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LIPID-PROTEIN INTERACTIONS AND CALCIUM UPTAKE IN INTESTINAL BRUSH BORDER MEMBRANE VESICLES

Allan Rodney Merrill

Thesis submitted to the School of Graduate Studies of the University of Ottawa in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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MATHEMATICS MAY BE COMPARED TO A MILL OF EXQUISITE WORKMANSHIP, WHICH GRINDS YOU STUFF OF ANY DEGREE OF FINENESS; BUT NEVERTHELESS, WHAT YOU GET OUT DEPENDS ON WHAT YOU PUT IN: AND AS THE GRANDEST MILL IN THE WORLD WILL NOT EXTRACT WHEAT FLOUR FROM PEASCODS, SO PAGES OF FORMULAE <OR GRAPHS> WILL NOT GET A DEFINITE RESULT OUT OF LOOSE DATA.

T.H. HUXLEY

To my loving wife

who has persevered

throughout the course of this study

and who has always encouraged and supported me.

TABLE OF CONTENTS

Abstract v
Acknowledgements vii
List of Tables viii
List of Figures x
List of Abbreviations xiii

INTRODUCTION 1

I. SOME CHARACTERISTICS OF THE INTESTINAL EPITHELIAL CELL PLASMA MEMBRANE 2
A. Properties pertaining to the isolation of the brush border membrane and the basolateral membrane 2
B. Morphological aspects of the isolated BBMV 3
C. Lipid composition of BBMV 4
D. Proteins and glycoproteins of BBMV 8

II. MODIFICATION OF THE LIPID COMPOSITION OF THE BRUSH BORDER MEMBRANE 10
A. Uptake of lipids by the brush border membrane 10

III. EFFECTS OF LIPID MODIFICATION ON STRUCTURE--FLUORESCENCE ANISOTROPY STUDIES 15
A. Monitoring changes in membrane fluidity using fluorescence anisotropy 15
B. Fluorescence anisotropy studies in brush border membranes 16
C. Effect of fatty acids on BBMV structure as measured by fluorescence anisotropy 21

IV. EFFECT OF FLUIDITY CHANGES ON MEMBRANE FUNCTION--BBMV FUNCTION 24
A. Effect of fatty acids on membrane function--some general examples 24
B. Effects of fatty acids on brush border membrane functions 26

V. SOME ASPECTS OF THE MOLECULAR BASIS FOR Ca^{2+} UPTAKE BY THE BRUSH BORDER MEMBRANE 29
A. Calcium uptake and the Ca^{2+}-binding proteins of the brush border membrane 31
1. Calbindin (CaBP) 31
2. Alkaline phosphatase-Ca^{2+}-ATPase complex 32
3. Intestinal membrane Ca^{2+}-binding protein 33
4. Calmodulin as mediator of intestinal Ca^{2+} transport 35
B. Terbium as a luminescent probe for the Ca^{2+} uptake system--Ca^{2+}-binding proteins 40

VI. AIMS OF THE PRESENT INVESTIGATION 44

Chapter 1: **CHARACTERIZATION OF BBMV LIPID AND MARKER ENZYME CONTENT--A COMPARISON OF Ca^{2+} AND Mg^{2+} PRECIPITATION METHODS** 45

I. INTRODUCTION 45

II. MATERIALS 46

III. METHODS 46
A. Preparation of brush border membranes 46
B. Marker enzyme assays 48
C. Lipid extraction and separation 48
D. Analytical procedures 49
E. Variation in the results 50

IV. RESULTS 51
A. Marker enzyme and protein content 51
B. Fatty acid content 54
C. Total lipid composition 56
D. Phospholipid composition 57

V. DISCUSSION 60

Chapter 2: **SOME PROPERTIES OF THE $Ca2^{+}$ UPTAKE PROCESS IN RABBIT INTESTINAL BRUSH BORDER MEMBRANES** 63

I. INTRODUCTION 63

II. MATERIALS 63

III. METHODS 63
A. Preparation of BBMV 63
B. D-glucose uptake 64
C. Ca^{2+} Uptake 65
D. Valinomycin-induced membrane potential 66
E. Measurement of free Ca^{2+} concentrations 66
F. Total Ca^{2+} determination 67
G. Sample size 68

IV. RESULTS 68

V. DISCUSSION 87

Chapter 3: **EFFECT OF EXOGENOUS FATTY ACIDS AND METHYL OLEATE ON Ca^{2+} UPTAKE IN BBMV** 94

I. INTRODUCTION 94

II. MATERIALS 96

III. METHODS 97
A. Preparation of methyl oleate 97
B. Preparation of fatty acid and methyl oleate solutions 97
C. Uptake of fatty acids and methyl oleate by BBMV 98
D. Analytical procedures 98

IV. RESULTS 98

V. DISCUSSION 111

Chapter 4: **RELATION BETWEEN Ca^{2+} UPTAKE AND FLUIDITY IN RABBIT BBMV** 117

I. INTRODUCTION 117

II. MATERIALS 118

III. METHODS 118
A. Fluorescence measurements 118
B. Absorbance measurements 119
C. Extractions and reconstitution of total BBMV lipids 120

IV. RESULTS 120

V. DISCUSSION 128

Chapter 5: **PROBING FOR Ca^{2+}-BINDING SITES IN INTESTINAL BRUSH BORDER MEMBRANES WITH A FLUORESCENT LANTHANIDE** 134

I. INTRODUCTION 134

II. MATERIALS 135

III. METHODS 135
A. Fluorescence measurements 135
B. Determination of Tb^{3+} concentration 136
C. Separation of vesicle bound Tb^{3+} from free 137

IV. RESULTS 138

V. DISCUSSION 151

Chapter 6: **PARTIAL CHARACTERIZATION OF Ca^{2+}-BINDING PROTEINS FROM PORCINE INTESTINAL BRUSH BORDER MEMBRANES** 160

I. INTRODUCTION 160

II. MATERIALS 161

III. METHODS 162
A. Preparation of porcine BBMV 162
B. Extraction of BBMV with detergent 163
C. Delipidation of brush border membrane protein extract with 1-butanol 164
D. Assay of Ca^{2+}-binding protein activity 165
E. Sephadex G-150 chromatography 166
F. Ion-exchange chromatography 166
G. FPLC of brush border membrane protein 167
H. Polyacrylamide gel electrophoresis 168
I. Isoelectric focussing 169
J. Phosphodiesterase assay 170
K. Reductive methylation of brush border membrane proteins 171
L. Peptide fingerprint mapping of brush border membrane proteins and calmodulin 172
M. Triton X-114 phase-partitioning assay 173
1. Analysis of ^{125}I-calmodulin binding to BBMV 175
N. ^{125}I-calmodulin gel overlay technique 176
O. Chromatography assay for calmodulin 176
P. Protein determination 177

IV. RESULTS 177

V. DISCUSSION 208

CONCLUSIONS 216

APPENDIX 221

List of References 223
Curriculum vitae 240

ABSTRACT

Structure-function relationships existing in the intestinal brush border membrane were studied with a vesicular preparation of this plasma membrane. This was accomplished by establishing an adequate procedure for the preparation of rabbit brush border membrane vesicles based on their marker enzyme content and lipid composition. The uptake of Ca^{2+} by these vesicles was found to be time, temperature and substrate concentration dependent. Also, this process displayed saturability, did not depend on added energy sources and occurred optimally in a pH range of 7.5-8.0. The equilibrium uptake value for Ca^{2+} accumulation by the vesicles was several fold higher than could be accommodated by the intravesicular space. It was concluded from these results that this uptake process involved complete binding of Ca^{2+} to the membrane.

The binding of a variety of fatty acids to BBMV, increased with acyl chain-length and was not diminished by washing with high ionic strength buffer. This binding of fatty acid by the membrane was not accompanied by a decrease in endogenous lipid and therefore corresponded to a net incorporation into the membranes. The rate of Ca^{2+} uptake by BBMV was stimulated by treatment of the membranes with low concentrations of unsaturated fatty acids (0.05 mM) as well as with various concentrations of octanoic acid (0.10-3.0 mM) and inhibited by treatment with higher concentrations of unsaturated fatty acids (0.20-0.60 mM). Saturated fatty acids had no marked effect on Ca^{2+} uptake. On the other hand, binding of methyl oleate by rabbit BBMV resulted in a concentration-dependent enhancement of the Ca^{2+} uptake rate. The effect of the incorporation of these lipids on the fluidity of the membrane was assessed by fluorescence anisotropy with the hydrophobic membrane probe, diphenylhexatriene (DPH). Oleic acid, linoleic acid and methyl oleate decreased the fluorescence anisotropy of membranes labelled with DPH in a dose-dependent manner. In contrast, the saturated fatty acid, palmitic acid, had no effect on the DPH-reportable order of the membrane within the concentration range used. These results supported the concept that saturated and *cis*-unsaturated fatty acids dissolved in different lipid domains and this, in itself, appeared to be an important factor defining whether the biological function of the membrane was affected by the lipid uptake.

The existence of protein sites capable of binding Ca^{2+} was implicated from studies with the fluorescent lanthanide, Tb^{3+}, which can substitute for Ca^{2+} in metal binding sites. The binding of Tb^{3+} was affected by fluidity changes, brought about by incorporation of oleic acid and its methyl ester into the brush border membrane, in a manner similar to Ca^{2+} binding. However, not all of the bound Tb^{3+} could be

displaced by Ca^{2+} which may point to a class of binding sites peculiar to the lanthanide in addition to sites which bind either cation. These findings prompted further work geared towards the isolation and characterization of proteins from the brush border membrane that were responsible for binding Ca^{2+}. Two Ca^{2+}-binding activities were identified. On the basis of its similarity to bovine brain calmodulin with respect to molecular weight, isoelectric point, affinity for resins, and susceptibility to proteases, one of the Ca^{2+}-binding activities was identified as intestinal calmodulin. The other, partially-purified binding activity was not clearly identified. Furthermore, by a gel overlay technique, the presence of a large molecular weight, membrane-bound protein (M_r=107,000) was revealed in detergent extract of the brush border membrane. This membrane protein bound calmodulin with relatively high affinity (0.14 μM). The role of these proteins in the uptake of Ca^{2+} by the intestinal brush border membrane remains to be determined.

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LIST OF TABLES

I-1 Asymmetric Distribution of Cell Surface Membrane-Bound Enzymes in Rat Intestinal Epithelial Cells (proximal duodenum) 9

I-2 Lipid Fluidity of Preparations of Rat Jejunal Mucosa and Rat Liver as Assessed by the Fluorescence Anisotropy (r) of Various Fluorophores at 25°C 18

I-3 Fluorescence Anisotropy at 23°C of Lipid Fluorophores in Isolated Microvillus and Basolateral Membranes Prepared From the Proximal Half of the Small Intestine of the Rat 19

I-4 Arrhenius Plot Break Points of Enzyme and Transport Activities in Rat Enterocyte Plasma Membranes 20

1-1 Marker Enzyme and Protein Content of Brush-Border Membranes Prepared by Mg^{2+} and by Ca^{2+} Precipitation 52

1-2 Total Fatty Acid Composition of Brush-Border Membranes Prepared by Mg^{2+} and Ca^{2+} Additions 55

1-3 Lipid Composition of Brush-Border Membranes Prepared by Mg^{2+} and Ca^{2+} Additions 58

1-4 Phospholipid Composition of Brush-Border Membranes Prepared by Mg^{2+} and Ca^{2+} Additions 59

2-1 The Effect of Washing with Chelating Agents on Ca^{2+} Uptake 81

2-2 Effect of Metal Cations on Ca^{2+} Uptake 83

3-1 The Effects of Exogenous Fatty Acids on Ca^{2+} Uptake by Brush-Border Membrane Vesicles 101

3-2 Effect of Washing with CsBr on the Uptake of Oleic Acid by Brush-Border Membrane Vesicles 102

3-3 Effect of Methyl Oleate on Ca^{2+} Uptake 104

3-4 Fatty Acid Composition of Brush-Border Membranes Before and After Exogenous Fatty Acid Uptake 108

3-5 Effect of Fatty Acid Uptake on Cholesterol Content of Brush-Border Vesicles 110

5-1 The Effect of EGTA on the $Tb3^{+}$ Fluorescence of Methyl Oleate-Treated BBMV 149

6-1 Summary of the Purification Steps for Peak #1 (Sephadex G-150) Ca^{2+}-Binding Activity 187

6-2 Two Step Column Assay for Calmodulin 190

6-3 Protease Digests of Bovine Brain Calmodulin and Pig Intestinal Ca^{2+}-Binding Protein (Peak 1, G-150) 194

LIST OF FIGURES

2-01 D-glucose uptake by BBMV and the effect of osmolarity 69

2-02 The effect of time and temperature on Ca^{2+} uptake 71

2-03 The effect of Ca^{2+} concentration on uptake 72

2-04 Lineweaver-Burke plot of the data plotted in Figure 2-3 73

2-05 The effect of pH on calcium ion uptake 75

2-06 The effect of osmolarity on uptake 76

2-07 Effect of ionophore A23187 on uptake 78

2-08 Effect of ionophore A23187 on efflux 80

2-09 Effect of Tb^{3+} on Ca^{2+} uptake 84

2-10 The effects on Ca^{2+} uptake of a valinomycin-induced electrochemical potential (inside negative) 86

3-01 Time course of oleic acid and methyl oleate incorporation 99

3-02 The effect of oleic acid on Ca^{2+} uptake 106

3-03 Scatchard plot of Ca^{2+} binding to BBMV 107

4-01 Fluorescence anisotropy versus absorbance 121

4-02 The effect of oleic acid on anisotropy 123

4-03 The effect of linoleic acid on anisotropy 124

4-04 The effect of methyl oleate on anisotropy 126

4-05 Effect of palmitic acid on anisotropy 127

4-06 The lipid extracts of fatty acid-treated brush border membrane vesicles 129

5-01 Various emission spectra 139

5-02 Time course of Tb^{3+} binding to BBMV 140

5-03 Effect of Tb^{3+} concentration and oleic acid treatment on binding of lanthanide to BBMV 142

