorylated intermediate exists for the placental enzyme as well, no attempt was made to study this aspect.

The ability to predict accurately the velocity of Pi release in the presence of ATP and ADP indicates the wider use that can be made of this formula. It also suggests that ATP and ADP are hydrolyzed by the same site and that product inhibition due to an increase in ADP concentration is negligible.

In addition, if one is interested in the contribution of each substrate to the total activity, it can be derived easily since each term in the formula represents the respective contributions.

Though it is possible to adopt this equation to predict the activity of an unsaturated enzyme, the prediction will be less accurate than for the saturated enzyme.

SUMMARY

The velocity of the reaction of a Ca^{2+} Mg^{2+}-stimulated ATPase in placental plasma membranes of the guinea pig was found to depend on the $[Mg^{2+}]/[Ca^{2+}]$ concentration ratio. The observed activation curve of the enzyme while changing the concentration ratio agrees with the theoretical equation, derived for the case in which two ions activate the same site while the enzyme is saturated. Because of the good agreement, it is concluded that the two divalent cations, Ca^{2+} and Mg^{2+}, activate this placental ATPase at the same site. The regulatory effect of $[Mg^{2+}]/[Ca^{2+}]$ on the placental ATPase activity, can be visualized by a three dimensional model. The equation may be used to determine whether or not two substrates compete for the same site, and what is the relative contribution to the total activity by each substrate. This was demonstrated with ATP and ADP, which are both hydrolyzed at the same site.

The equation is not specific for this enzyme and it can be used for other enzymes as well, provided the necessary constants are available, or can be calculated.

CHAPTER IV

COMPARISON BETWEEN ALKALINE PHOSPHATASE AND Ca^{2+}-ATPase

INTRODUCTION

Alkaline phosphatase is one of the most studied placental enzymes, and its specific activity level serves as a general index of placental function (Curzen and Morris, 1968). However, its physiological role and even its naturally occurring substrate have not been defined. It has been suggested that in the intestine Ca^{2+}-ATPase activity is just another manifestation of alkaline phosphatase (Haussler et al., 1970; Russel et al., 1972). Since Ca^{2+}-ATPase is implicated in Ca^{2+}-transport, alkaline phosphatase can be matched loosely with Ca^{2+}-transport. While this study does not oppose this suggestion regarding the physiological role of alkaline phosphatase, it does argue that the two enzymes are not identical. As described in previous chapters, it was found that the two enzymes are located in the microsomal fraction. Since no specific inhibitor is available for either enzyme, a different approach was taken to separate the two enzymes by selective purification. The dissociation of these two phosphatase activities (ATPase and monophosphatase), is made by comparing key properties of the two throughout the process of purification of alkaline phosphatase. If the two enzymes

are not identical, the purified alkaline phosphatase fraction should not demonstrate Ca^{2+}-ATPase activity.

An additional subject described in this chapter is the specific activity profile of Ca^{2+}-ATPase in developing guinea pig placenta throughout the gestation period. Its relation with the reported transplacental calcium flux and fetal calcium deposition (Twardock and Austin, 1970; Twardock, 1967) is considered. The logic behind this study is that since the placenta is a dynamic organic which responds to the changing requirements of the fetus, it may be expected that the placental enzymatic picture will be modified accordingly to meet the changing requirements of the fetus for calcium.

MATERIALS AND METHODS

Purification of Guinea Pig Placental Alkaline Phosphatase

Alkaline phosphatase was purified by the method of Ghosh and Fishman (1968), in a procedure involving three major steps: 1) Butanol extraction to disrupt the lipoprotein association; 2) Ammonium sulphate precipitation; 3) Gel filtration using sephadex G-200.

Lipids were extracted from the membrane preparation by addition of butanol (20 ml butanol to 50 ml membrane preparation). The enzyme passes to the aqueous phase with slow stirring for 1 hr at 4°C and then for 10 min at 37°C. The extract was centrifuged (Sorvall RC2-B) for 30 min at 14,600 x g and the aqueous phase was centrifuged again for 30 min at 35,000 x g. The supernatant (which contains the enzyme) was

dialyzed overnight against 50 mM Tris-HCl pH 8.6 (10 volumes) at 5°C-6°C. The proteins were then precipitated with 90% $(NH_4)_2\ SO_4$, extracted with 40% $(NH_4)_2\ SO_4$ and reprecipitated with 80% $(NH_4)_2\ SO_4$. The resultant precipitate was suspended in 50 mM Tris-HCl pH 8.6 and dialyzed overnight under the same conditions as the previous dialysis. The column was prepared as described by Ghosh and Fishman (1968) using a 60 x 2.5 cm column and sephadex G-200 gel. The enzyme solution was layered on the top of the column, eluted with 50 mM Tris-HCl pH 8.6 and collected in 10 ml fractions.

The protein concentration of the fractions was determined by reading abosrbence at 280 nm (Perkin-Elmer spectrophotometer, Coleman-124).

Alkaline phosphatase activity was measured as described in the Methods section of Chapter I, using carbonate-bicarbonate buffer (100 mM) except when Ca^{2+} was added to the incubation medium and glycine buffer (50 mM) was used.

Estimation of the Gestational Age of the Guinea Pigs

Pregnant guinea pigs at different stages of gestation were handled as described previously (Chapter I). The fetuses were weighed and the average weight per litter was used to estimate the placental age by using growth tables given by Draper (1920). Each placenta was processed separately and the membrane preparation of each placenta was assayed for Ca^{2+}-ATPase (see Chapters I and III for details). The mean and the standard error of the specific activity of Ca^{2+}-ATPase for each litter (3-5 fetuses) were calculated.

RESULTS

Purification of Alkaline Phosphatase

The elution profile of alkaline phosphatase activity and protein is presented in Figure 28. The alkaline phosphatase activity peak appeared between fractions No. 11-16; the highest total activity and specific activity (500 μmole/mg protein) was found in fraction No. 14.

Comparison Between Alkaline Phosphatase Activity and Ca^{2+}-ATPase Activity

The eluate was tested for Ca^{2+}-ATPase activity under the standard conditions for this enzyme (5 mM Ca^{2+}, 5 mM ATP, 70 mM Na^{+}, and 20 mM Tris-HCl pH 8.2); no measurable activity could be detected in any fraction. Studying the Ca^{2+}-ATPase activity in the previous steps revealed that the butanol extraction abolished completely the Ca^{2+}-ATPase activity. However, though Ca^{2+}-ATPase activity could not be detected, ATP was hydrolyzed to a certain extent by the purified alkaline phosphatase fraction. the pH profile of ATP hydrolysis (Figure 29), shows that the pH optimum for ATP hydrolysis shifted from pH 8.2-8.5 (in the membrane preparation) (Figure 16) to pH 9.0-9.5; and only slight activation by 10 mM Ca^{2+} was observed at the peak (pH 9.0-9.5). Ca^{2+} was completely ineffective as a stimulator at a lower pH range. Mg^{2+} showed basically the same profile with somewhat better efficiency as a stimulator of enzyme activity.

The pH profile of p-nitrophenyl-phosphate hydrolysis by fractions No. 13-14 was typical of alkaline phosphatase with a pH optimum of 10.0-10.2, and only Mg^{2+} stimulated hydrolysis. (Figure 30). The specific

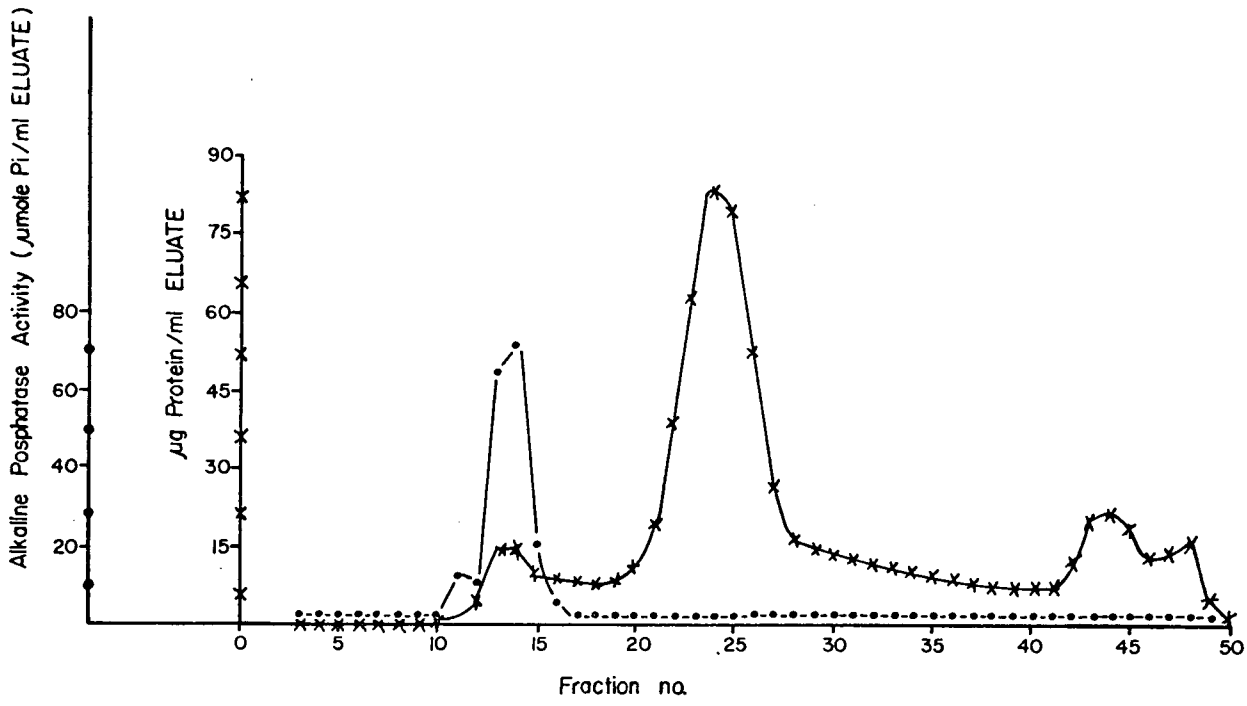


Figure 28. The elution profile of alkaline phosphatase and protein on Sephadex G-200 gel filtration carried out by the method of Ghosh and Fishman (1968). X —— X, protein; ●——●, enzyme activity. The protein was eluted with 50 mM Tris-HCl pH 8.6 and collected in 10 ml fractions.

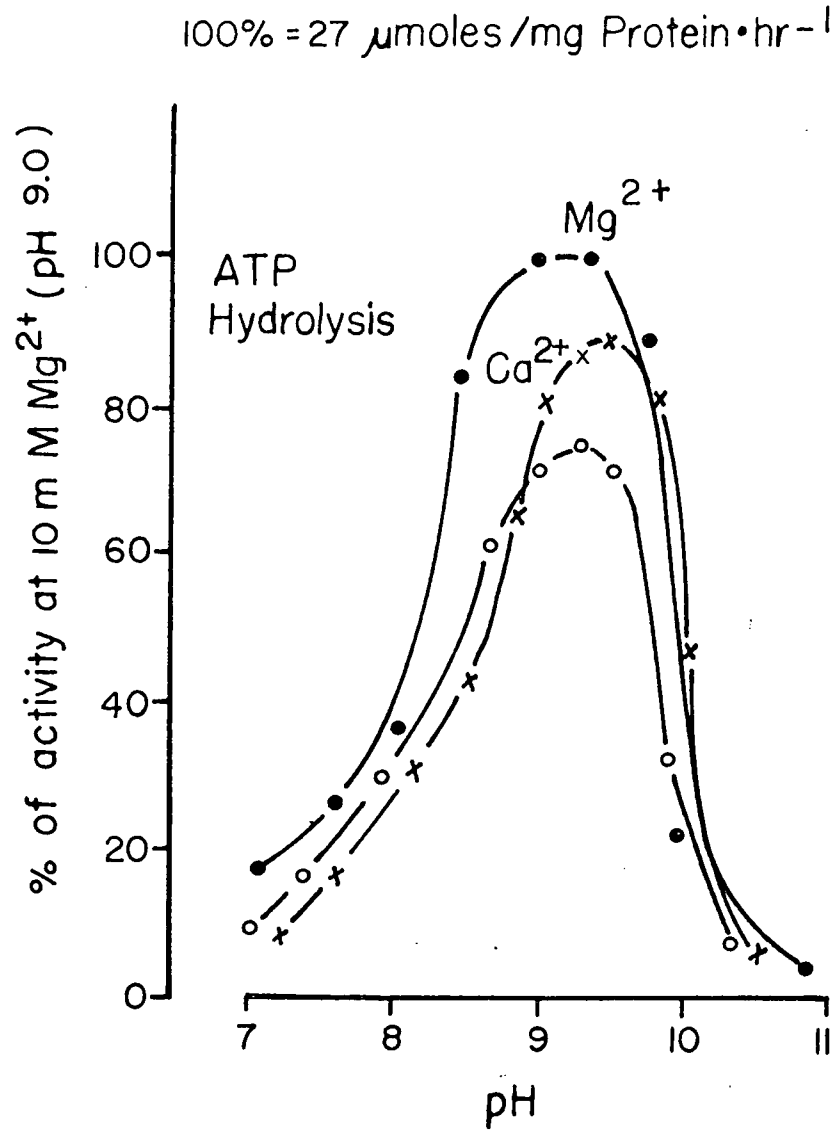


Figure 29. The effect of pH on ATP hydrolysis by the purified alkaline phosphatase (fraction no. 13-14). o —— o, without divalent cations; X —— X, with 10 mM Ca^{2+}; ●—— ●, with 10 mM Mg^{2+}. ATP concentration was 5 mM.

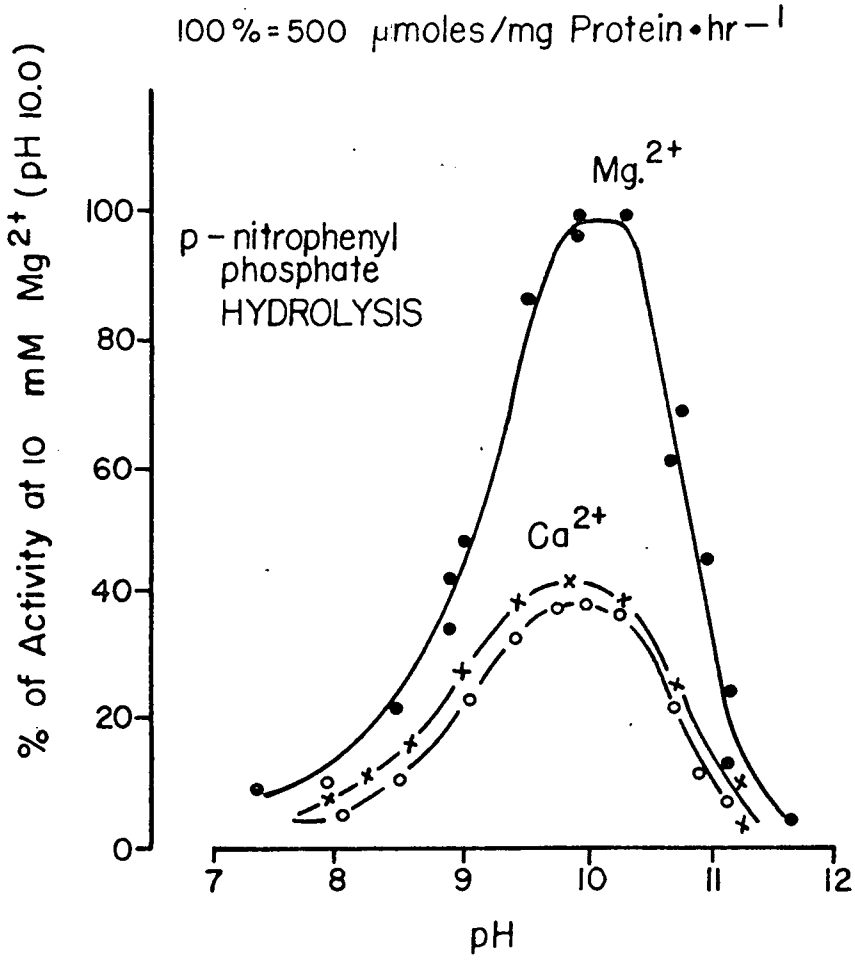


Figure 30. The effect of pH on p-nitrophenyl phosphate hydrolysis by the purified alkaline phosphatase (fractions no. 13-14). o —— o, without divalent cations; X —— X, 10 mM Ca^{2+}; ● —— ●, with 10 mM Mg^{2+}. The samples were incubated in the presence of 5 mM p-nitrophenyl phosphate.

activity at the peak was 500 μmole/mg protein compared with only 27 μmole/mg protein (5.4% efficiency) when ATP served as a substrate under the same conditions (pH 10.0, 10 mM Mg^{2+}). The stability on storage of the two enzyme activities in the membrane preparation was compared. Alkaline phosphatase was very unstable when stored at 4°C, and less than 10% of the initial activity was left after 7 days (Figure 31). Ca^{2+}-ATPase, on the other hand, lost only 10% of its initial activity during the same period (Figure 31) and no further decrease in activity of Ca^{2+}-ATPase was observed up to 2 months at 4°C. The results of the comparison study between alkaline phosphatase and Ca^{2+}-ATPase activities in the membrane preparation and the purified alkaline phosphatase fraction, are summarized in Table IV.