5-04 Effect of Tb^{3+} concentration and methyl oleate treatment on binding of lanthanide to BBMV 144

5-05 Displacement of Tb^{3+} from BBMV by Ca^{2+} 146

5-06 The effect of EGTA on Tb^{3+} binding by BBMV 148

5-07 Titration of BBMV with Tb^{3+} as measured by DPA complexing 150

6-01 Calcium-binding assay standard curve using Sephadex G-25 spin column method 178

6-02 Optimization of solubilization conditions for Ca^{2+}-binding activity using the non-ionic detergent, Lubrol-PX 180

6-03 Sephadex G-150 elution profile of porcine BBMV extract 182

6-04 Ion-exchange chromatography of Sephadex G-150 peak 1 Ca^{2+}-binding activity 184

6-05 A. Electrophoretic analysis of Sephadex G-150 peak 1 Ca^{2+}-binding protein at various steps in purification 185

B. Isoelectric focussing gels of purified pig Ca^{2+}-binding protein 185

6-06 The Ca^{2+}-dependent activation of PDE by calmodulin and by purified pig Ca^{2+}-binding protein (G-150, peak 1) 188

6-07 SDS-PAGE of calmodulin and pig Ca^{2+}-binding protein after the calmodulin two-step assay procedure 191

6-08 Peptide fingerprint maps of bovine brain calmodulin and pig Ca^{2+}-binding protein 193

A. Tracing of an autoradiogram of an elastase digest of ^{14}C-calmodulin and ^{3}H-pig Ca^{2+}-binding protein 193

B. Tracing of an autoradiogram of a chymotrypsin digest of ^{14}C-calmodulin and ^{3}H-pig Ca^{2+}-binding protein 193

6-09 A. Extraction of BBMV with detergents 195

B. Porcine BBMV extracted with increasing ionic strength 195

6-10	^{125}I-calmodulin binding to Triton X-114 solubilized BBMV	198
6-11	A. ^{125}I-calmodulin gel overlay incubated in the presence of 1 mM Ca^{2+}	200
	B. Autoradiogram of gel A (above)	200
6-12	A. ^{125}I-calmodulin gel overlay incubated in the presence of 1 mM EGTA	201
	B. Autoradiogram of gel A (above)	201
6-13	Ion-exchange chromatography of peak 2 (G-150) Ca^{2+}-binding activity	203
6-14	Isoelectric focussing of peak 2 (G-150) activity after further purification on DEAE-Sephacel	204
6-15	Chromatofocussing of DEAE-Sephacel Ca^{2+}-binding activity (peak 2, G-150)	205
6-16	Electrophoretic protein pattern of various fractions eluted from the FPLC chromatofocussing column (Figure 6-15)	206
6-17	Flow chart summary of the purification steps for the two major Ca^{2+}-binding activities in porcine BBMV	207

LIST OF ABBREVIATIONS

$1,25(OH)_2$-D_3	the active form of vitamin D, i.e., vitamin D_3
107K	107,000 daltons
110K	110,000 daltons
Å	angstrom
ATP	adenosine triphosphate
BBMV	brush border membrane vesicles
BBOT	2,5-di(tert.-butyl-2-benzoxazolyl-) -thiophene
bis-acrylamide	N,N'-methylene-bis acrylamide
CaBP	intestinal calcium-binding protein
CAPS	3-(cyclohexylamino)-1-propane-sulfonic acid
CHAPS	3-[3-(cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate
CoA	coenzyme A
cpm	count per minute
cyclic AMP	cyclic adenosine monophosphate
DEAE-Sephacel	diethylaminoethyl-Sephacel
DMPC	dimyristoylphosphatidylcholine
DOPC	dioleoylphosphatidylcholine
DPA	dipicolinic acid
DPH	1,6-diphenylhexatriene
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ESR	Electron Spin Resonance
FPLC	Fast Phase Liquid Chromatography
HCHO	formaldehyde
HEPES	N'-2-hydroxyl-ethylpiperazine-N'-2-ethanesulfonic acid
IMCal	intestinal membrane calcium binding protein
K_a	association constant
K_d	dissociation constant
K_m	Michaelis-Menton constant
lysoPC	lysophosphatidylcholine
mA	milliampere
MES	2-(N-morpholino)ethanesulfonic acid
mg	milligram
M_r	relative molecular weight
MW	molecular weight
NIH	National Institute of Health
nmol	nanomole
PC	phosphatidylcholine
PCS	Phase Combining System
PDE	phosphodiesterase
PE	phosphatidylethanolamine
pmol	picomole

PMSF	phenyl methyl sulfonyl fluoride
S.D.	standard deviation
S.E.	standard error
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TIU	tyrosine inhibitor units
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)amino methane

INTRODUCTION

This thesis is concerned with structure and function relationships in a biological membrane, the intestinal brush border membrane of columnar epithelial cells which line the luminal surface of the small intestine. Primarily considered in this study, is the potential role that exogenous fatty acids play in modulating the structure of this membrane as well as a biological activity found therein i.e., the Ca^{2+} uptake process. The approach taken has been to alter the lipid composition of rabbit intestinal brush border membranes *in vitro* and to observe the effect this change has on membrane fluidity and Ca^{2+} uptake of these membranes. BBMV, prepared from rabbit intestine, have not been extensively studied. Hence, preparation procedures to isolate vesicles with reproducible properties had to be developed, followed by the characterization of their Ca^{2+} uptake process. These studies led to investigations in order to define in detail the molecular basis for Ca^{2+} uptake and binding by brush border membranes. In this introduction, a review of the information pertaining to this experimental approach will be presented.

I. SOME CHARACTERISTICS OF THE INTESTINAL EPITHELIAL CELL PLASMA MEMBRANE

A. Properties pertaining to the isolation of the brush border membrane and the basolateral membrane

The plasma membrane of intestinal epithelial cells can be functionally divided into two parts; the luminal (brush border membrane) and contraluminal (basolateral membrane) areas. These two membranes exhibit differences in protein and lipid composition. These distinctions are reflected in various biochemical parameters: different buoyant density - determined by lipid to protein ratio - and different surface properties such as surface receptors, surface charge density, and surface polarity - this last property being related to the degree of glycosylation of membrane proteins and lipids. There results from these compositional traits, differences in resistance to osmotic shock or shearing forces (Schmitz et al., 1973) and membrane fragments of different sizes may be formed from the two membranes during homogenization procedures. This, together with differences in buoyant densities, provides the basis for the separation of the two membranes and makes possible the preparation of purified brush border membrane fragments by the technique of differential centrifugation. Upon the addition of certain divalent cations (Schmitz et al, 1973) the brush border membrane is separated from the cytoskeletal apparatus as well as from other contaminating cellular

organelles and membranes, which precipitate. After homogenization, the brush border membrane fragments spontaneously reseal to form intact vesicles suitable for transport and binding studies (Hopfer, 1984).

B. Morphological aspects of the isolated BBMV

When brush border membranes are isolated they form what appear to be, under the electron microscope, closed vesicle structures. Electron micrographs (Murer and Hildmann, 1984) of brush border membranes *in situ* have revealed that the external membrane surface is coated with a fuzzy structure, probably representing the glycocalyx, which can be clearly recognized in high magnification thin section pictures, especially after staining with ruthenium red. However, upon preparation of BBMV most of the glycocalyx is removed (Biber et al., 1981). This was concluded on the basis of biochemical and microscopical examination of the isolated membranes.

The orientation of these isolated brush border membranes (inside-out versus right-side out), was determined by Haase et al. (1978) who used two approaches to study this problem in vesicles isolated from rat proximal tubules and from intestinal epithelia: freeze-fracture electron microscopy and immunological techniques. Both methods led to the conclusion that almost all (98%) of the brush border vesicles were oriented right-side out.

C. Lipid composition of BBMV

Relatively few detailed studies have been concerned with characterizing the lipid composition of the brush border membrane. This plasma membrane is complex with respect to its lipid composition and several workers have reported that the microvillus membrane of intestinal epithelial cells is rich in cholesterol and glycolipids, but poor in phospholipids (Kawai, et al., 1974; Hauser et al., 1980; Mansbach et al., 1982; Pind and Kuksis, 1987). The major phospholipids in both rabbit and rat brush border membrane are choline- and ethanolamine- containing. The former, consisting largely of phosphatidylcholine (PC) and sphingomyelin, make up about 45% (40% in the rat brush border membrane) of the total lipid phosphorus, the latter about 38-40% (Hauser et al., 1980). However, the relative proportion of these two major phospholipids is somewhat different as reported for mouse brush border membranes by Kawai et al. (1974), being 49% phosphatidylethanolamine (PE) and 25% PC. The fatty acids of the phospholipids in the mucosal membrane are characterized by a relatively high degree of unsaturation with a weight ratio of unsaturated to saturated fatty acid being 1.40 in the rabbit brush border membrane (Hauser et al., 1980).

Although the lipid composition of the brush border membrane has not completely been analyzed, it is clearly evident from available reports that its composition differs

remarkably from that of the basolateral membrane (Kawai et al., 1974; Hauser et al., 1980; Yakymyshyn et al., 1982; Christiansen and Carlsen, 1981; Mansbach et al., 1982; Verger et al., 1982). The lipid content of the mouse brush border membranes was found to be approximately 0.61 mg per mg protein while the proportion of lipid in the basolateral membranes was reported to be much larger, 0.95 mg per mg protein (Kawai et al., 1974). These same investigators found the molar ratio of cholesterol, phospholipid, and glycolipid to be 1:1:1 in microvillus membranes and 1:2.5:0.3 in basolateral membranes. Cholesterol is much more abundant in the microvillus membrane than in the basolateral membrane with approximately 70% of the neutral lipid fraction of the former being cholesterol; the remaining fraction consisted primarily of diglycerides and free fatty acids. Brasitus and Schachter (1980) reported the cholesterol:phospholipid ratio for the rat microvillus membrane to be 0.87:1 while for the basolateral membrane this ratio was 0.62:1. Also, glycolipids are surprisingly abundant in the microvillus membrane but not nearly so in the basolateral membrane (Kawai et al., 1974).

Most methods for the isolation of BBMV use as precipitant of undesired organelles such as Golgi apparatus membranes, nuclei, microsomes, mitochondria and basolateral membranes, either 10 mM $CaCl_2$ or $MgCl_2$ (Schmitz et al., 1973; Selhub and Rosenberg, 1981; Max et al., 1978; Kessler

et al., 1978; Stieger and Murer, 1983; Hauser et al., 1980; Yakymyshyn et al., 1982; Christiansen and Carlsen, 1981; Del Castillo and Robinson, 1982). A report by Hauser et al. (1980) indicated, however, that the better method for preparing BBMV from rabbit small intestine involved precipitation with $MgCl_2$ rather than $CaCl_2$. In their study, the use of the latter precipitating agent apparently caused an activation of brush border membrane phospholipase A which resulted in a relatively high accumulation of free fatty acids and lysophospholipids. They also found that the level of phospholipid breakdown decreased substantially when $MgCl_2$ was used as the precipitant instead of $CaCl_2$.

Reports of other investigators, (Mansbach et al., 1982; Verger et al., 1982; Subbaiah and Ganguly, 1970; Bonnefis et al., 1977; and Bonnefis et al., 1978), however, did not verify the findings of Hauser et al., the values reported for the content of lysophospholipids in BBMV preparations being substantially lower than those published by Hauser et. al. In fact, in studies where investigators made use of Ca^{2+} as the precipitant, the lysoPC content in rat BBMV was found to be between 3% and 8%, (Chapell and Gilles-Baillien, 1983; Brasitus and Schachter, 1980), and no or very little lysoPE was found in these membranes.

The accumulation of lysophospholipids derived from PE and PC is difficult to envision since there appears to be some controversy concerning the substrate specificity and

the mode of action of the phospholipase(s) of microvilli membranes. Indeed, recent reports (Mansbach et al., 1982; Verger et al., 1982) indicated a strong preference of phospholipase A for phosphatidylglycerol, although the substrate specificity of the enzyme was broad. These authors proposed that the intestinal enzyme may be specialized for degrading bacterial lipid which often contains large amounts of phosphatidylglycerol. Additionally, a previous study by Subbaiah and Ganguly (1970) had shown that at the pH range used for preparing BBMV (7.0-7.5) mainly phospholipase B activity was detected (when the enzyme was tested against PC) with little lysoPC accumulation.

A possible explanation for the divergent results obtained by Hauser et al. is that an endogenous phospholipase A of broad specificity is present in the brush border membrane which would not normally degrade endogenous lipids even in the presence of Ca^{2+} unless the membrane integrity was sufficiently affected. Membrane damage by conditions such as freezing and thawing, as used by Hauser et al., could conceivably activate an endogenous phospholipase A in the BBMV.

In a quest for the preparation of a high quality BBMV fraction displaying a minimum breakdown of lipids due to intrinsic phospholipase A activation it was deemed

necessary to conduct a study to evaluate available methods involving precipitation with either Mg^{2+} or Ca^{2+}.

D. Proteins and glycoproteins of BBMV

The microvillus membrane from rabbit small intestinal cells contains nearly 50% protein by weight (Hauser et al., 1980), most of which is constituted by enzymes. The electrophoretic patterns on polyacrylamide gels of brush border membranes isolated from rat intestine demonstrate the presence of at least 25-30 protein bands and about 10 glycoprotein bands (Starita-Geribaldi et al., 1977; Sigrist-Nelson et al., 1977). Their molecular weights range from 25,000 to over 400,000. Interestingly, the presence of prominent bands with high molecular weight seems to be a unique characteristic of the brush border membrane, unlike other plasma membranes.