Ca^{2+}-ATPase Specific Activity as a Function of Placental Age

The results obtained from 21 guinea pigs with 3-5 fetuses per litter revealed that Ca^{2+}-ATPase activity increased up to the 50th day, leveled between the 50th and the 60th day, and dropped after the 60th day (Figure 32).

DISCUSSION

It has been demonstrated clearly that the purified alkaline phosphatase does not possess any Ca^{2+}-ATPase activity, and nor do any of the other eluted fractions. The only ATPase activity present is not dependent on Ca^{2+} for hydrolyzing ATP. It seems very likely from

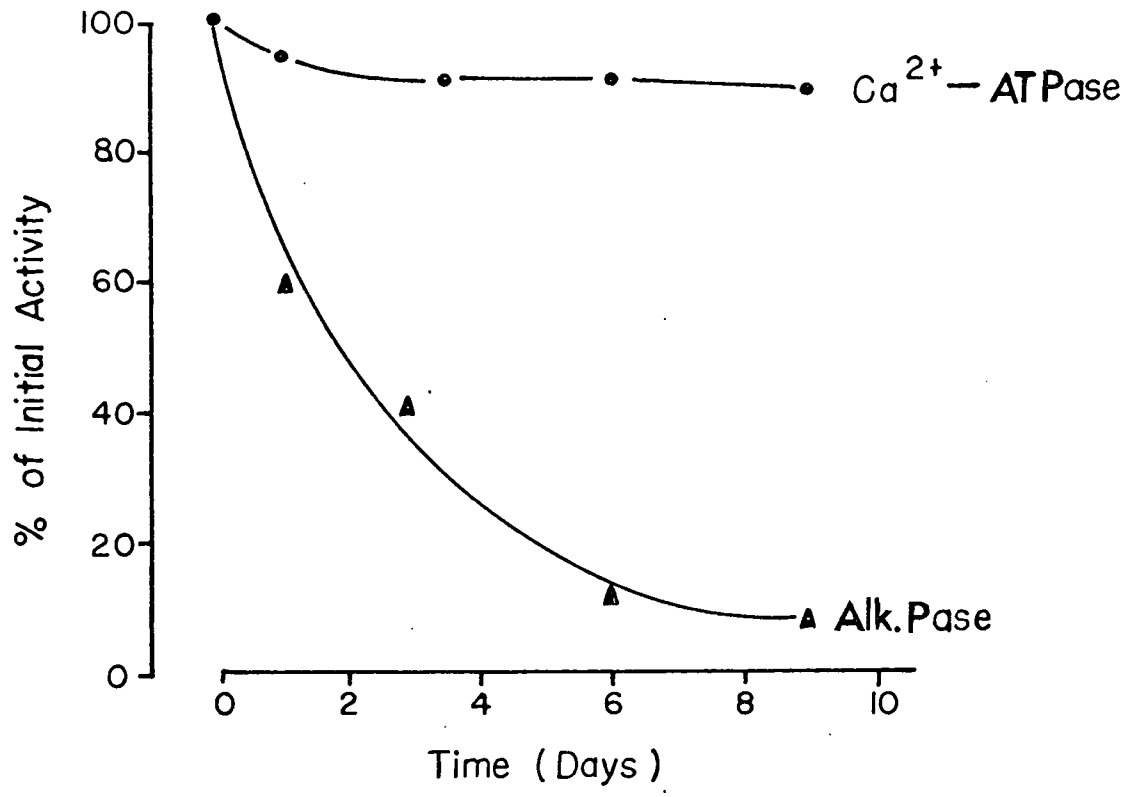


Figure 31. The effect of storage at 4°C on enzyme activity.
●——● , Ca^{2+}-ATPase; ▲——▲ , alkaline phosphatase.

Table IV. Comparison between alkaline phosphatase activity and ATPase activity in the membrane preparation and the purified alkaline phosphatase fraction.

Substrate	Preparation	pH optimum	μmoles Pi release per mg protein per hour at 37°C			Stability at 4°C
			No divalent cation	Net activation by (1) and (2) (1) 10 mM Mg^{2+}	Net activation by (1) and (2) (2) 10 mM Ca^{2+}	
5 mM p-nitrophenyl phosphate	Membrane preparation	10.0-10.2	5**	12**	0**	Very unstable
(Alkaline phosphatase activity)	Purified alkaline phosphatase fraction	10.0-10.2	200***	300***	0**	
5 mM Na_2ATP	Membrane preparation	8.2- 8.5	0.75*	30*	50*	Very stable
(ATPase activity)	Purified alkaline phosphatase fraction	9.0- 9.5	20*	7*	3*	

*
**Tris-HCl buffer (20 mM)
***Glycine buffer (50 mM)
Carbonate-bicarbonate buffer (100 mM)

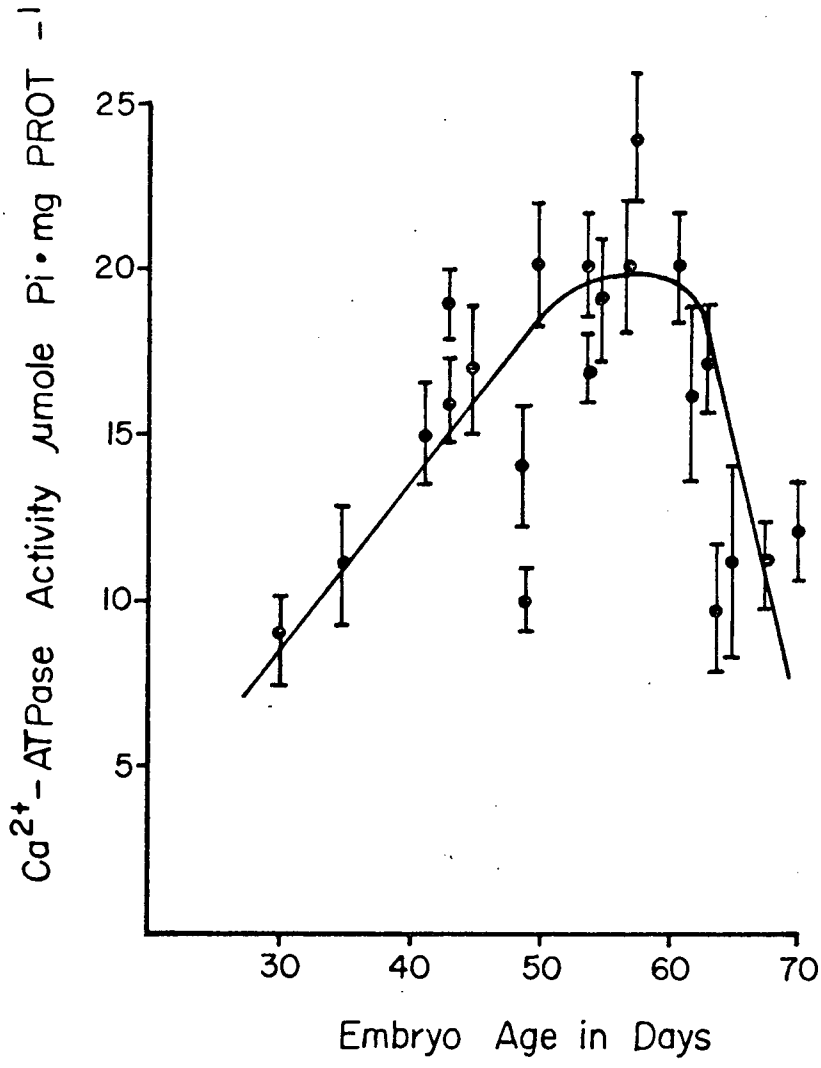


Figure 32. Ca^{2+}-ATPase specific activity as a function of embryo age. Each experimental value represents the mean $\pm$ S.E. for each litter.

the pH profile that ATP is being hydrolyzed by alkaline phosphatase and not by ATPase. Felix and Fleisch (1974) investigated the possibility that purified alkaline phosphatase from calf bone might demonstrate (Ca^{2+}-Mg^{2+})-ATPase activity. They found that the only activation of ATP hydrolysis by Ca^{2+} was obtained in the absence of Mg^{2+} and always was substantially lower than the activation by Mg^{2+}. Their conclusion was that calf bone alkaline phosphatase is not a "true" (Ca^{2+}-Mg^{2+})-ATPase, as it has been suggested for the intestinal alkaline phosphatase (Curzen and Morris, 1968; Haussler et al., 1970).

It has been shown that the extraction of the lipids with butanol from the placental plasma membranes abolished completely the Ca^{2+}-ATPase activity of the preparation. Emmelot and Bos (1968) found that (Na^{+}, K^{+})-ATPase activity depended on the lipid component of the preparation. Similar results were reported by MacLennan (1970) for the Ca^{2+}-ATPase of the sarcoplasmic reticulum. He was able to restore 50% of the original activity by adding phospholipid to Ca^{2+}-ATPase which was previously digested with phospholipase C. The exact role of the lipid is not known, however, it might be that the lipoprotein association keeps the protein in the conformation required for enzymic activity. The lack of dependence of alkaline phosphatase on the lipid, and its instability on storage at 4°C compared with the very high stability of the Ca^{2+}-ATPase, are additional indications that Ca^{2+}-ATPase is not just a manifestation of alkaline phosphatase. Another argument against this hypothesis can be drawn from the work of Manning et al. (1970), who found that alkaline phosphatase from the guinea pig placenta is very heat-sensitive (16 min

at 55°C resulted in 95% inactivation). On the other hand, the Ca^{2+}-ATPase was inactivated only at temperatures above 70°C (Figure 23).

The peak of Ca^{2+}-ATPase activity in the developing placenta appears to occur between the 50th and 60th day of pregnancy, with an increase in activity up to the 50th day and a decrease after the 60th day. A similar profile was reported for alkaline phosphatase in the guinea pig (Hard, 1946). Twardock (1967) reported that trans-placental calcium transport in the guinea pig (in vivo study), peaks around the 55th day of pregnancy, and drops thereafter. Thus this profile is in agreement with the Ca^{2+}-ATPase activity profile in the developing placenta. However, these profiles cannot account for the continuous increase of calcium deposition in the fetus throughout pregnancy (Twardock, 1967).

SUMMARY

The purified alkaline phosphatase fraction does not possess any Ca^{2+}-ATPase activity. Disruption of the lipoprotein association of the membranes by butanol destroyed completely the Ca^{2+}-ATPase activity, while increasing the alkaline phosphatase activity. In the membrane preparation Ca^{2+}-ATPase is very stable on storage at 4°C, while alkaline phosphatase activity decayed very rapidly. The specific activity profile of Ca^{2+}-ATPase throughout the gestation period revealed a peak between the 50th and the 60th day of pregnancy, similar to the alkaline phosphatase profile. The peak of enzyme activity corresponds with the reported trans-placental Ca^{2+} flux, but not with Ca^{2+}-deposition in the fetus.

In conclusion, it was demonstrated very clearly that Ca^{2+}-ATPase and alkaline phosphatase from the guinea pig placenta are two separate enzymes.

DIVISION III

Ca^{2+}-UPTAKE BY PLACENTAL PLASMA MEMBRANE VESICLES

CHAPTER V

Ca^{2+}-UPTAKE BY PLACENTAL PLASMA MEMBRANE VISICLES

INTRODUCTION

Transplacental calcium transport is asymmetrical. Presumably the ion must be transferred across at least the two plasma membranes which comprise the trophoblastic layer that separates the maternal and the fetal circulations. Studying Ca^{2+}-transport in such a system as one unit is extremely difficult if not impossible. However, active Ca^{2+}-transport can be studied with less difficulty if it occurs across a single plasma membrane, which is clearly a necessary step for any asymmetrical active transport. To be able to measure such transport one must have a system in which Ca^{2+} will be concentrated. This requirement is met by the placental membrane preparation which consists predominantly of vesicles (Plates 3, 4) of plasma membrane origin.

In previous chapters it was demonstrated that the placental plasma membrane vesicles contain Ca^{2+}-ATPase. This enzyme is sufficiently similar to other Ca^{2+}-ATPases involved in active Ca^{2+}-transport, to suggest that these vesicles may also be involved.

Ca^{2+}-uptake by sarcoplasmic reticulum vesicles has been studied by many investigators for almost two decades, but numerous questions

are still unresolved, and to date there is no widely accepted model for Ca^{2+}-transport. Part of the literature on this subject is reviewed by Inesi (1972). The relevant data will be compared with the findings of this study in the discussion.

This chapter was designed to answer very simple questions:

1) Can the placental plasma membrane vesicles accumulate calcium; if so, how efficiently?

2) Is the uptake dependent on ATP hydrolysis?

3) What is the effect of the external Ca^{2+} concentration on Ca^{2+}-uptake?

It is realized that many more questions must be answered before a firm conclusion can be made concerning the relationship of the Ca^{2+}-related properties of the placental plasma membranes to the active transport of Ca^{2+}. However, positive answers to the basic questions may suggest that the system is operating generally in the same manner as the sarcoplasmic reticulum (Inesi, 1972) and cardiac microsomes (Repke and Katz, 1972).

Before any measurement of Ca^{2+}-uptake can be made, the term uptake must be defined and distinguished from binding. The most widely used working definitions of Ca^{2+}-uptake and binding are given by Entman et al. (1973) and Repke and Katz (1972). Binding is defined as Ca^{2+} accumulation in the presence of ATP but in the absence of a calcium-precipitating anion (oxalate or phosphate). This process is rapid and reaches a maximum within 1 min. Uptake is Ca^{2+} accumulation in the presence of ATP and calcium-precipitating anion. This is a slower process.

When calculating uptake the binding should be subtracted. This is done by extrapolating the uptake to time zero.

In this study binding and uptake are defined differently. Bound calcium is accessible for an immediate exchange and can be displaced by a competitor. Bound calcium is measured by labeling the membrane with $^{45}Ca^{2+}$ (in the absence of ATP) and displacing it with "cold" calcium. Uptake is represented by the fraction of $^{45}Ca^{2+}$ which cannot be rapidly displaced by a large excess of $^{40}Ca^{2+}$.

The flow dialysis system described in Chapter I was adapted for measuring Ca^{2+}-uptake.

MATERIALS AND METHODS

Measurement of Ca^{2+}-uptake

Placental plasma membrane vesicles were incubated at 26°C in 20 mM Tris-HCl buffer pH 8.0, 100 mM NaCl, 10^{-5}M $^{45}Ca^{2+}$ and $^{40}Ca^{2+}$ as indicated, with or without 5 mM ATP (final volume 1.5 ml). At the end of the incubation period 3 x 0.1 ml aliquots were taken for the determination of Pi release. One ml of the incubation medium was introduced to the upper chamber of the flow dialysis cell, 0.5 ml of 1 M $^{40}Ca^{2+}$ was added, and the diffusion rate was measured. The diffusion rate of the control without ATP was also measured. The difference in the diffusion rates represents the fraction of calcium which is not available for displacement; thus Ca^{2+}-uptake can be calculated. After each measurement the upper chamber was emptied and washed three times with the

effluent buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl) before the next sample was introduced. ^{45}Ca counting was carried out as described in Chapter I, protein was determined by the Lowry method (1951), and Pi released by the Gomori (1942) method.

RESULTS

Evaluation of the Flow Dialysis Method for Measuring Ca^{2+}-uptake

The usefulness of the method for measuring calcium uptake was tested by incubating vesicles for 2 hours in the presence of ATP. Displacement of $^{45}Ca^{2+}$ by 3×10^{-1} M $^{40}Ca^{2+}$ was measured in the flow dialysis cell. The results are represented in Figure 33. It is evident that in the presence of 5 mM ATP, 5 mM Ca^{2+} and the placental plasma membrane vesicles (Figure 33, column B), part of the $^{45}Ca^{2+}$ (25%) is not available for displacement after 2 hrs of incubation at 24°C. From the results of the various controls it is concluded that this fraction of non-displacable Ca^{2+} represents uptake by the vesicles and is not the result of nonspecific unexchangable binding to any of the constituents of the incubation fluid. Thus the suitability of the modified flow dialysis method for Ca^{2+}-uptake studies is demonstrated.