Table I-1 lists some of the enzyme activities localized in the brush border membrane. Whether the enzymes of this membrane are attached to or inserted within the outer surface of the membrane has not been completely defined (Ugolev et al., 1979). Typical marker enzymes for this membrane are sucrase and alkaline phosphatase, with the former considered as the best indicator of the brush border membrane.

TABLE I-1

Asymmetric Distribution of Cell Surface Membrane-Bound Enzymes in Rat Intestinal Epithelial Cells (proximal duodenum)

	Basolateral membranes	Brush border membranes
	Percentage distribution	
Na^+,K^+-ATPase	100	
Adenylate cyclase	100	
Sucrase		100
Aminopeptidase M		100
γ-Glutamyl transpeptidase		100
Ca^{2+}-ATPase	66	34
Acid phosphatase[a]	28	4
Mg^{2+}-ATPase[a]	20	6
Guanylate cyclase[a]	11	63
Alkaline phosphatase	35	65

[a]The sum is not 100% because the remaining enzyme activity is found to be associated with intracellular structures. Experiments suggest that anion-stimulated Mg^{2+}-ATPase is also a constituent of brush border membranes (Humphreys et al. 1980; Humphreys and Chou 1979).

II. MODIFICATION OF THE LIPID COMPOSITION OF THE BRUSH BORDER MEMBRANE

A. Uptake of lipids by the brush border membrane

A number of studies to date have been concerned with the uptake of lipids by the intestinal brush border membrane and have involved the incorporation of free fatty acids. The abundance of investigations appears to stem from the interest in the digestion and absorption process.

It is known that hydrolysis of long-chain triglycerides by pancreatic lipase is an obligatory step before their subsequent absorption as fatty acids can occur (Go et al., 1970). The absorption of fatty acids by the gut membrane is believed to be a passive process that is increased by the presence of bile salt micelles (Westergaard and Dietschy 1976). These investigators proposed that the principal role of the micelle in facilitating lipid absorption is to overcome unstirred layer resistance, while the actual process of fatty acid absorption occurs through a monomer phase in equilibrium with the micelle. Studies with BBMV, however, have indicated the involvement of lipid aggregates in the uptake process (Proulx et al., 1984).

Absorption of fatty acids and alcohols is a passive process, being linearly related to concentration, independent of metabolic energy supply, and not influenced by potential competitive inhibitors (Sallee and Dietschy, 1973). Also, lipid uptake clearly indicates a partitioning process with the lipid bilayer of the membrane because of

the influence of acyl chain length on uptake (Proulx et al., 1985; Sallee, 1978). However, despite the strong experimental evidence for the concept of passive uptake of lipids in the mucosal membrane, several reports have suggested that lipid uptake may include binding or carrier-mediated transport (Mishkin, et al., 1972; Mishkin et al., 1975; Stremmel et al., 1985). A fatty acid binding protein was identified in the cytosolic fraction of the enterocyte and it was suggested by Ockner and associates (1974) that this protein may assist in the transfer of fatty acids from the brush border membrane to the smooth endoplasmic reticulum. More recently, Stremmel et al. (1985) isolated a fatty acid-binding protein from purified rat brush border membranes. They succeeded in partially characterizing this protein and discovered that it preferentially bound oleic, palmitic, arachidonic and linoleic acids but not the oleate ester of cholesterol, phosphatidylcholine or taurocholate. Intriguingly, this binding of fatty acids by the protein could be distinguished from direct partitioning of the acids into the microvillus membrane. Further studies are needed to determine the level of involvement of this protein in fatty acid binding as well as it's role in fatty acid absorption <u>in vivo</u>.

Many of the earlier studies involving the uptake of fatty acids by intestinal cells were conducted using a number of classical methods including everted intestinal

of fatty acids, electrolytes, ions etc., outside the sac and their uptake by the intestinal epithelium is quantified by measuring the uptake of these solutes with time. Alternatively, the tissue is analyzed directly for uptake.

One such study was carried out by Profirov (1981), who incubated intestinal segments from chicken duodena with liposome suspensions consisting of different cholesterol/phospholipid ratios. He found that this treatment led to changes in the brush border membrane cholesterol/phospholipid ratio.

In another study, Chow and Hollander (1978) used everted intestinal sacs of the rat to demonstrate that the absorption of arachidonic and linoleic acid proceeded by dual mechanisms of uptake: at the low micromolar range of concentrations, facilitated diffusion was prominent, whereas at millimolar concentrations, simple diffusion was the dominant mechanism of absorption. The absorption of these two fatty acids was increased with thinning of the unstirred water layer and a decrease in pH.

The research group headed by Sallee (University of Texas, Dallas) carried out a number of investigations on the rate of uptake and permeability of various fatty acids by rat intestinal epithelia (Sallee et al., 1972; Sallee and Dietschy, 1973; Sallee 1974). In these studies it was reported that the uptake of fatty acids and bile salts in the rat jejunum was perfectly linear with respect to

rate of uptake and permeability of various fatty acids by rat intestinal epithelia (Sallee et al., 1972; Sallee and Dietschy, 1973; Sallee 1974). In these studies it was reported that the uptake of fatty acids and bile salts in the rat jejunum was perfectly linear with respect to concentration, demonstrated no competitive inhibition, had low temperature dependency, and was insensitive to metabolic inhibition, indicating that the uptake proceeds by passive diffusion. Also, it was shown that uptake of short-chain fatty acid monomers was rate limited by the cell membrane but diffusion through the unstirred water layer became increasingly important as the chain length increased.

Bennet-Clark (1978) found that the initial rates of oleic acid uptake were slower than the initial rates of mucosal esterification of these absorbed fatty acids to form triglyceride. These results substantiated the earlier findings of Marubbio et al. (1974), who showed that intracellular pools of oleic acid effectively regulate oleic acid uptake.

Also, the feeding of a high fat diet for two weeks led to the enhancement of the uptake of fatty acid and cholesterol (Thomson and Dietschy, 1983); absorption of these lipids was also higher after feeding of a low- rather than a high-cholesterol diet and was lower after feeding a low- rather than a high-protein diet. Although the diet-related changes may be partially attributed to variation in

the surface area of the membrane or to possible changes in the effective resistance of the intestinal unstirred water layer, the lipid composition of the brush border membrane may also be affected, leading to differences in the membrane permeability properties.

More recently some studies have focussed on the uptake of fatty acids involving less complicated systems i.e., BBMV (Sallee, 1978; Maenz and Forsyth, 1982; Proulx et al., 1985). Sallee found that the fatty acid uptake process by rat BBMV reaches equilibrium very rapidly, which was later confirmed by Proulx et al. (1985), using rabbit BBMV. However, Sallee used a BBMV preparation that was probably more contaminated by organellar membranes as has been since demonstrated by Steiger and Murer (1983). Nonetheless, Sallee found that the partition coefficient increased with fatty acid chain length by a factor which corresponded to an incremental free energy of -820 cal/ml per methylene group. The findings of Proulx et al. (1985) indicated that the rates of fatty acid incorporation into rabbit BBMV were too rapid to be effectively measured such that only equilibrium or near equilibrium values could be reported for uptake. These investigators found that fatty acid binding to the membrane increased with chain length up to 16 carbons, i.e., the degree of uptake could be directly related to the relative solubility of the fatty acid in the apolar phase of the membrane. However, in all these aforementioned studies

no attempt was made to correlate the uptake of fatty acids with an effect on the structure and biological function(s) of the brush border membrane.

III. EFFECTS OF LIPID MODIFICATION ON STRUCTURE--FLUORESCENCE ANISOTROPY STUDIES

A. Monitoring changes in membrane fluidity using fluorescence anisotropy

In concert with asserting the effect of lipids on the biological activity of a membrane function it was desirable to quantify, by some sensitive method, any simultaneous alterations in the "fluidity"[1] of the membrane system under study. In fact, in order to understand more completely specific modulatory properties of lipids on biological function, one must be able to describe the structure of the lipid phase, the topological distribution of lipids in membranes as well as the physical constraints imposed by the actual lipid:protein ratios. These aspects may be investigated by a biophysical technique. Shinitzky (1984) arguably claimed that "the most sensitive and direct tool for evaluation of lipid fluidity parameters on a

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[1]The term "fluidity" as applied to model bilayers and natural membranes is used here to express the relative motional freedom of the lipid molecules or substituents thereof. When assessed by the estimation of steady-state fluorescence anisotropy of the fluorophore, diphenylhexatriene, changes in the anisotropy may be due to alterations in the correlation time (rate of motion) and/or maximal hindered anisotropy of the probe (angular range of motion). The term "fluidity" is used here to describe both kinds of alterations indiscriminately.

submacroscopic level is fluorescence polarization or anisotropy."

Steady-state anisotropy measurements with hydrophobic probes such as diphenylhexatriene (DPH) had been used to derive the "microviscosity" of the bilayer (Shinitzky, 1984). This approach was very approximate for a lipid probe undergoing restricted motion in a partially-ordered environment such as a membrane bilayer. Time-resolved fluorescence experiments have shown that this is the case; since the anisotropy depends on the rotational correlation time and an order parameter (Janig, 1979; Pottel et al. 1986). In steady state fluorescence experiments, the relative contributions of motion and order are difficult to evaluate. Hence, it appears wiser to consider the fluorescence anisotropy as an empirical measure of fluidity or mobility restriction (Shinitzky, 1984).

B. <u>Fluorescence anisotropy studies in brush border membranes</u>

The interactions of the proteins and lipids in the microvillus membrane is not well understood although it is increasingly recognized that such interactions can influence protein functions in many membrane types. Brasitus et al. (1979) reported that rat microvillus membrane lipid had relatively low fluidity and exhibited a characteristic thermotropic transition at 26 ± 2°C as determined by fluorescence anisotropy with DPH. The lipid fluidity of the plasma membrane was decreased relative to that of the other

organelles and this was generally true for the microvillus membrane as well. This trend was seen in comparisons of rat enterocyte total homogenates versus purified microvillus membranes and in corresponding fractions prepared from rat liver (Table I-2). There is considerable evidence that the microvillus membrane is much less fluid than the basolateral membrane (Brasitus and Schachter, 1980). The fluorescence anisotropy values for some fluorescent lipid probes revealed this difference (Table I-3).

Protein-lipid interactions in the enterocyte plasma membrane have been explored further by examination of Arrhenius plots of membrane enzymes and transport activities in relation to their lipid thermotropic transitions. Table I-4 lists the break points observed for a number of microvillus membrane protein activities. These enzyme activities can be categorized into two groups. The first includes the digestive enzymes lactase, maltase, sucrase, leucine amino peptidase and γ-glutamyl transpeptidase which do not appear to experience the effects of bulk lipid transition on function (Brasitus and Schachter, 1980). These are designated 'extrinsic' activities of the membrane. Each activity of the second group, including Ca^{2+}- and Mg^{2+}-dependent ATPases, p-nitrophenyl phosphatase, guanylate cyclase and sodium-dependent transport of D-glucose, show a change in slope of the Arrhenius plot in the temperature

TABLE I-2

Lipid Fluidity of Preparations of Rat Jejunal Mucosa and Rat Liver as Assessed by the Fluorescence Anisotropy (r) of Various Fluorophores at 25°C

Tissue	Fluorescence probe	Preparation	Fluorescence anisotropy(r)	Ref.[a]
Jejunal mucosa	Diphenylhexatriene	Total homogenate	0.241	1
		Microvillus membrane	0.302	
	12-Anthroyloxy stearate	Total homogenate	0.108	
		Microvillus membrane	0.155	1
	Retinol	Total homogenate	0.275-	1
		Microvillus membrane	0.298	
Liver	Diphenylhexatriene	Total homogenate	0.154	2
		Plasma membrane	0.267	

[a]Reference: 1, Schachter and Shinitzky, 1977; 2, Livingstone and Schachter, 1980.

TABLE I-3

Fluorescence Anisotropy at 23°C of Lipid Fluorophores in Isolated Microvillus and Basolateral Membranes Prepared From the Proximal Half of the Small Intestine of the Rat

Fluorophore	Fluorescence anisotropy (r)		Fluorescence lifetime (τ_F) (ns)	
	Microvillus membrane	Basolateral membrane	Microvillus membrane	Basolateral membrane
Diphenylhexatriene	0.285	0.219	11	11
2-Anthroyloxystearate	0.134	0.108	12	12
12-Anthroyloxystearate	0.109	0.081	14	16
Retinol	0.275	0.221	8	8
Dansylphosphatidyl-ethanolamine	0.162	0.142	14	14

Data from Brasitus and Schachter (1980)

TABLE I-4

Arrhenius Plot Break Points of Enzyme and Transport Activities in Rat Enterocyte Plasma Membranes

Membrane	Activity	Break point temperature (°C)	Ref.[a]
Microvillus	Lactase	none	1
	Maltase	none	
	Sucrase	none	
	γ-Glutamyl transpeptidase	none	
	Leucine aminopeptidase	none	
	p-nitrophenylphosphatase	29 ± 0.7	
	Calcium ATPase	26 ± 0.1	
	Magnesium ATPase	28 ± 1.5	
	Na^+-dependent glucose transport	27 ± 1.3	
	Guanylate cyclase	30 ± 1.0	2
Basolateral	5'-Nucleotidase	28 ± 2.1	3
	Adenylate cyclase		
	Basal	30 ± 1.6	
	NaF-stimulated	30 ± 1.5	
	PGE_1-stimulated	29 ± 0.6	
	$(Na^+ + K^+)$-dependent ATPase	22 ± 1.5	
	K^+-dependent p-nitrophenyl-phosphatase	20 ± 1.0	
	Magnesium-dependent ATPase	20 ± 1.3	

[a]References: 1, Brasitus et al., 1979; 2, Brasitus and Schachter, 1980; 3, Brasitus and Schachter, 1980.

range 26-30°C and are deemed `intrinsic' membrane activities.