The Effect of Incubation Time on Ca^{2+}-uptake and ATP Hydrolysis

Calcium uptake and ATP hydrolysis by the placental plasma membrane vesicles showed similar kinetic behaviour (Figure 34A). No Ca^{2+}-uptake was detected in the absence of ATP. The ratio between ATP

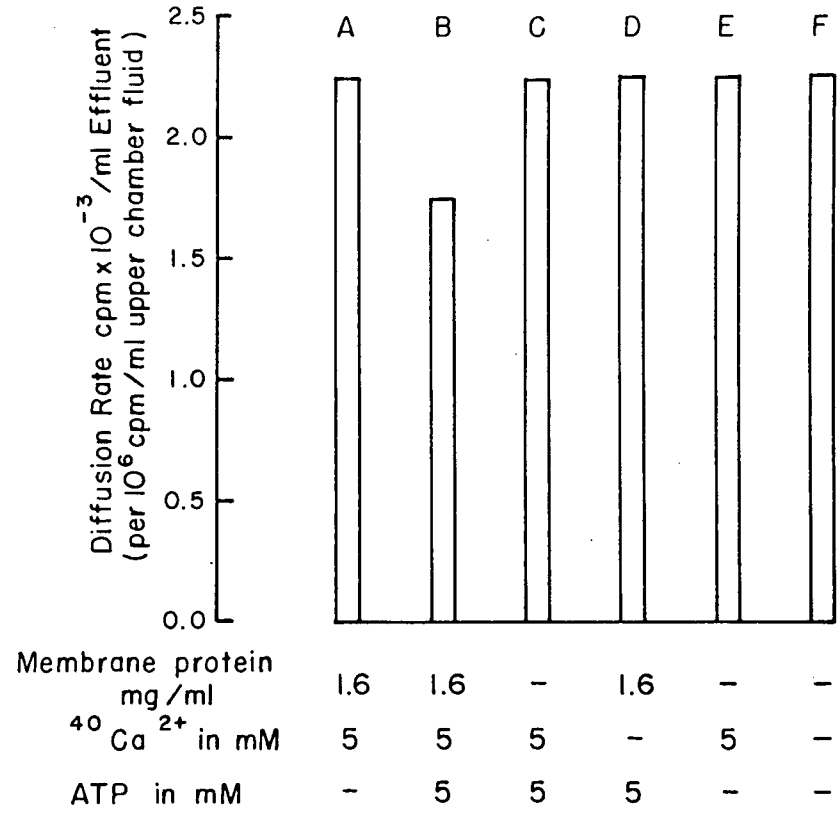


Figure 33. The effect of the major constituents of the incubation medium on ^{45}Ca displacement by $3 \times 10^{-1}M$ $^{40}Ca^{2+}$. The samples were incubated for two hours in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, $10^{-5}M$ $^{45}Ca^{2+}$, and different combinations of ATP, $^{40}Ca^{2+}$, and protein as indicated. $^{40}Ca^{2+}$ ($3 \times 10^{-1}M$) was added after two hours of incubation at 24°C, and the diffusion rate obtained was divided by the cpm concentration in the upper chamber to give cpm/ml effluent per 10^6 cpm/ml upper chamber fluid.

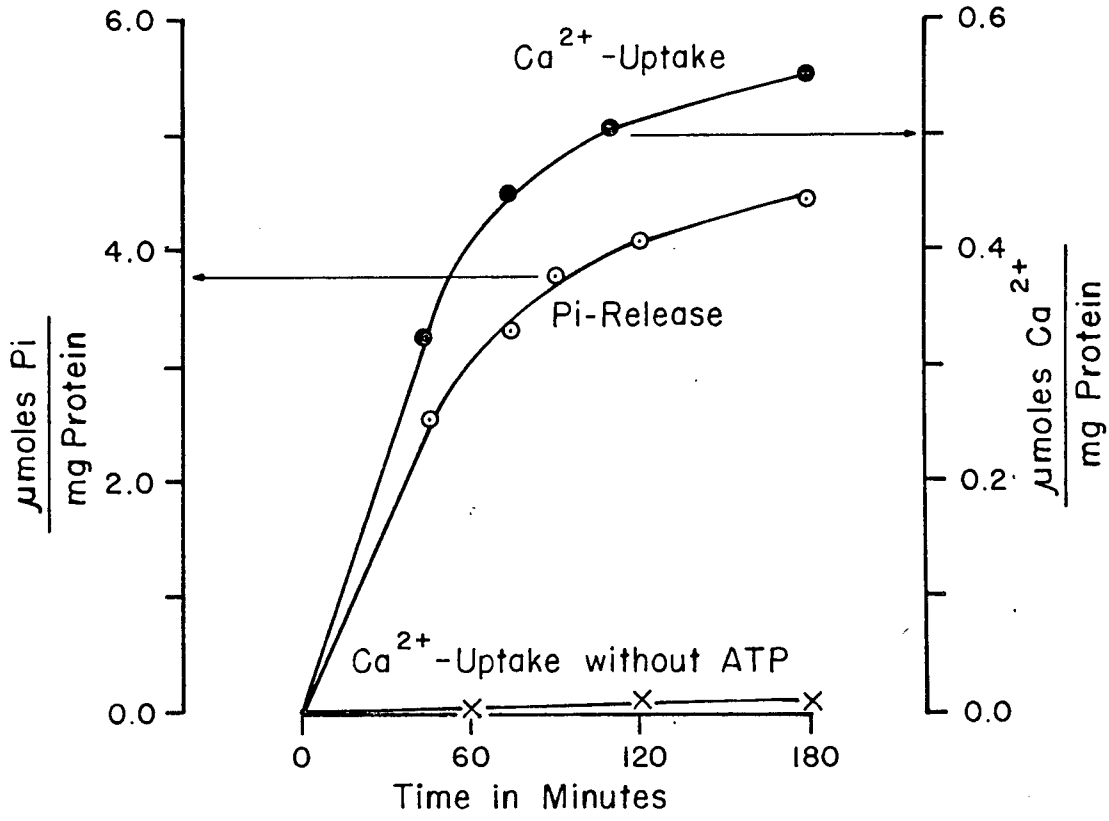


Figure 34A. The effect of incubation time on Ca^{2+}-uptake and ATP hydrolysis. The samples were incubated under standard conditions. Protein concentration was 1.78 mg/ml.

● —— ●, Ca^{2+}-uptake in the presence of 5 mM ATP and 5 mM Ca^{2+}

o —— o, Pi release under the same conditions

x —— x, Ca^{2+}-uptake in the absence of ATP

hydrolysis and Ca^{2+}-uptake was 7.7 (Figure 34B). Thus for every 7.7 μmole ATP hydrolyzed 1 μmole Ca^{2+} was taken up. This ratio was constant throughout the incubation time. These results demonstrate Ca^{2+}-uptake in the absence of calcium-precipitating ion (except for the Pi released). However, the process was slow (even after 2 hrs of incubation steady-state conditions were not reached) and required high protein concentration (1.5-2.0 mg protein/ml).

An attempt to improve the efficiency of Ca^{2+}-uptake by sonication (sonic dismembrator, Artek N.Y.) for up to 30 min at 40 watts, 20 Kh, did not affect the efficiency of Ca^{2+}-uptake. However, it was observed that Ca^{2+}-uptake by freshly prepared vesicles was very low. "Aging" the vesicles by storing for 2 weeks at 4°C improved substantially the efficiency of Ca^{2+} uptake, and the Pi/Ca ratio dropped to 4 (in the presence of 5 mM ATP and 5 mM Ca^{2+}).

The Effect of External Ca^{2+} Concentration on Ca^{2+}-uptake and Pi/Ca Ratio

At Ca^{2+} concentrations below 10^{-3}M, Ca^{2+}-uptake was very low (Figure 35A) (less than 0.05 μmoles/mg protein per 2 hrs) and Pi/Ca ratio values were very high, indicating extremely low efficiency (Figure 35B). Ca^{2+}-uptake showed a sharp increase at Ca^{2+} concentrations between 10^{-3}M-10^{-2}M (Figure 35A), with a similar increase in Ca^{2+}-uptake efficiency. The Pi/Ca ratio dropped to 2 at 10^{-2}M Ca^{2+} (Figure 35B).

Estimation of Calcium Concentration in the Vesicles

The estimation of the intravesicular calcium concentration requires the knowledge of vesicular volume. An attempt to measure this

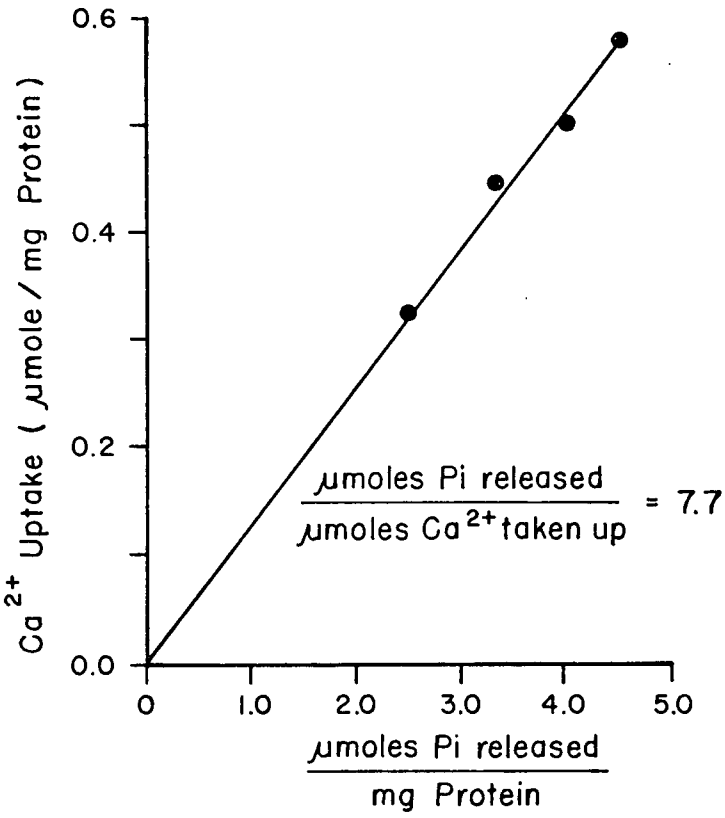


Figure 34B. The relationship between Pi release and Ca^{2+}-uptake. The data to construct this figure were obtained from Figure 34A.

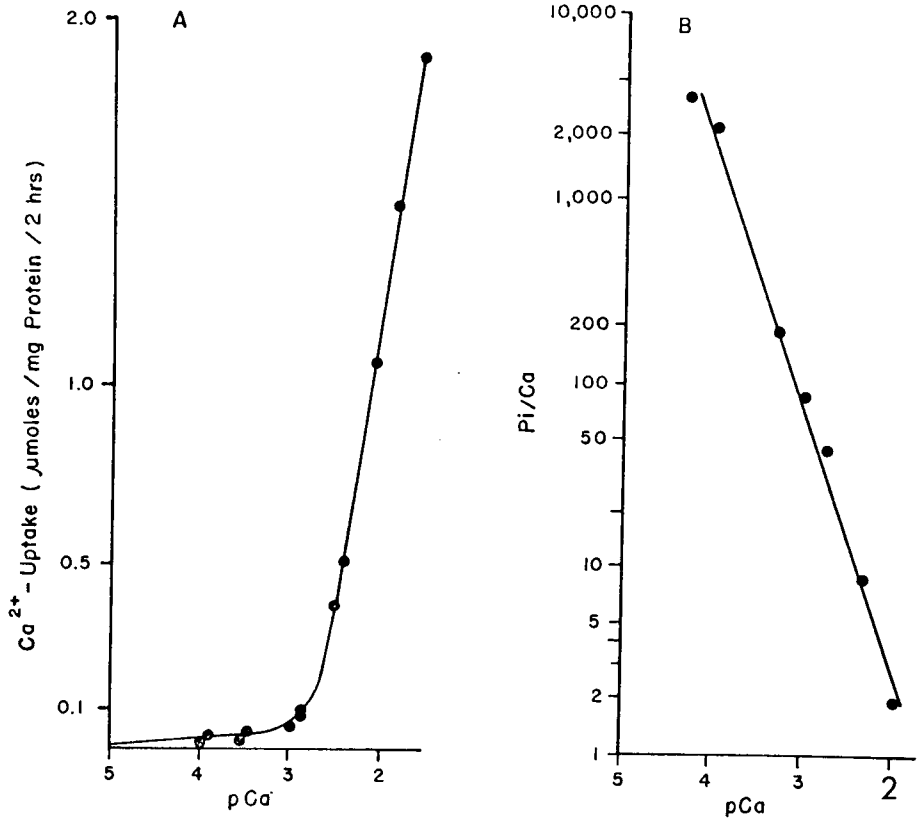


Figure 35A. The effect of Ca^{2+} concentration on Ca^{2+}-uptake. The samples were incubated for two hours under the standard conditions with different concentrations of Ca^{2+}. The results are expressed in µmoles Ca^{2+} accumulated per mg protein. Protein concentration was 1.72 mg/ml.

B. The effect of Ca^{2+} concentration on Pi/Ca ratio. Pi release is expressed in µmoles/mg protein (was measured in the same samples which were used for constructing Figure 35A), and Ca^{2+}-uptake in µmoles/mg protein.

volume with ^{14}C labelled sorbitol (which does not penetrate membranes) was not successful. It was found that after centrifugation of 5 ml of a suspension of vesicles for 30 min at 35,000 x g, the sorbitol concentration was lower in the supernatant (top 4 ml) than in bottom 1 ml which contained the vesicles. Thus some binding of sorbitol to the vesicles made this method for measuring vesicular volume ineffective. The vesicular volume of a sarcoplasmic reticulum preparation, using ultracentrifugation, was estimated to be 10 µl per mg membrane protein (Weber et al., 1966). Using this approximate figure, the intravesicular Ca^{2+}-concentration was calculated to be 190 mM (1.9 µmole/10 µl = mg protein) after 2 hrs of incubation. The initial Ca^{2+} concentration was 10 mM and after two hours 20% was taken up by the vesicles, so that the final external Ca^{2+} concentration dropped to 8 mM. Thus the intravesicular calcium was concentrated approximately 24-fold (190/8 = 23.8). The data was obtained from Figure 35A. When the vesicles were incubated in the presence of 5 mM Ca^{2+} and 5 mM Pi, the vesicles concentrated calcium 39-fold (calculated from Table V).

Phosphate Effect on Ca^{2+}-uptake

To exclude the possibility that Ca^{2+} accumulation in the presence of ATP is due to the release Pi which acts as a calcium-precipitating ion, samples were incubated with 0.3 and 5 mM $Na_2HPO_4 \cdot 7H_2O$ in the absence of ATP under the standard conditions (5 mM Ca^{2+} for two hours). No Ca^{2+}-uptake was found. When the samples were incubated with Pi (5 mM) and ATP (5 mM) a marked increase in Ca^{2+}-uptake was observed and the Pi/Ca ratio dropped from 4.0 to 1.7. The results so obtained are presented in Table V.

Table V. The effect of phosphate on Ca^{2+}-uptake. The samples were incubated for 2 hours in 20 mM Tris-HCl pH 8.0 100 mM NaCl, 5 mM Ca^{2+}, ATP and Pi as indicated. Protein concentration was 2.1 mg/ml.

Additions	Ca^{2+}-uptake µmoles/mg protein	Pi release µmoles/mg protein	Pi/Ca $\frac{\mu moles\ Pi}{\mu moles\ Ca}$
5 mM ATP	0.52	2.2	4.0
5 mM ATP + 0.3 mM Pi	0.52	2.2	4.0
5 mM ATP + 5 mM Pi	1.2	2.2	1.70

Table VI. The effect of 5.4 mM Mg^{2+} on ^{45}Ca displacement (diffusion rate) by 3 x 10^{-1}M $^{40}Ca^{2+}$ and Ca^{2+}-uptake. The samples were incubated for two hours at 24° C in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10^{-5}M ^{45}Ca, and 2.2 mg membrane protein, in final volume of 1.85 ml. The concentrations of Ca^{2+}, Mg^{2+}, and ATP were varied as indicated.

Additions					
mM Ca^{2+}	5.4	5.4	5.4	0.02	0.02
mM Mg^{2+}	5.4	-	5.4	-	5.4
mM ATP	-	5.0	5.0	5.0	5.0
Diffusion rate $\frac{\text{cpm/ml effluent}^{-1}}{\text{per } 10^6 \text{ cpm/ml upper chamber fluid}}$	2,540	2,020	1,990	2,520	2,330
Ca^{2+}-uptake $\frac{\text{nmoles } Ca^{2+}}{\text{mg protein}}$	-	934	986	-	1.4

Mg^{2+} Effect on Ca^{2+}-uptake

The effect of Mg^{2+} (5.4 mM) on Ca^{2+}-uptake was studied when the vesicles were incubated in the presence of high (5.4 mM) Ca^{2+}, and low (2×10^{-5}M) Ca^{2+}. Mg^{2+} (5.4 mM) had no effect on Ca^{2+}-uptake when the external medium contained 5.4 mM Ca^{2+}. However, when the external medium contained only 2×10^{-5}M Ca^{2+}, the diffusion rate dropped from 2,520 for the control (without Mg^{2+}) to 2,330 (cpm/ml effluent per 10^6 cpm/ml upper chamber fluid) when Mg^{2+} (5.4 mM) was present, indicating that the vesicles accumulated about 1.4 nmoles Ca^{2+} per mg protein. The results are presented in Table VI.

DISCUSSION

The flow dialysis method, which was used successfully for studying Ca^{2+}-binding (Chapter I), was also found suitable for measuring Ca^{2+}-uptake. Taking advantage of the flow dialysis system, a different and more simple definition of uptake was possible; Ca^{2+} taken up is the Ca^{2+} which cannot be rapidly displaced by a high concentration of competitor, due to accumulation in the vesicles (in the presence of ATP). This definition enables one to distinguish between Ca^{2+} bound to the external surface of the vesicles and the Ca^{2+} accumulated in the vesicles at all stages of the incubation period. This definition contrasts with other definitions of binding and uptake (Entman et al., 1973; Repke and Katz, 1972) in which the distinction between binding and uptake is possible only at time zero. Thus any event during the incubation period that

alters the binding of calcium to the external surface of the vesicles from the time zero value may distort the measurement of uptake. For example, the hydrolysis products of ATP (which are not present at time zero) can form a complex with Ca^{2+} (e.g. Ca-ADP). The concentration of such complexes will be time dependent and so will their binding to the external surface of the vesicles. Thus taking measurements later than time zero without being able to distinguish between binding and uptake, can overestimate Ca^{2+}-uptake. Since the definition presented here enables one to distinguish between binding and uptake throughout the incubation period, such overestimation of Ca^{2+}-uptake is avoided.