Schachter and Shinitzky (1977) carried out an extensive study of rat intestinal microvillus membranes using fluorescence polarization. Although these workers did not treat the membranes with lipid their results are interesting and enlightening with respect to protein and lipid structure. It was found that the degree of fluorescence polarization of DPH was exceptionally high in these membranes. Both the lipids and proteins were found to contribute to the reported values. Another set of experiments was completed by Brasitus et al. (1980), who compared the technique of differential scanning calorimetry (DSC) with fluorescence polarization and found that the fluorescence polarization experiments detected the lower critical temperature of the lipid transition in rat BBMV. Calorimetry of the BBMV preparations showed a reversible lipid thermotropic transition in the range of 23-29°C. It was concluded that fluorescence polarization and DSC gave comparable results with the latter technique reporting a broader phase transition.

C. <u>Effect of fatty acids on BBMV structure as measured by fluorescence anisotropy</u>

Studies dealing with the effects of incorporated fatty acids on the structure of the brush border membrane as monitored by a physical technique were few and the need for a more complete study was evident. Only two investigations

to date have dealt with this subject. Bikle et al. (1984) incorporated the methyl esters of cis- and trans-vaccenic acid into chicken BBMV and found that both cis- and trans-vaccenic acid methyl esters lowered the degree of polarization of the BBMV. This effect was seen whether the BBMV had been isolated from vitamin D-replete or -deficient chicks. In this study, however, the amount of the methyl esters that was incorporated was not determined, neither was more than one concentration of these fatty acid derivatives used, so it could not be determined as to whether the effect was concentration dependent or not. A related study was conducted by Putkey et al. (1982) who fed chicks a diet deficient in essential fatty acids and compared the isolated brush border membranes of these with membranes from control animals. Essential fatty acid deficiency resulted in a significant alteration of the fatty acid patterns of the mucosal lipids. In the essential fatty acid-deficient group the levels of linoleic acid were reduced to 50% of the essential fatty acid-containing groups. Arachidonic acid levels remained low, while myristic, palmitoleic and eicosatrienoic acid appeared in measurable amounts in the essential fatty acid-deficient groups, but were absent in the essential fatty acid-containing groups. The results demonstrated that essential fatty acid-deficiency in the chick resulted in an incorporation of the short chain fatty acid, palmitoleic and eicosatrienoic acid in compensation

for the loss of linoleic acid. It was suggested by these workers that this substitution might reflect a compensation mechanism, similar to that of the unsaturated fatty acid auxotroph of Escherichia coli, which enables the intestinal mucosal membranes to maintain their physical and transport properties in vivo despite an alteration in the fatty acid composition. As a result of this compensatory mechanism, no change was seen in the lipid fluidity of the brush border membranes when studied by ESR using I(12,3) as a spin label.

From an assessment of the above-mentioned studies it became very obvious that a more detailed study of fatty acid uptake by the intestinal mucosal membrane was needed. A complimentary in vitro approach to the uptake of fatty acids by isolated brush border membrane vesicles was required in order to circumvent difficulties resulting from compensatory mechanisms operating in vivo. This in vitro approach would make possible, for example, the evaluation of transitory effects of fatty acids on the overall structure of the membrane as monitored by fluorescence anisotropy. In this kind of study, different concentrations of various fatty acids, differing in chain length and degree of unsaturation, could be used in order to examine possible correlations between fatty acid structure and amounts taken up with alterations in membrane fluidity.

IV. EFFECT OF FLUIDITY CHANGES ON MEMBRANE FUNCTION--BBMV FUNCTION

A. Effect of fatty acids on membrane function--some general examples

There exists a number of studies which were conducted to assess the effect of free fatty acids on membrane function. Klausner et al. (1980) found that free fatty acids readily intercalate into the membrane and produce significant changes in the packing of the lipid molecules. These membrane alterations can be divided into two patterns: 1) *cis*-unsaturated fatty acids disorder the membrane interior and order the head group region. 2) *trans*-unsaturated and saturated fatty acids cause little or no alteration in the bilayer interior and order the head group region. Accordingly, Wetzker et al. (1983) found that oleic acid regulated the Ca^{2+}-ATPase of red blood cell membranes in a concentration-dependent fashion, low concentrations caused an activation of the pump whereas high concentrations inhibited the ATPase. This regulation of Ca^{2+}-ATPase by oleic acid was attributed to the ability of this fatty acid to bind to the pump itself.

In another study, Simmonds et al. (1982) incorporated purified (Ca^{2+} + Mg^{2+})-ATPase with mixtures of fatty acids and lipids. These investigators found that oleic acid inhibited ATPase reconstituted with dioleoylphosphatidylcholine (DOPC). The effects could be reversed by addition of high concentrations of bovine serum

albumin to complex the fatty acid. However, the addition of oleic acid to the ATPase reconstituted with dimyristoylphosphatidylcholine (DMPC) initially resulted in enhanced enzyme activity and then, at higher concentrations of fatty acid, led to decreased activity. The authors speculated that the fatty acid binding at the phospholipid/protein interface led to a decrease in activity; whereas, binding at non-annular sites led to an increase in activity, depending on the phospholipid in the system. It is important to realize that this study was conducted with the fully-uncoupled ATPase and so reflected direct effects of fatty acids on the ATPase.

Another interesting study was done by Colard et al. (1980) who measured adenylate cyclase activity of the rat liver plasma membrane simultaneously with the incorporation of acyl chains into the membrane phospholipids using oleoyl CoA, linoleoyl CoA or arachidonoyl CoA. The basal, fluoride-, and glucagon-stimulated adenylate cyclase activities were increased by the incorporation of linoleate into the plasma membrane phospholipids. Oleoyl CoA did not alter the adenylate cyclase activities whereas arachidonoyl CoA, at high concentration, decreased the adenylate cyclase activity. These findings indicated a specific effect of phospholipid molecular species containing linoleate on this membrane-bound enzyme.

It has also been established by Pilch et al. (1980) that glucose transport in adipocyte plasma membranes was dependent on the fluidity of the membrane and the rate of transport of this sugar was modified with fluidizing agents, one of which was cis-unsaturated fatty acids. However, similar experiments with intestinal brush border membranes failed to show an effect of increased fluidity on glucose transport (Proulx, unpublished observations).

B. Effects of fatty acids on brush border membrane functions

In this area of study, investigations have been performed using BBMV from a variety of species; however, most have involved rat and chicken membrane preparations and have been correlated with the effects of vitamin D. Kreutter et al. (1983) succeeded in altering the lipid composition of the brush border membrane by raising chicks on a vitamin D, essential fatty acid-deficient diet and measured Ca^{2+} absorption from duodenal sacs *in situ* and Ca^{2+} uptake into brush border membrane vesicles *in vitro*. It was found that the rate of Ca^{2+} uptake increased with an increase in the linoleic acid content of the brush border membrane as measured by both *in situ* and *in vitro* techniques. However, it must be recognized that these results were complicated by the pathological effects of fatty acid-deficiency.

A study by Fontaine et al. (1981) was reported in which the effects of cis- and trans-vaccenic acid and their

corresponding methyl esters on the rates of Ca^{2+} uptake were evaluated. It was reported that methyl *cis*-vaccenic acid caused an increase in the rate of Ca^{2+} uptake into vesicles from vitamin D-deficient chickens but not in those from vitamin D-treated animals. Conversely, methyl *trans*-vaccenic acid caused a decrease in the rate of Ca^{2+} uptake in vesicles from vitamin D-treated chickens but no change in vesicles from vitamin D-deficient controls. The results of this study were not validated by a later study conducted by Bikle et al. (1984), who found that both methyl esters of *cis*- and *trans*-vaccenic acid markedly increased Ca^{2+} uptake at all temperatures studied, the *trans* isomer being more effective.

Putkey et al. (1982) determined the effects of essential fatty acid-deficiency on vitamin D-mediated intestinal Ca^{2+} transport in chickens. Essential fatty acid-deficiency did not affect the increase in serum Ca^{2+} in response to vitamin D. Similarly, the increased Ca^{2+} transport (*in vivo*) in response to vitamin D was not altered by essential fatty acid deficiency. It was seen that vitamin D administration enhanced Ca^{2+} absorption 4-5 fold in both essential fatty acid-containing and essential fatty acid-deficient groups. In contrast to both serum Ca^{2+} levels and Ca^{2+} transport *in vivo*, the vitamin D-dependent modulation of Ca^{2+} flux as measured *in vitro* was dependent on the essential fatty acid status of the chick.

A study of the intestinal secretagogue, ricinoleic acid, was reported by Maenz and Forsyth (1982), who discovered that this fatty acid acted as a Ca^{2+} ionophore when incubated with pig BBMV. The result was a marked stimulation in the accumulation of Ca^{2+} by the vesicles at low Ca^{2+} concentration (10 µM) which paralleled the effect seen in the same preparations with the Ca^{2+} ionophore, A23187. These results implied, potentially, a very interesting function for transiently-incorporated fatty acids in the brush border membrane.

As alluded to in the above-stated studies the effects of vitamin D on the mucosal cell membrane have been well documented (Adams et al., 1970; Fontaine et al., 1981; Rasmussen et al., 1979). It was proposed by Rasmussen et al. (1982) that the initial, acute action of this hormone was to alter the fluidity of the brush border membrane which was correlated with an increase in Ca^{2+} transport both *in vitro* and *in vivo* (Matsumoto et al., 1980; Bikle et al., 1984). However, despite the evidence favoring the regulation of brush border membrane fluidity by cholecalciferol, the actual mechanism by which this occurs in the membrane remains obscure.

Obviously, there was a general lack of experiments strictly aimed at correlating the uptake of fatty acids by BBMV with changes in structure and function. Therefore, part of the experimental approach of this thesis was to

examine the effect of an alteration in the lipid composition of the brush border membrane, procured by modifying the fatty acid content *in vitro* on the uptake of Ca^{2+} as well as the fluidity of the membranes. Such relationships between fluidity and Ca^{2+} uptake are still not firmly established. In addition to providing insight into the lipid-protein interactions of this membrane, studies of this nature are apt to shed light on the mechanism whereby this physiologically-important cation is moved across the brush border membrane.

V. SOME ASPECTS OF THE MOLECULAR BASIS FOR Ca^{2+} UPTAKE BY THE BRUSH BORDER MEMBRANE

The transfer of Ca^{2+} from the gut lumen to the lamina propria can be divided into three distinct steps: the transfer of Ca^{2+} across the brush border membrane; transfer through the cell cytoplasm; and extrusion across the basolateral membrane of the cell. The process whereby Ca^{2+} is moved across the brush border membrane involves a rather steep concentration gradient, the Ca^{2+} concentration inside the cell being rather small; and since cations do not readily diffuse across bilayers, it has been proposed that this translocation occurs by facilitated diffusion and does not require cellular energy in the form of ATP (Bronner et al., 1984; Bikle et al., 1983; Wilson and Lawson, 1980; Miller and Bronner, 1981; Miller et al., 1982). Although the brush border membrane is equipped with an ATPase-

alkaline phosphatase complex, it is unlikely that ATPase activity is involved as a Ca^{2+} pump since the affinity of this enzyme for Ca^{2+} is low (Ghijsen et al., 1980). Nonetheless, it has been suggested by some that Ca^{2+} fluxes across the brush border membrane are energy dependent being stimulated by ATP (Somermeyer et al., 1983; Maenz and Forsyth, 1983). Contradictory to the findings of the latter two investigators is the report of Gmaj et al. (1979), who previously found that a primary active ATP-driven pump was present in the basolateral plasma membrane but not the brush border membrane in rat kidney.

Studies performed with BBMV isolated from rat duodena have demonstrated that the uptake of Ca^{2+} in these membranes is saturable and involves mostly binding (Bronner et al., 1984; Bikle et al., 1983; Wilson and Lawson, 1980). This binding process seems to depend on the association of Ca^{2+} with interior sites of the membranes in the rat (Miller and Bronner, 1981; Miller et al., 1982) but to a lesser extent in the chick, in which case, Ca^{2+} is largely found in the free state trapped inside the vesicles (Rasmussen, et. al., 1979). Miller and Bronner (1981), using rat BBMV, succeeded in characterizing both high and low affinity Ca^{2+}-binding sites by Scatchard analysis and identified the lower affinity sites, with a reported K_d of 3.5 mM, as phospholipid in nature. These investigators suggested that

the higher affinity sites were located on Ca^{2+}-binding proteins with a K_d of 1.5 µM.

A. Calcium uptake and the Ca^{2+}-binding proteins of the brush border membrane

1. Calbindin (CaBP)

In addition to its vitamin D-dependency, the concentration of this small, cytosolic protein in the intestine varies directly with the efficiency of intestinal Ca^{2+} absorption under a wide variety of physiological and nutritional circumstances (Wassermann and Corridino, 1973). Two general types of CaBP have been identified, the avian type of 28,000 M_r and the mammalian type of about 9,000-11,000 M_r. The former binds 4 atoms of Ca^{2+} per molecule and the latter, 2, with high affinity. The apparent K_a of both types is about 2 x 10^6 M^{-1} (Bruns et al., 1977; Dorrington et al., 1974). The amino acid sequence of bovine intestinal Ca^{2+}-binding protein (Fullmer and Wassermann, 1981) and porcine (Hofman et al., 1979) have been sequenced and the three-dimensional structure of the bovine CaBP have been determined crystallographically to 2.3 Å (Szebenyi et al., 1981).

Some microscopic (Taylor and Wassermann, 1970) and biochemical studies (Corradino et al., 1976) suggested a subcellular localization of CaBP in the microvillus membrane and implicated CaBP as being responsible for the movement of Ca^{2+} across this membrane. However, Thorens et al. (1982) demonstrated by using light

and electron microscopy immunolabelling techniques, that CaBP was present in the cytosol and euchromatin of absorptive cells, which was compatible with a role for the protein in the regulation of intracellular Ca^{2+} concentration.

One proposal for the function of CaBP was that it translocated Ca^{2+} from the apical to the basal region of the intestinal cell (Schachter, 1969). This idea was feasible since CaBP, with its high affinity, could decrease or prevent the sequestration of Ca^{2+} by mitochondria and endoplasmic reticulum, maintaining intracellular Ca^{2+} at nontoxic levels during its transcellular passage.

2. Alkaline phosphatase-Ca^{2+}-ATPase complex

The involvement of alkaline phosphatase-Ca^{2+}-ATPase in Ca^{2+} uptake and absorption has been proposed by many (Morrissey et al., 1978; Feher and Wassermann, 1979; Schiffle and Binswanger, 1980). The reason for this is clear. (1) Enzyme activity correlates with the degree of Ca^{2+} absorption in different parts of the intestine; (2) such correlations were observed between enzyme activity and Ca^{2+} transport under different conditions (Shiffle and Brunswanger, 1980); and (3) the activity of a Ca^{2+}, Mg^{2+}-dependent ATPase present in intestinal brush borders was shown to be vitamin D-dependent (Melancon and DeLuca, 1970).

However, the involvement of alkaline phosphatase in Ca^{2+} transport is no longer as generally accepted since it was shown that the K_m for Ca^{2+} activation of the enzyme is much larger than the K_m for Ca^{2+} transport and the enzyme shows a lack of specificity towards divalent cations. Furthermore, it is now established that the increase in the synthesis of alkaline phosphatase caused by vitamin D lags well behind that of Ca^{2+} transport (Bikle 1979).

3. Intestinal membrane Ca^{2+}-binding protein

One of the most extensive investigations of vitamin D-dependent Ca^{2+}-binding proteins of intestinal brush border membranes has been conducted by Kowarski and Schachter (1980). By following the vitamin D-dependent binding of Ca^{2+} in membrane extracts and in fractions obtained from separation procedures, these workers were able to develop a purification procedure for one of the binding proteins. This procedure consisted of gel filtration on Sephadex G-150 followed by spheroidal hydroxylapatite column chromatography. In eluates of the Sephadex G-150 column they observed a major peak of activity in a high molecular-weight fraction (200,000-250,000), and this same fraction contained Ca^{2+}-ATPase and p-nitrophenyl phosphatase activities. This Ca^{2+}-binding activity was resolved on columns of spheroidal hydroxylapatite. The resulting purified Ca^{2+}-binding protein was named IMCal, an acronym

for intestinal membrane Ca^{2+}-binding protein. Initial characterization of IMCal revealed a M_r of 200,000, which could be dissociated to yield units of M_r 20,500. This Ca^{2+}-binding activity was previously shown to correlate positively with known features of the intestinal Ca^{2+} transport mechanism i.e., distribution in the small intestine, effects of vitamin D, dietary Ca^{2+}, and rat age (Kowarski and Schachter, 1975).

However, there exists, at present, some discrepancies in the findings of Kowarski and Schachter as reported by themselves and when compared with the results of others. Kowarski and Schachter reported that the vitamin D-dependent increase in this Ca^{2+}-binding complex required protein synthesis. This does not fit with the findings of Bikle et al. (1978) and Rasmussen et al. (1979) who demonstrated that the initial response of Ca^{2+} transport to vitamin D did not require protein synthesis. Still, another problem exists when considering the affinity of IMCaL for Ca^{2+}. In 1975, IMCal was described as having an apparent K_d of 2×10^{-5}M, and was postulated to act by transferring Ca^{2+} to the higher affinity CaBP of the cytoplasm (Kowarski and Schachter, 1975). However, more recently, the apparent dissociation constant for IMCaL was reported to be 3.7×10^{-7}M as compared to 2.25×10^{-6}M for soluble CaBP (Kowarski and Schachter, 1980). These relative affinities suggest the need for a modulating factor that would promote the transfer

of Ca^{2+} from IMCaL to CaBP for such a Ca^{2+} transport mechanism to function effectively.

4. Calmodulin as mediator of intestinal Ca^{2+} transport

Bikle and Munson (1985) have proposed the attractive hypothesis that calmodulin, a Ca^{2+}-binding protein whose total concentration in the intestinal epithelial cell was not altered by $1,25(OH)_2$-D_3, might mediate the hormone's action on Ca^{2+} transport. In this scheme, $1,25(OH)_2$-D_3 effected a redistribution of calmodulin within the cell rather than new protein (calmodulin) synthesis. In support of this model Bikle and Munson (1985) observed an increase in the calmodulin content of the brush border membrane following $1,25(OH)_2$-D_3 administration which coincided with an increased capacity of the purified BBMV to accumulate Ca^{2+}. Calmodulin antagonists blocked the enhanced Ca^{2+}-accumulating activity by brush border membrane vesicles (BBMV) from chicks which received $1,25(OH)_2$-D_3.

It was a requirement in the above-stated scheme, that $1,25(OH)_2$-D_3 increased the ability of BBMV to bind calmodulin. The idea of the existence of proteins that bind calmodulin was not a novel one for several such proteins have been identified including erythrocyte plasma membrane Ca^{2+}-ATPase (Niggli et al., 1979), calcineurin (Wallace et al., 1980), and phosphorylase b (Cohen et al., 1978).

Munson and Bikle (1985) evaluated the effect of $1,25(OH)_2$-D_3 on calmodulin-binding proteins in BBMV. A protein that binds calmodulin in the absence of Ca^{2+} entry into the cell, should do so independently of changes in intracellular Ca^{2+} concentration; otherwise, the process would more likely be secondary to the changes in Ca^{2+} entry rather than modulating these changes. Also, the binding of such a protein to calmodulin should be enhanced by $1,25(OH)_2$-D_3 administration independent of protein synthesis.

A 110K polypeptide was identified as part of the lateral bridges linking the actin filaments of the underlying cytoskeleton to the brush border membrane. It has been proposed as a likely candidate for binding of calmodulin. This protein appeared to meet the aforementioned requirements since its ability to bind calmodulin increases after $1,25(OH)_2$-D_3 administration coincident with an increased ability of BBMV to accumulate Ca^{2+}. This ability of the 110K polypeptide to bind calmodulin did not require Ca^{2+} and was not inhibited by the protein synthesis inhibitor, cycloheximide.

Other investigators have described a Ca^{2+}-independent calmodulin-binding protein of similar molecular weight to that described by Munson and Bikle in chick intestinal brush border membrane (Glenney and Weber, 1980; Verner and Bretscher, 1985; Howe and Moosekar, 1983).

Localization studies suggested that this protein formed a bridge between the plasma membrane and the microfilament core of the microvillus (Glenney et al., 1982; Coudrier et al.,1981). A recent study observed that this protein had myosin-like ATPase activity (Collins and Borysenko, 1984) but its function in the microvillus was still unclear. Glenney and Glenney (1985) have proposed that this protein was an integral membrane protein, where it could conceivably serve in Ca^{2+} transport. They propose this function from their findings that extraction of this 110K-calmodulin complex from the brush border required detergent and that detergent was necessary for maintaining the solubility of the complex.

Evidence for the hydrophobic nature of the 110K protein could be deduced from the preferential partitioning of the protein into the hydrophobic phase in the phase partitioning assay of Bordier (Bordier, 1981) and in its ability to insert into artificial liposomes. In this regard, Glenney and Glenney (1985) have recently demonstrated a complex pH-sensitive interaction involving Ca^{2+} and calmodulin binding to this protein, which may facilitate its role as a transporter of Ca^{2+} across the membrane into the cytosol.

On the other hand, Verner and Bretscher (1985) presented evidence that this protein was bound to the microvillus cytoskeleton, from which it could be dissociated

by ATP and was, therefore, not an integral part of the membrane. To explain the results of Verner and Bretscher, Conzelman and Moosekar (1986) suggest that the 110K-calmodulin complex may lack hydrophobic domains involved in membrane insertion, either as a result of proteolysis or loss of boundary lipid. In fact, Conzelman and Moosekar (1986) showed that the difference in behavior of the 110K-calmodulin complex following ATP treatment (peripheral membrane protein behavior) or detergent extraction (integral membrane protein behavior) is due to the method used to solubilize it.

It is possible, though speculative at present, that the binding of calmodulin to the 110K subunit may help to maintain a three-dimensional conformation of the latter protein and upon the dissociation of calmodulin the hydrophobicity of the 110K protein is altered i.e., calmodulin binding may regulate the exposure of a hydrophobic insertion sequence involved in membrane binding. It is, however, also possible that calmodulin binding to this protein may simply minimize aggregation and sedimentation behavior. Whatever the <u>in vivo</u> manner of association that the 110K-calmodulin has with the microvillus membrane the aforementioned studies suggest that this complex is intricately associated with the membrane and such an association implies a probable function, and

potential role in the Ca^{2+} transport process in the brush border membrane.

Bikle and Munson (1986) conducted further studies of the Ca^{2+}-accumulating ability, calmodulin content, and calmodulin-binding protein content of the intestinal cell as a function of eluted villus cell fraction corresponding to the depth of cell location on the villus. It was well known that there existed a gradient of cells along the villus of the small intestine (Bikle et al., 1977); alkaline phosphatase activity was highest in the cells near the tip of the villi. In cells eluted from the duodenum of the small intestine of chickens this corresponded to fraction two eluted upon the addition of a cell dissociation buffer, the active agent being EDTA. Bikle and Munson (1986) found that the increase in Ca^{2+}-accumulating ability of cells from the villus tip compared with the villus base correlated extremely well with the calmodulin content and calmodulin binding-protein content of these cell fractions. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of these cell extracts revealed increases in calmodulin and calmodulin-binding protein content per cell. Additionally, they found that administration of active vitamin D to chickens 2 and 4 h before cell harvesting led to an increase in the calmodulin-binding protein content in cell fractions taken from the

villus tip. No stimulation was observed in cells from the other cell fractions.

Taken together these studies indicate that calmodulin concentration and Ca^{2+}-accumulating ability are highest in BBMV from cells near the tip of the villus and fall in parallel as BBMV from cells from more basal regions of the villus are evaluated. The correlation of both calmodulin binding and calmodulin content with Ca^{2+}-accumulating ability in BBMV suggests that this protein may be part of the mechanism that controls Ca^{2+} transport across the brush border membrane. As well, the stimulation of calmodulin binding to this protein by $1,25(OH)_2\text{-}D_3$, first detected in BBMV from cells near the villus tip and not seen in cells from the base of the villus, adds some credibility to this rather interesting hypothesis.

In the current study the presence of Ca^{2+}-binding proteins in brush border membrane will be sought. This approach should provide information as to the identity of the proteins in the brush border that are involved in Ca^{2+} binding, such proteins being potential candidates for, at least, part of the Ca^{2+} transport machinery in this membrane.

B. Terbium as a luminescent probe for the Ca^{2+} uptake system--Ca^{2+}-binding proteins

There are 12 lanthanide ions (Ln^{3+}) which possess qualities that make them suitable probes for Ca^{2+} in Ca^{2+}-binding proteins. In forming complexes with macromolecules,

both Ca^{2+} and Ln^{3+} prefer similar ligands, oxygen or nitrogen donor atoms (Richardson, 1982). In cellular and subcellular systems, Ln^{3+} can often perform some of the same roles as Ca^{2+}, occasionally more effectively and also sometimes inhibit more specifically processes that require Ca^{2+} (Maezzi et al., 1983). It is frequently found that Ln^{3+} interacts to varying degrees with different Ca^{2+}-related processes, thus providing a tool to help distinguish among multiple roles played by Ca^{2+} in biological systems. For example, the fluorescent lanthanide, Tb^{3+} competes with Ca^{2+} for binding sites on proteins in erythrocyte ghosts and lymphoid cells (Mikkelsen and Wallach, 1974).

Terbium as a probe for Ca^{2+} binding to proteins has been widely used because of the spectral properties of this lanthanide and also due to its similarity to Ca^{2+} with respect to ionic radius and binding properties (Martin and Richardson, 1979). Fluorescence of Tb^{3+} may be observed by direct excitation of the metal ion's chromophore. Alternatively, an indirect mechanism which involves optical excitation of ligand chromophores (ligand to metal ion) and electronic energy transfer to the Tb^{3+} atom may be used. Due to the weak absorptivity of Tb^{3+} in the visible and near UV spectral regions direct excitation of Tb^{3+} requires either an intense excitation source or a relatively high concentration (> 0.01 M) of the lanthanide (Dockter, 1983) in order to detect a fluorescent signal from the Tb^{3+}. On

the other hand, indirect excitation of Tb^{3+} emission may be accomplished using somewhat lower intensities of exciting light and lower Tb^{3+} concentrations if the ligand environment includes a highly absorptive chromophore capable of acting as an efficient electronic energy donor to the Tb^{3+} acceptor emitter species. In protein solutions, Tb^{3+} luminescence may be excited by energy transfer from nearby aromatic side chain chromophores which are excited directly by near UV radiation. The overall process is described as follows: (1) Absorption of the exciting light by an aromatic side chain of phenylalanine, tyrosine, or tryptophan in the 250-300 nm range. (2) Resonance energy transfer from an excited aromatic side chain to a nearby Tb^{3+}, followed by strongly enhanced Tb^{3+} luminescence in the green (535-545 nm) spectral region.

Martin and Richardson (1979) investigated the occurrence of enhanced Tb^{3+} luminescence in 40 proteins, most of which, but not all, were known to interact with Ca^{2+}. In the majority of the proteins, Tb^{3+} was bound more strongly than Ca^{2+}. Though excitation spectra indicated energy transfer from tryptophan was most common, transfer from tyrosine and phenylalanine side chains also occurred. For instance, results for Tb^{3+} binding to both prothrombin and its amino terminal fragment I corresponded closely to independent studies for Ca^{2+} binding, even to the extent of apparently mimicing the cooperativity in binding of the

first few equivalents of metal (Bajaj et al., 1975). This correspondence strongly suggested that Tb^{3+} substituted for Ca^{2+} in specific binding sites and was not being bound at alternative sites.