The first question which this study was designed to answer related to the ability of the vesicles of the placental plasma membrane to accumulate calcium. Clearly these vesicles are capable of accumulating calcium, even without a calcium precipitating ion (except for the Pi released due to ATP hydrolysis). After two hours of incubation the concentration of calcium in the vesicles was calculated to be 190 mM and in the external medium 8 mM. Thus calcium was concentrated approximately 24-fold. A 40-fold increase in Ca^{2+} was observed when the vesicles were incubated in the presence of 5 mM Ca^{2+} and 5 mM Pi. In this study the use of oxalate as calcium-precipitating ion was avoided, since it would result in a major deviation from physiological conditions.

Calcium accumulation by the sarcoplasmic reticulum was calculated to reach a concentration of 50 mM (500-fold over the external Ca^{2+} concentration) in the absence of oxalate (Hasselbach, 1964). Meisner (1973) calculated the total calcium concentration the vesicles of sarcoplasmic

reticulum to be 30-35 mM, and the free calcium concentration 13-18 mM (Inesi, 1972). In less specialized calcium accumulating systems such as platelet membranes, the intravesicular Ca^{2+} concentration can reach only 1 mM, a 10-fold increase over the external Ca^{2+} concentration, in the absence of oxalate and in the presence of ATP (Robblee et al., 1973). Evidently the calcium concentration within the placental plasma membrane vesicles (as measured in the absence of oxalate) exceeds that of the sarcoplasmic reticulum and platelet membrane vesicles. However, the concentrating capability falls far behind that of the sarcoplasmic reticulum, although it is higher than that of the platelet membrane vesicles.

The stoichiometry of Ca^{2+}-uptake was somewhat different from one preparation to another, and fluctuated between 4-8 ATP molecules hydrolyzed for each Ca^{2+} molecule taken up (measured in the presence of 5 mM Ca^{2+}). A constant ratio of 2 molecules of Ca^{2+} taken up for each ATP molecule hydrolyzed, has been repeatedly reported for sarcoplasmic reticulum (Martonosi and Feretos, 1964; Weber et al., 1966; and Hasselbach and Makinose, 1972). However, when the vesicles are leaky, higher Pi/Ca ratios (4-8) were reported for the sarcoplasmic reticulum (Racker and Eytan, 1973; Huxtable and Bressler, 1973). For platelet membranes the Pi/Ca ratio is between 10-20 (Robblee, 1973). The lowest Pi/Ca ratio observed in this study was 1.8 (when the incubation medium contained 10 mM Ca^{2+}). The high Pi/Ca ratio can be explained as follows: 1) The percentage of closed vesicles as seen by electron microscopy is approximately 50%. 2) It is quite possible that some of the closed

vesicles are inside-out; thus no accumulation of Ca^{2+} takes place. 3) The calcium which is complexed with ATP is not taken up by the vesicles. Only free Ca^{2+} is available for uptake; when the Ca^{2+} concentration is 10^{-3}M very little calcium is available. On the other hand, Ca-ATP is the substrate for the Ca^{2+}-ATPase, with Km = 0.25 mM. Thus, with increasing Ca^{2+} concentration, ATP hydrolysis will approach a maximum before significant Ca^{2+}-uptake occurs. This might explain the logarithmic decrease in Pi/Ca ratio as the external Ca^{2+} concentration rises. All of the above factors and others will tend to increase the apparent Pi/Ca ratio so that the actual Pi/Ca may be much lower and might even be as low as was reported for the sarcoplasmic reticulum (0.5) (Weber et al. 1966).

It was noted that in the presence of 5 mM Pi, but in the absence of ATP, no Ca^{2+} was accumulated in the vesicles after two hours of incubation. This indicates that the vesicles are fairly impermeable to calcium.

The exact role of ATP was not revealed in this study. However, the profile of ATP hydrolysis as a function of incubation time was the same as the profile of Ca^{2+}-uptake, and no uptake of Ca^{2+} was detected in the absence of ATP. As has been mentioned already, the most obvious role of ATP is to serve as the source of energy for the uptake process. Nakamura and Konishi (1974), observed that for brain microsomes, though no Ca^{2+}-uptake occurred without ATP, Ca^{2+} uptake did not follow the profile of Pi release. Their conclusion was that the brain microsomes exhibit ATP-dependent Ca^{2+}-uptake without the participation of Ca^{2+},

Mg^{2+}-dependent ATPase. They suggested that the role of ATP is to provide the phosphate for the formation of the phospholipid triphosphoinositide through the action of the enzyme diphosphoinositide kinase (EC 2.7.1.68). The stability constant of the complex between this phospholipid and Ca^{2+} is higher (10-fold) in the presence of ATP than in its absence (Hendrickson and Reinertsen, 1969), and this might explain the role of ATP in Ca^{2+}-uptake by brain microsomes. The profiles of Pi release and Ca^{2+}-uptake by placental plasma membrane vesicles show the same kinetic behaviour, unlike the case of brain microsomes. Thus it is unlikely that the role of ATP in the uptake process is other than acting as a source of energy.

The increase in Ca^{2+}-uptake in the presence of 5 mM Pi and 5 mM ATP, is most likely due to precipitation of calcium-phosphate in the vesicles, thereby decreasing the leakage of Ca^{2+}.

A positive effect of Mg^{2+} on Ca^{2+}-uptake was noted only at low Ca^{2+} concentration, and is interpreted as follows. Mg^{2+} frees Ca^{2+} from the complex with ATP. Since Ca^{2+} is the substrate of the uptake system, there is more Ca^{2+} available. The enzymes can still be activated by Mg-ATP so that the overall result will be an increase in Ca^{2+}-uptake. However, this is true only when the Ca^{2+} concentration is very low compared with that of Mg^{2+}. At 5 mM Ca^{2+} and 5 mM Mg^{2+} the increase in Ca^{2+}-uptake was not significant.

Ca^{2+}-uptake by the sarcoplasmic reticulum is dependent on Mg^{2+}, as is the ATPase activity (Martonosi and Feretos, 1964; Weber et al., 1966; Hasselbach and Makinose, 1972). No such dependency was noted in this study, provided that calcium concentration was above 10^{-3}M.

In conclusion, the present study demonstrated that the placental plasma membrane vesicles are capable of accumulating Ca^{2+}, and this appears to be dependent on ATP hydrolysis by the placental Ca^{2+}-ATPase.

SUMMARY

The placental plasma membrane vesicles are capable of accumulating up to 190 mM Ca^{2+}. This is 24-fold higher than the external Ca^{2+} concentration.

This process is dependent on ATP hydrolysis by the placental Ca^{2+}-ATPase.

The Pi/Ca ratio is dependent on the external Ca^{2+} concentration, and reaches the value of 2 at 10 mM Ca^{2+}.

Phosphate (5 mM) can double Ca^{2+}-uptake when measured in the presence of 5 mM Ca^{2+}.

Mg^{2+} increased Ca^{2+}-uptake only at a low Ca^{2+} concentrations, and had no significant effect at 5 mM Ca^{2+}.

GENERAL CONCLUSIONS

The objective of this thesis was to investigate the Ca^{2+}-related properties of the placental plasma membranes, and to fill a gap created by the fact that there is no full description of these properties for any plasma membranes involved in asymmetrical calcium transport. This section will attempt to correlate these properties with each other and to speculate on their possible role in calcium transport across the placenta.

The effect of Ca^{2+} concentration on Ca^{2+}-binding, and the velocities of Ca^{2+}-ATPase and Ca^{2+}-uptake, was the key parameter which was investigated. Figure 36 shows this effect. It is evident that at a Ca^{2+} concentration of 10^{-4}M only Ca^{2+}-binding and Ca^{2+}-ATPase reached significant level. Ikemoto (1974) found that, in the sarcoplasmic reticulum, the high affinity sites for Ca^{2+} have a Ks = 4×10^{-6}M. This figure was the same as the Km of the Ca^{2+}-ATPase. He concluded that high affinity sites are involved in activation of the enzyme. In this study the Km for Ca^{2+} (2.5×10^{-4}M) with respect to ATPase activity does not correspond with either of the dissociation constants for the Ca^{2+}-binding sites ($Ks_1 = 3 \times 10^{-5}$M, $Ks_2 = 10^{-3}$M). There is no evidence that these sites are involved in the activation of Ca^{2+}-ATPase. However, theoretically only the high affinity sites could take part, since ATP forms a complex with Ca^{2+} ($Ks \sim 10^{-4}$M) which will dissociate most of the Ca^{2+} from the low affinity sites.