The binding sites for Ca^{2+} in the brush border membrane have not yet been identified although evidence exists, as mentioned earlier in this section, for the presence of proteins capable of binding this cation. However, phospholipids have also been implicated as possessing low affinity binding sites for this ubiquitous ion. The use of Tb^{3+} as a probe for Ca^{2+}-binding sites on proteins, which are close enough to aromatic amino acid residues for "resonance energy transfer"[2] to occur, may facilitate the identification of sites that are protein in nature. A preliminary study of the binding of Tb^{3+} to rat BBMV was reported by Ohyashiki et al. (1979), who revealed the presence of protein sites capable of binding Tb^{3+} in porcine BBMV. Further studies using Tb^{3+} as a probe to locate Ca^{2+}-binding sites on proteins in the brush border membrane, along with the effect of fluidity changes on these

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[2]The term "resonance energy transfer" as used here refers to the process whereby a molecule distinct from Tb^{3+} (i.e., an aromatic amino acid residue) absorbs light of a specific wavelength and becomes excited to a higher energy level. If this energy level is close enough to that of the $^5D_4$ excited energy level of Tb^{3+} and if the physical distance is not too great, the energy can then be transferred from the first molecule (the donor) to Tb^{3+} (the acceptor) by a radiationless process. (For more details see Dockter 1983).

sites, should prove fruitful for the further characterization of the binding proteins of this system.

VI. AIMS OF THE PRESENT INVESTIGATION

The aims of this study are generally to further clarify structure-function relationships in intestinal brush border membranes. In relation to this, the specific aims are:

(1) To examine different procedures for the preparation of pure brush border membranes from rabbit intestine.

(2) To characterize the properties of a Ca^{2+} uptake system found in these membranes.

(3) To study the uptake of fatty acids by the BBMV preparations and ascertain their effects on the Ca^{2+} uptake system of the brush border membrane.

(4) To characterize any structural changes occurring in the membranes of the BBMV as monitored by the physical technique of fluorescence anisotropy and to correlate these changes with those in lipid composition and Ca^{2+} uptake.

(5) To attempt to identify the protein(s) responsible for Ca^{2+} binding by the BBMV and thus obtain some degree of understanding of the molecular basis for uptake of this cation by BBMV.

Chapter 1

CHARACTERIZATION OF BBMV LIPID AND MARKER ENZYME CONTENT--A COMPARISON OF Ca^{2+} AND Mg^{2+} PRECIPITATION METHODS

I. INTRODUCTION

Spurious conclusions concerning the characteristics of transport or binding systems may be made when studies are conducted using membrane preparations which are contaminated by other cellular membranes (Hopfer, 1984). Unpublished results obtained in this laboratory, relating to the preparation of BBMV from various species, suggested that preparations obtained with Mg^{2+} as the precipitant were not as pure as those prepared with Ca^{2+} as the precipitant, although current reports in the literature seemed to suggest an indiscriminate use of either cation as precipitant in the isolation of BBMV.

In a quest for the preparation of a high quality BBMV fraction displaying a minimum breakdown of lipids due to intrinsic phospholipase A activation, a study was conducted to evaluate available methods involving precipitation with either Mg^{2+} or Ca^{2+}. The purity of the fractions was assessed on the basis of lipid composition and marker enzyme content.

II. MATERIALS

Common reagents and solvents used in these experiments were ACS reagent grade and were used without further purification, except in the case of petroleum ether, which was distilled just prior to use. Heptadecanoic acid, fatty acid standards and phospholipid standards were obtained from Sigma Chemical Company, St. Louis, Mo.; as were N'-2-hydroxy-ethylpiperazine-N'-2ethanesulfonic acid (Hepes), ethylene glycol bis(β-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA), silica, aprotinin, leupeptin, phenylmethylsulfonylfluoride (PMSF), butylated hydroxytoluene (BHT), 2,5-di (5-tert.-butyl-2-benzoxaxzolyl)-thiophene (BBOT), and cholesterol standard.

III. METHODS

A. Preparation of brush border membranes

Female, New Zealand white rabbits were used for the isolation of BBMV. These were weaned at 4-6 weeks of age and fed standard Purina rabbit chow up to 12 h before the time of death. For each experiment, from two to four rabbits were routinely sacrificed at 12-16 weeks of age, at which time the animals weighed 4.5-6.0 lbs. The animals were killed by cervical dislocation without anaesthesia and the small intestine was removed immediately, everted, and thoroughly washed with physiological saline. The epithelial cells lining the lumen of the small intestine were removed

by scraping the luminal surface of the intestine with a microscope slide taking care not to remove the underlying muscle tissue. These cells, primarily from the duodenum and jejunum, were homogenized and the plasma membrane was purified according to the method of Selhub and Rosenberg (1981). Briefly, the mucosal scrapings (cells) were homogenized for 30s at 0°C in 15 volumes (w/v) of 0.05 M mannitol and the homogenate was centrifuged for 10 min at 10,000 x g. Upon removing the supernatant, the pellet fraction was homogenized for 1 min with a fresh mannitol solution (7 volumes) and then combined with the first supernatant, and the pooled suspension was filtered through a cheesecloth. $CaCl_2$ (solid) was added to the filtrate to a final concentration of 10 mM, ($MgCl_2$ or $MgCl_2$ + EGTA were substituted for $CaCl_2$ in some experiments as outlined by Kessler et al., 1978 and Hauser et al., 1980, respectively), and, after standing in an ice bath with occasional stirring for 20 min, the suspension was centrifuged for 10 min at 4,000 x g. The pellet was discarded and the supernatant was subjected to another centrifugation (15 min at 40,000 x g). The pellet fraction was resuspended in 100 mM mannitol, 10 mM Hepes titrated with Tris to pH 7.4 (4 ml/g of original scrapings), and was homogenized with a Sorvall homogenizer (setting #9), by applying ten strokes up and down. The homogenate was centrifuged (40,000 x g, 15 min) and the

pellet fraction was resuspended in 10 ml of the mannitol/Hepes solution.

To guard against potential protease degradation of the proteins in the brush border membrane, three different protease inhibitors were added to the homogenate and to any required wash solutions; namely, leupeptin (1 µg/ml), aprotinin (0.11 tyrosine inhibitor units, TIU/ml), and phenyl methyl sulfonyl fluoride (30 µM).

B. Marker enzyme assays

The purified plasma membrane preparations were assayed for various marker enzymes to determine the degree of purification and level of contamination from other cellular organelles. Succinate dehydrogenase was assayed as marker of mitochondria by the method of King (1967); glucose-6-phosphatase, as indicator of endoplasmic reticulum, was measured according to Harper (1963), ($Na^+ + K^+$)-ATPase, a marker of basolateral membranes, was determined as described by Scharschmidt et al. (1979). Marker enzymes for the brush border membrane, alkaline phosphatase and sucrase, were assayed according to the methods of Forstner et al. (1968) and Dahlquist (1964), respectively. Protein concentrations were determined by the method of Lowry et al. (1951).

C. Lipid extraction and separation

Lipids were extracted according to the method of Bligh and Dyer (1959) and separated by two dimensional thin

layer chromatography (TLC) on silica gel H coated plates, 20 x 20 cm. The method of Bowyer and King (1977) was used in which the coated plates were washed with methanol and activated by heating for 1 to 2 h at 110°C. The chromatograms were developed in the first dimension with chloroform-methanol-acetic acid-water (55:35:3:2), and in the second dimension with chloroform-acetone-methanol-acetic acid-water (45:16:15:11:6). The lipids were visualized with iodine vapors except when oxidation had to be avoided. In the latter case, visualization was made possible by spraying with 0.1% BBOT, a fluorescent dye, used at a concentration of 10 mg/100 ml.

D. Analytical procedures

Total lipid phosphorus was determined by the method of Vaskovsky et al. (1975). Glycolipids were determined by measuring the total sphingosine content of the lipid extracts (Naoi et al., 1974) and subtracting sphingosine due to sphingomyelin. Cholesterol content of the extracts was determined by the method of Bowyer and King (1977).

Free fatty acids were separated from other lipids by TLC as described above. Fatty acid content from lipid extracts was determined as methyl esters formed by heating the samples containing dry, 2.5% methanolic HCl at 70°C for 1 h under nitrogen. The antioxidant, butylated hydroxytoluene (BHT) 0.01%, was added to the samples to

prevent the oxidation of the unsaturated fatty acids. The methyl derivatives were analyzed with a Varian 6000 gas chromatograph, equipped with a flame ionization detector, employing a 1.83 meter x 2 mm packed glass column (10% SP-2330 on Chromosorb W, AW 100/20 mesh). The settings for the separation were: injection temperature (260°C), ion temperature (300°C), nitrogen flow rate (7.5 ml/min), column pressure (68.9 kPa) and temperature program was 160°C for 20 min followed by 8°C/min to 200°C. Heptadecanoic acid, 200 µg, was added to each sample as an internal standard.

TLC analysis of the neutral lipid fraction revealed cholesterol and free fatty acids as the major components with only trace amounts of acylglycerols and cholesterol esters being detected. Therefore, the cholesterol content of the extracts was determined without prior chromatography and without correction for esterified cholesterol. The free fatty acid measurements when calculated on the basis of the product of cholesterol content and the molar cholesterol/free fatty acid ratio gave less variable results. This ratio, which was constant for any given sample run in duplicate or triplicate, was calculated by analyzing the cholesterol and free fatty acids recovered from the same TLC plate.

E. Variation in the results and sample size.

The results shown throughout this thesis represent

averages ± standard error obtained from 3-4 determinations each from four to six animals unless otherwise specified.

IV. RESULTS

A. Marker enzyme and protein content

Table 1-1 lists the various marker enzymes for the brush border membrane (sucrase and alkaline phosphatase); the basolateral membrane ((Na^+ + K^+)-ATPase); the mitochondrial membrane (succinate dehydrogenase); and the endoplasmic reticulum membrane (glucose-6-phosphatase). The specific activities of these enzymes found in the homogenate of the epithelial cell and in the Mg^{2+}- and Ca^{2+}-prepared BBMV are compared. Mg^{2+}-prepared BBMV differ from Ca^{2+}-prepared BBMV in that the former has been prepared by the addition of 10 mM Mg^{2+} to precipitate contaminating cellular components from the brush border membrane whereas, the latter has been prepared by the addition of 10 mM Ca^{2+}. It can be noted that the Ca^{2+} preparation was made in the presence and the absence of three different protease inhibitors; aprotinin, an inhibitor of trypsin and chymotrypsin proteases; leupeptin, which inhibits Ca^{2+} dependent proteases; and PMSF which acts as a more general protease inhibitor. The protease inhibitors were used to determine if protease activity had any bearing on the differences seen in the quality of these two types of preparations. These results indicate that there is indeed a

TABLE 1-1

Marker Enzyme and Protein Content of Brush-Border Membranes Prepared by $Mg2^{+}$ and by Ca^{2+} Precipitation

	Homogenate	Mg^{2+}-prepared BBM	Ca^{2+}-prepared BBM	
		-Inhibitors	+Inhibitors[a]	-Inhibitors
Proteins	1085 ± 17	51 ± 3(93±1)	52 ± 3(86±3)	47 ± 1(92±1)
Sucrase	61 ± 5	748 ± 21(83±3)(69)	936 ± 52(82±3)(86)	929 ± 12(86±3)(77)[b]
Alkaline phosphatase	79 ± 2	902 ± 106(81±2)(65)		1150 ± 98(86±2)(73)[c]
(Na+ + K^{+})-ATPase	48 ± 7	125 ± 5(94±3)(12)	35 ± 6(89±3) (3.5)	19 ± 4(100±2)(2.5)[b]
Succinate dehydrogenase	91 ± 7	6 ± 1		4 ± 1
Glucose-6-phosphatase	81 ± 3	95 ± 10		108 ± 17

[a]Leupeptin (1 μg/ml) apotinin (11 TIU/ml) and PMSF (30 μM) were added throughout the isolation procedure.
[b]Difference between Mg^{2+}- and Ca^{2+}-prepared membranes: $P<0.01$.
[c]Difference between Mg^{2+}- and Ca^{2+}-prepared membranes: $P<0.05$.

Proteins are expressed as mg recovered in each fraction. Enzyme activities are expressed as nmol/mg per min. Values are averages ± S.E. 6-8 determinations on membranes from 4-6 animals. Values in the first parentheses are average percent total recoveries ± S.E. obtained from analysis of all the fractions which include a Ca^{2+} or Mg^{2+} precipitate, a brush-border membrane fraction (BBM), a post-BBM supernatant and BBM wash. Values in the second parenthesis represent percent of the recovered activity found in each fraction.

difference between the two preparations with the Ca^{2+}-prepared BBMV showing less contamination from basolateral membranes. This fact is supported by comparing the data for the specific activity of $(Na^{+} + K^{+})$-ATPase, a marker for the basolateral membrane, which is 125 nmol/mg per min in the Mg^{2+}-prepared BBMV as contrasted with its activity of 19 nmol/mg per min in the Ca^{2+}-prepared BBMV (Table 1-1). Contamination of the two preparations by mitochondrial membranes was minimal and similar in the two preparations, 6 nmol/mg per min and 4 nmol/mg per min for the Mg^{2+} and Ca^{2+} preparations, respectively. Likewise, there was no significant difference between the purity of the two preparations with respect to contamination from the endoplasmic reticulum membrane (95 nmol/mg/min and 108 nmol/mg/min for the Mg^{2+}- and Ca^{2+}-prepared BBMV, respectively). It is important to note that the specific activity of the marker enzymes for the brush border membrane in the two preparations differed significantly, the enrichments of alkaline phosphatase and sucrase being some 20-30% greater in the Ca^{2+}-prepared membranes. This, again, demonstrates the superiority of the Ca^{2+} preparation over the Mg^{2+} preparation.

When comparing differences in specific activity of the marker enzymes in the two preparations it is of paramount importance that the differences are not due to a specific activation of proteases or a selective inhibition

of marker enzyme activity in the presence of one or the other cation. It can be seen from Table 1-1 that the total enzyme recoveries are very similar for sucrase (83% and 86% for Mg^{2+}- and Ca^{2+}-prepared BBMV, respectively), alkaline phosphatase (81% and 86% for Mg^{2+}- and Ca^{2+}-prepared BBMV, respectively) and (Na^{+} + K^{+})-ATPase (94% and 100% for Mg^{2+}- and Ca^{2+}-prepared BBMV, respectively) as are the total protein recoveries (93% and 92% respectively for Mg^{2+}- and Ca^{2+}-prepared BBMV) when either precipitation method is used. In fact, the (Na^{+} + K^{+})-ATPase activity was significantly higher in the Mg^{2+}-prepared BBMV even when recovery of the total activity in the Ca^{2+}-prepared fractions was complete (-inhibitors, 100 ± 2 nmol/mg/min). Consequently, the differences in the two preparations can not be attributed to anything other than the actual quality of the preparation and the Ca^{2+} preparation on this basis is significantly superior.

B. Fatty acid content

Table 1-2 compares the fatty acid content of the Mg^{2+}- and Ca^{2+}-prepared BBMV. It can be seen from this data that the fatty acid content of the two types of preparations do not differ significantly and, as well, the total fatty acid content on a per mg protein basis is very similar despite the differences in marker enzyme content (Table 1-1) and lipid composition (Tables 1-3 and 1-4, below), between the two preparations. Although published

TABLE 1-2

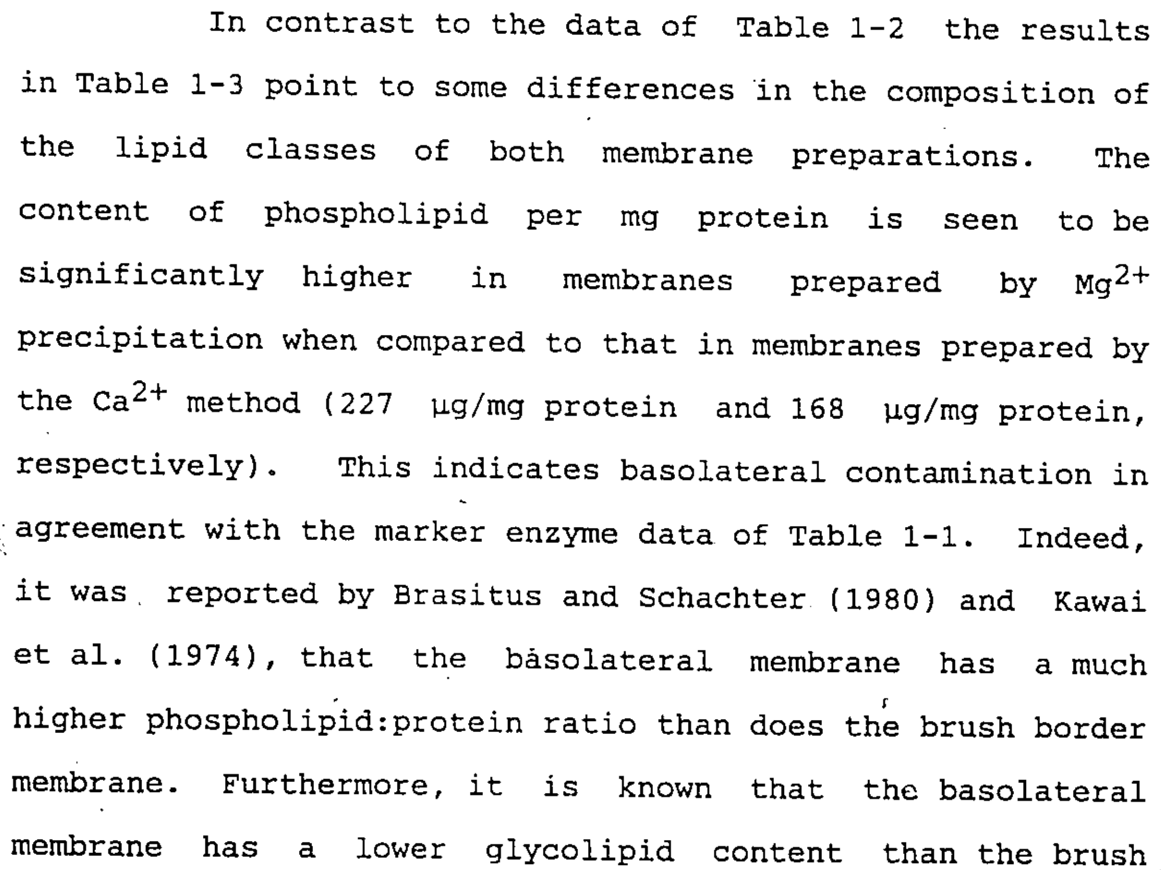
Total Fatty Acid Composition of Brush-Border Membranes Prepared by Mg^{2+} and by Ca^{2+} Additions

Fatty Acid	Membrane Fraction Mg^{2+}-Prepared % of Total*	 Ca^{2+}-Prepared % of Total*
C16:0	12	12
C18:0	30	31
C18:1	12	12
C18:2	35	34
C18:3	4	4
C20:3 + C20:4	2	2
Others	5	5

*The total fatty acid content for Mg^{2+}- and Ca^{2+}-prepared membranes was 103 µg/mg protein and 91.2 µg/mg protein respectively. Percentages represent averages of duplicate determinations from pooled lipid samples obtained from 5 rabbits.

data is lacking concerning the fatty acid composition of the brush border membrane in the rabbit, data published by Kawai et al. (1974) for mouse brush border membranes, however, is very complimentary with that shown in Table 1-2. It is interesting to note the relatively high amounts of unsaturated fatty acids found in this membrane, i.e., oleic and linoleic acid (C18:1 and C18:2, respectively), the latter corresponding to approximately one-third of the fatty acid content of the brush border membrane. All in all, the results from Table 1-2 do not suggest any difference in the total fatty acid content between the two preparations.

C. <u>Total lipid composition</u>



In contrast to the data of Table 1-2 the results in Table 1-3 point to some differences in the composition of the lipid classes of both membrane preparations. The content of phospholipid per mg protein is seen to be significantly higher in membranes prepared by Mg^{2+} precipitation when compared to that in membranes prepared by the Ca^{2+} method (227 µg/mg protein and 168 µg/mg protein, respectively). This indicates basolateral contamination in agreement with the marker enzyme data of Table 1-1. Indeed, it was reported by Brasitus and Schachter (1980) and Kawai et al. (1974), that the basolateral membrane has a much higher phospholipid:protein ratio than does the brush border membrane. Furthermore, it is known that the basolateral membrane has a lower glycolipid content than the brush

border membrane, (Kawai et al., 1974). The data in Table 1-3 indicate that Mg^{2+}-precipitated BBMV have a lower glycolipid sphingosine content than the Ca^{2+}-precipitated BBMV, which again reveals the presence of basolateral membrane contamination in the former.

Cholesterol content of the two preparations was similar while the free fatty acid content was more variable in the Ca^{2+}-precipitated membranes but not significantly different from that of the Mg^{2+}-precipitated membranes. These data are not surprising because it is known that the free fatty acid and cholesterol contents of the basolateral and brush border membrane are quite similar (Kawai et al., 1974).

D. Phospholipid composition

The phospholipid composition of brush border membranes from both methods of preparation are shown in Table 1-4 along with data from another method of preparation which uses EGTA together with Mg^{2+}. It is interesting to note that little or no lysophospholipid was found in any of the three preparations. The lysolipid content was the highest in the Ca^{2+}-prepared membranes, as might be expected if Ca^{2+} were to activate an endogenous phospholipase A, but accounted for no more than 6% of the total phospholipid content. Looking further, there is a major difference in the phosphoacylglycerol composition between the Ca^{2+}-prepared membranes and either of the Mg^{2+} preparations. The

TABLE 1-3

Lipid Composition of Brush-Border Membranes
Prepared by Mg^{2+} and by Ca^{2+} Addition

	Membrane fraction (μg/mg protein)	
	Mg^{2+}-prepared	Ca^{2+}-prepared
Cholesterol	61 ± 1	60 ± 2
Free fatty acids	32 ± 10	34 ± 6
Lipid phosphorus	227 ± 12	168 ± 5[a]
Glycolipid sphingosine	138 ± 4	157 ± 1[a]

[a]Difference between Mg^{2+}-prepared and Ca^{2+}-prepared membranes: $P < 0.005$

The values represent averages ± S.E. of 5-11 determinations on extracts from 5-9 rabbits.

TABLE 1-4

Phospholipid Composition of Brush-Border Membranes Prepared by Mg^{2+} or by Ca^{2+} Additions

	Fraction (µg/mg protein)		
	Mg^{2+}-prepared[a]	Mg^{2+}-EGTA-prepared[b]	Ca^{2+}-prepared[a]
Phosphatidylethanolamine	108 ± 2	127	85 ± 2[c]
Phosphatidylcholine	59 ± 4	82	37 ± 1[c]
Sphingomyelin	31 ± 1	26	13 ± 1[c]
Phosphatidylinositol	14 ± 2	21	10 ± 1
Phosphatidylserine	31 ± 2	30	24 ± 1[c]
Lysophosphatidylethanolamine	2 ± 1	nil	5 ± 1
Lysophosphatidylcholine	3 ± 1	nil	6 ± 1

[a] Values represent averages ± S.E. of 7-11 determinations obtained from four rabbits.
[b] Values represent the average of two determinations obtained with a single rabbit.
[c] Difference between Mg^{2+}-prepared and Ca^{2+}-prepared membranes: $P < 0.01$.

phosphatidylethanolamine-phosphatidylcholine (PE/PC) ratio that was obtained for the Ca^{2+} preparation was consistently higher than for either the Mg^{2+} or the Mg^{2+}-EGTA preparation. A report by Kawai et al. (1974) indicated a PE/PC ratio of 2.0-3.0 for the mouse brush border membrane and a lower ratio of 0.5-0.9 for the basolateral membrane. On this basis, the results in Table 1-4 point to the heavier contamination of the Mg^{2+}-prepared BBMV with basolateral membranes substantiating the conclusions made from the data in Tables 1-1 and 1-3.

V. DISCUSSION

The literature is replete with procedures for the isolation of the brush border membrane from epithelial cells from various animal species (Schmitz et al., 1973; Selhub and Rosenberg, 1981; Max et al., 1978; Kessler et al., 1978; Stieger and Murer, 1983; Hauser et al., 1980; Christiansen and Carlsen, 1981; and Kawai et al., 1974), with some investigators using Mg^{2+} while others make use of Ca^{2+} as the precipitant in the isolation procedure. The approach that was taken in this study was to find a method of preparation yielding the highest quality membrane fraction possible in the minimum time. Preparations obtained by centrifuging through a Percoll gradient (Yakymyskyn et al., 1982) or by applying fractions to a Sepharose column (Carlsen and Christiansen, 1983) are time consuming and may

lead to loss of enzyme or other functional activities without significantly improving the quality of the brush border membrane preparation.

The data presented in Tables 1-1 to 1-4 indicate that preparation of BBMV by Ca^{2+} precipitation yields purer fractions than those obtained by Mg^{2+} precipitation. However, the use of Ca^{2+} as precipitant would have been avoided if, indeed, it resulted in the activation of phospholipase A and serious degradation of membrane phospholipids. Indeed, Hauser et al. (1980) suggested that Ca^{2+}-prepared BBMV are inferior because of Ca^{2+} activation of endogenous phospholipases resulting in a much higher fatty acid and lysophospholipid content in this type of preparation. The data in Table 1-3 refute this claim and contradict those presented by Hauser. However, there is a plausible explanation which lies in the handling of the intestinal tissue after the death of the animals. Hauser et al. (1980) routinely froze their tissue and prepared their membrane fractions after thawing of the cells. Such measures could conceivably lead to activation of endogenous membrane-bound phospholipase A. In addition, Pind and Kuksis (1987) have recently found that a specific phospholipase A can be activated in rat intestinal cells only after freezing and thawing of the cells, and when prepared fresh within a single day, BBMV isolated with Ca^{2+} as precipitant do not possess high levels of

lysophospholipids. Accordingly, the BBMV prepared in this study were always prepared from fresh tissue with the vesicles being isolated on the same day.

With respect to the conclusions derived from Table 1-4, that the high PE/PC ratio obtained with the Ca^{2+}-prepared membranes was indicative of purer BBMV, it must be realized that on the basis of this ratio alone it is not possible to determine the degree and relative purity of the different preparations. This is because this ratio has been shown to change with the age of the animal and to be dependent upon the segment of the intestine from which the membranes originate (Schwartz et al., 1985; Brasitus et al., 1984; Pang et al., 1983).

All in all, the results of this section demonstrate the superiority of the preparation obtained by the addition of Ca^{2+} as the precipitant. For this reason, the Ca^{2+} method for preparing BBMV to be used in transport and binding studies was adopted.

CHAPTER TWO

SOME PROPERTIES OF THE Ca^{2+} UPTAKE PROCESS IN RABBIT INTESTINAL BRUSH BORDER MEMBRANES

I. INTRODUCTION

Most studies conducted on the uptake of Ca^{2+} by brush border membrane vesicles have involved the use of chick and rat intestinal tissue while little has been revealed concerning the uptake of this cation in other species (Murer and Hildeman, 1981; Maenz and Forsyth, 1982). This study is concerned with the elucidation of some of the properties of the Ca^{2+} uptake system as it exists in the rabbit BBMV.