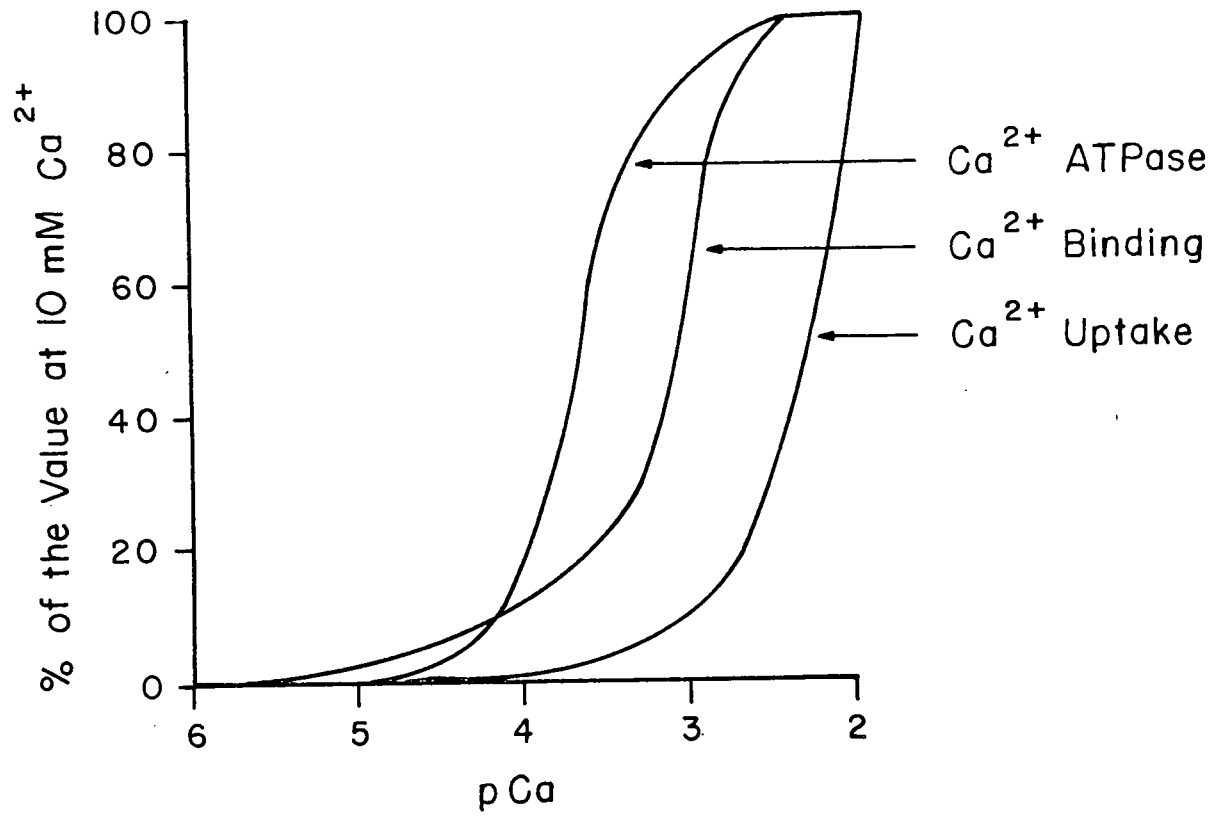


Figure 36. The effect of Ca^{2+} concentration on Ca^{2+}-binding and on the velocities of ATP hydrolysis and on Ca^{2+}-uptake.

100% Ca^{2+}-binding = 250 nmoles Ca^{2+}/mg protein

100% ATP hydrolysis = 4 μmoles Pi/mg protein

100% Ca^{2+}-uptake = 1.9 μmoles Ca^{2+}/mg protein

Protein concentration in the incubation medium was 2 mg/ml.

The presence of high affinity sites for Ca^{2+} was reported for membranes which are involved in active transport of Ca^{2+} (see Chapter I). The suggested role for these sites is binding Ca^{2+} passively as the first step in Ca^{2+} transport. However, because no specific inhibitor is available, either for the sites or for Ca^{2+}-ATPase, the role of these sites remains speculative.

The ability of the placental plasma membrane vesicles to accumulate Ca^{2+} has been demonstrated. This accumulation is dependent on ATP hydrolysis by the placental Ca^{2+}-ATPase. The involvement of Ca^{2+}-ATPase in Ca^{2+}-uptake by the sarcoplasmic reticulum is well documented (see Chapter V). In sarcoplasmic reticulum a single protein acts as both ATPase and ionophore in promoting Ca^{2+}-uptake activity (Warren et al., 1974). The present study suggests that the substrate of Ca^{2+}-ATPase is Ca-ATP complex, while only free Ca^{2+} can be transported. Thus in the presence of ATP at Ca^{2+} concentration below 10^{-3}M there was very little free Ca^{2+} and Ca^{2+}-uptake was insignificant.

The specificity of the Ca^{2+}-related properties was studied by introducing Mg^{2+} into the incubation medium and analysing its effect on the measured values. The affinity of Ca^{2+} for the high affinity sites was 10-fold higher than for Mg^{2+}. Thus if these sites face the extracellular fluid where the Ca^{2+} concentration is higher than the Mg^{2+} concentration these sites will be occupied by Ca^{2+}. However, if the high affinity sites face the cytosol where the Ca^{2+} concentration is much lower than the Mg^{2+} concentration, these sites will be occupied to a large extent by Mg^{2+}.

The effect of $[Ca^{2+}]/[Mg^{2+}]$ concentration ratio on Ca^{2+}-ATPase activity was pronounced. From the kinetic behaviour of the enzyme as a function of the $[Ca^{2+}]/[Mg^{2+}]$ ratio, it is concluded that the two ions activate the enzyme at the same site. The derived formula enables one to predict very precisely the velocity of the reaction under any combination of Ca^{2+} and Mg^{2+} concentrations. There was no requirement for Mg^{2+}, and the reaction could proceed with either of the two ions, although Ca^{2+} was more efficient. The Ca^{2+}-ATPase of sarcoplasmic reticulum has an absolute requirement for Mg^{2+} (MacLennan, 1970) and no activation by Ca^{2+} was obtained in the absence of Mg^{2+}. The same is true for Ca^{2+}-uptake by the sarcoplasmic reticulum (Martonosi and Feretos, 1964; Weber et al., 1966; Hasselbach and Makinose, 1972).

Magnesium did not affect Ca^{2+}-uptake by the vesicles of placental plasma membranes when the Ca^{2+} concentration was 5 mM. At 2×10^{-5}M Ca^{2+} the effect of Mg^{2+} on Ca^{2+}-uptake was pronounced compared with Ca^{2+}-uptake in the absence of Mg^{2+}. However, the actual value was very low relative to Ca^{2+} uptake at 10^{-2}M Ca^{2+} (1.4 nmoles/mg compared with 1.9 µmoles/mg). One can speculate that under physiological conditions two systems are active: 1) a low Ca^{2+} concentration, Mg^{2+} dependent system with low efficiency, and 2) a high Ca^{2+} concentration, Mg^{2+} independent system with higher efficiency. The overall Ca^{2+}-related properties of the placental plasma membranes are independent of Mg^{2+} and the entire process from binding to the membrane through activation of the enzyme and finally to Ca^{2+}-uptake, appears to be dependent on Ca^{2+} alone. This situation appears unique to the placental plasma

membranes. The other systems described in the literature deal with the Ca^{2+}-related properties of membranes involved in intracellular Ca^{2+}-regulation.

The physiological role of this system in Ca^{2+}-transport across the placenta cannot be defined clearly. The objective of this thesis was to study the relation between Ca^{2+} and the membrane involved in Ca^{2+}-transport. However, one can speculate that the vesicles which are loaded with Ca^{2+}, may be an in vivo part of the continuous tubular network which has been seen with electron microscope in the trophoblast (Plates 1 and 2). Croley (1973) localized Ca^{2+} histochemically in this network within the human trophoblast. Since the human placenta and guinea pig placenta have the same basic structure (haemochorial) one can expect similar localization of Ca^{2+} in the guinea pig trophoblast. Thus fusion of segments of this network with the basal plasma membrane will provide the link between the maternal and the fetal circulation.

The Ca^{2+}-related properties of placental plasma membranes described in this thesis, provides possible explanations of the first steps in the asymmetrical transplacental Ca^{2+}-transport. Further investigation is required before the entire process is fully understood.

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