II. MATERIALS

The radiochemicals, $^{45}CaCl_2$ and D[1-^{14}C]glucose were purchased from New England Nuclear Corporation (Boston, MA). The Ca^{2+} ionophore, A23187, and D(+)-cellobiose, the sodium salts of 2-(N-morpholino)ethanesulfonic acid (Mes) and 3-(cyclohexylamino)-1-propanesulfonic acid (Caps) were purchased from Sigma Chemical Co. (St. Louis, MO). Spectroanalyzed HCl along with the 100 mM Ca^{2+} standard solution were obtained from Fischer Scientific Co. (Whitby, Ont.).

III. METHODS

A. Preparation of BBMV

The Ca^{2+} precipitation method of Selhub and Rosenberg (1981) was used to prepare BBMV on a routine basis.

since their method yielded membranes which were of sufficient purity to allow use in transport and binding studies (cf. Chapter 1).

B. D-glucose uptake

D-glucose uptake was measured by modifying slightly, the procedure of Hopfer et al. (1973). Briefly, freshly prepared vesicles, kept at 0°C on ice until use, (110 µg membrane protein) were incubated together with 100 µM [1-^{14}C]D-glucose, 100µM $MgCl_2$, and 150 mM NaSCN in 10 mM Hepes-Tris buffer, pH 7.5 in a final volume of 40 µl at 25°C for up to 10 minutes (in kinetic studies) and 60 minutes (in equilibrium studies). The incubation mixtures were terminated by the addition of a 25-fold dilution of ice-cold stop solution consisting of 100 mM mannitol, 150 mM NaCl, 0.2 mM phlorizin, and 55 mM $MgCl_2$ in 10 mM Hepes-Tris buffer pH 7.5. The suspensions were rapidly filtered through pre-wetted (with stop solution) 0.45 µm nitrocellulose filters. The filters were immediately washed twice with 2.5 ml of the ice-cold stop solution and allowed to air dry in 20 ml plastic scintillation vials (Fischer Scientific, Whitby, Ont.). A scintillation cocktail consisting of Phase Counting System (PCS, Amersham Corp., Oakville Ont.)-toluene 1:1 (v/v) mixture was added to the counting vials and the samples were counted in a Beckman LS-150 liquid scintillation counter using the channel ratio method for quench correction.

C. <u>Ca^{2+} uptake</u>

For the determination of Ca^{2+} uptake[1] into rabbit BBMV the method of Miller and Bronner (1981) was employed with some modifications. Essentially, the uptake was initiated by the addition of 10 µl of BBMV (30 µg membrane protein) to 25 µl of $^{45}CaCl_2$ (specific activity, 40,000 cpm/nmol), 0.36 mM final concentration. This concentration of Ca^{2+}, which was not saturating, was chosen because higher concentrations have been shown to cause fusion of membranes (Papahadjopoulos, 1974) and to lead to rigidifying effects on bilayer structure. This Ca^{2+} concentration was sufficiently great to allow easily measurable rates of uptake since these rates were usually examined for relatively short periods of time (1-5 min). Considering that less than 4 % of the labelled Ca^{2+} was taken up by the BBMV during the rate studies, it was initial rates that were assessed and compared throughout. Incubations maintained at 25°C were terminated at various times by the addition of 40 volumes of ice-cold stop solution containing 100 mM mannitol, 10 mM Hepes-Tris buffer, 5 mM EDTA, 20µM $LaCl_3$, pH 7.5. Subsequent to rapid filtration under low vacuum the 'stopped' reaction mixture containing the trapped BBMV was washed twice with 2.5 ml of ice-cold stop solution, the

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[1]The term, uptake, as used in this thesis refers to binding and transport because these processes could not be clearly differentiated experimentally, the two operating simultaneously.

filters transferred to 20 ml scintillation vials and allowed to air dry. To the vials was added 10 ml of the 1:1 toluene:PCS scintillation cocktail and the samples were counted for radioactivity in a Beckman LS-250 liquid scintillation counter. An external standard ratio method was used for quench correction.

D. Valinomycin-induced membrane potential

During the preparation procedure, BBMV were preloaded with 50 mM K_2SO_4 as described by Somermeyer et al. (1983). Aliquots of 100 µg of brush border membrane protein in 10 µl incubation buffer were diluted 20-fold at time zero with 190 µl of $^{45}CaCl_2$ (0.36 mM Ca^{2+}, final concentration) containing 10 µg/ml valinomycin in 100 mM mannitol and 10 mM Hepes-Tris buffer, pH 7.5. Both control and treated samples possessed valinomycin but the control had no entrapped K_2SO_4. The final concentration of K_2SO_4 (2.5 mM) had, by itself, no effect on Ca^{2+} uptake into the vesicles (results not shown).

E. Measurement of free Ca^{2+} concentrations

Free Ca^{2+} concentrations were measured with an Orion Ionalyzer model 407 A/L equipped with an ion-selective electrode sensitive to 10^{-7} M Ca^{2+}. The analyzer was connected to a Fischer Recordall 5000 for some experiments while for others the scale was read directly. The Ca^{2+} selective electrode was calibrated using several dilutions

of a 100 mM Ca^{2+} standard solution (Fischer Scientific Ltd., Whitby, Ont.).

F. Total Ca^{2+} determination

Total membrane Ca^{2+} content was determined by atomic absorption analysis using an air-acetylene flame atomic absorption spectrophotometer, model 551 (Instrumentation Laboratories, Montreal, Que.) equipped with an integrator-microprocessor for sample calculations. It was imperative for this procedure that a high quality acid be used, one that was metal-free and ultra-analyzed. A hollow cathode lamp was operated at 7mA with the wavelength set at 479.5 nm. The procedure for the extraction of Ca^{2+} from BBMV was adapted from Carafoli and Lehninger (1971). This involved the addition of 1.5 ml of 0.5 N HCl to 10 mg of brush border membrane protein and placing the samples in a boiling water bath for 15 minutes to denature the proteins and dissociate the Ca^{2+} from the membrane. The cooled solution was centrifuged for 10 min at 10,000 x g, the supernatant withdrawn and the extraction procedure repeated. The second supernatant was pooled with the first. The volume of each sample was made up to 5 ml with 0.05 N HCl. To all samples and standards was added 0.1% (w/v) $LaCl_3$ to suppress the interferences by phosphates. The Ca^{2+} standard solution (500 mM in 0.05 N HCl) was purchased from Canlab (Missassauga, Ont.).

G. Sample size

All values reported in this study are the means ± S.D. from four to 12 determinations obtained from at least two different membrane preparations.

IV. RESULTS

D-glucose uptake displayed a typical overshoot phenomenon and proved to be an osmotically-sensitive process (Figure 2-1). The overshoot phenomenon seen in these vesicles was indicative of intact, closed structures that allow a sodium ion gradient to persist briefly during the time course of the experiment (Kessler et al., 1978).

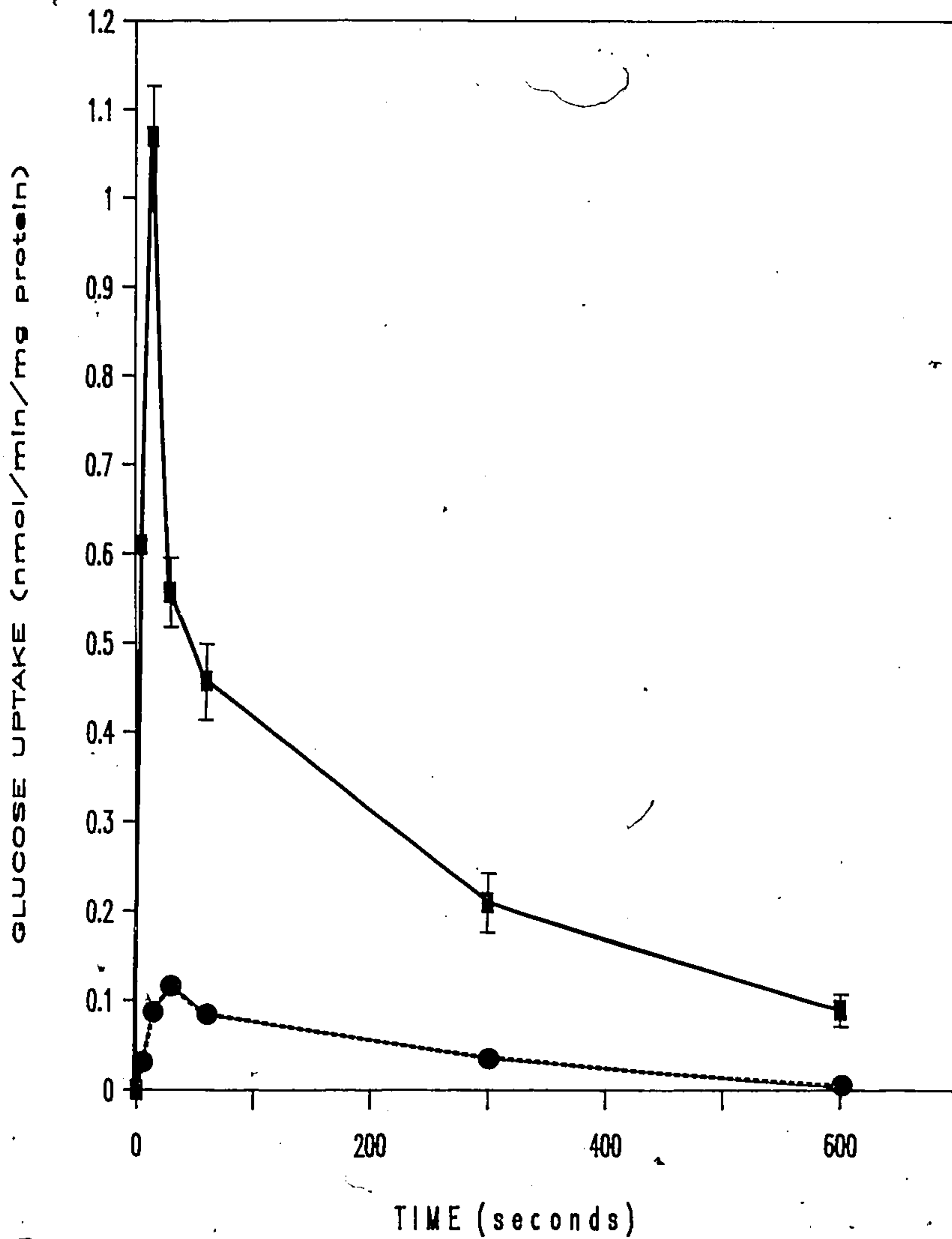
Since these vesicles were osmometers (Figure 2-1) they were sensitive to the osmolarity of their environment. This property has previously been used for determining to what extent the uptake of a permeant was dependent on the intravesicular volume (transport) or involved simply a binding phenomenon. Consequently, data shown in Figure 2-1 reveal that the uptake of D-glucose by these vesicles was a transport process, because in the presence of D-cellobiose, concentration outside being greater than the concentration inside, shrinkage of the vesicles occurred and the equilibrium uptake of this sugar was drastically reduced.

In the absence of cellobiose, equilibrium uptake was reached in 10 min and in the presence of 100 μM D-glucose this value corresponded to 90 pmol/mg brush border membrane

Figure 2-1

D-glucose uptake by BBMV and the effect of osmolarity.

^{3}H-D-glucose (specific activity, 3 · 10^5 DPM/nmole) uptake into BBMV was measured by stopping the incubation at various times and counting the filtered vesicle suspension for radioisotope content. The conditions for the assay were: 110 µg BBMV protein, temperature 25°C, NaSCN 150 mM, D-glucose 200 µM, and 100 mM mannitol, 10 mM Hepes/Tris buffer, pH 7.5 in a volume of 40 µl. The vesicular uptake of D-glucose was terminated by the addition of a 25 fold dilution of ice-cold stop solution. D-glucose uptake was determined in the presence of 300 mM D-cellobiose (●) and in its absence (■).


GLUCOSE UPTAKE (nmol/min/mg protein)
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
200
400
600
TIME (seconds)

protein. On the basis of this information the vesicular volume could be calculated as indicated in Table A-1 (see Appendix). This value falls within the range reported for BBMV prepared from mammalian microvilli of between 8.0×10^{-4} and 2.0×10^{-3} ml/mg brush border membrane protein (Alvarado et al., 1979; Kessler and Semenza, 1979; Lucke et al., 1979, Hammerman et al., 1980).

Figure 2-2 shows the pattern of Ca^{2+} uptake with time and the effect of temperature on the uptake process. This process was sensitive to the temperature of the incubation and was linear at a Ca^{2+} concentration of 0.36 mM for up to 5 minutes at 25°C. This process of Ca^{2+} uptake displayed saturability (Figure 2-3) and was comparable in this respect to the process which was defined in rat BBMV (Miller and Bronner, 1981). Figure 2-4, a Lineweaver-Burke plot of the data shown in Figure 2-3, gave a V_{max} of 7.60 nmol/mg per min with a K_m equal to 0.66 mM.

In the course of these incubations, free Ca^{2+} did not change appreciably, the fall in free Ca^{2+} under standard incubation conditions (0.36 mM Ca^{2+} and 0.85 mg protein/ml), due to total uptake including nonspecific absorption[2], being no greater than 3% and 7% for the rate and equilibrium

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[2]Nonspecific absorption as used in this context refers to the Ca^{2+} that is bound to the exterior of the vesicles and that is normally removed by the chelator wash procedure as performed for the determination of the Ca^{2+} uptake values at various time points.

Figure 2-2

The effect of time and temperature on Ca^{2+} uptake.

The uptake was initiated by the addition of 25 µl of 0.5 mM $^{45}CaCl_2$ to 10 µl (30 µg protein) of brush-border membranes and pursued at 5°C (●), 25°C (□), and 37°C (▲) for various times. Mannitol (100 mM) in 10 mM Hepes-Tris buffer (pH 7.5) was used to prepare the Ca^{2+} solution or membrane suspension used for the incubation mixture. The uptake was terminated by dilution and rapid filtration.

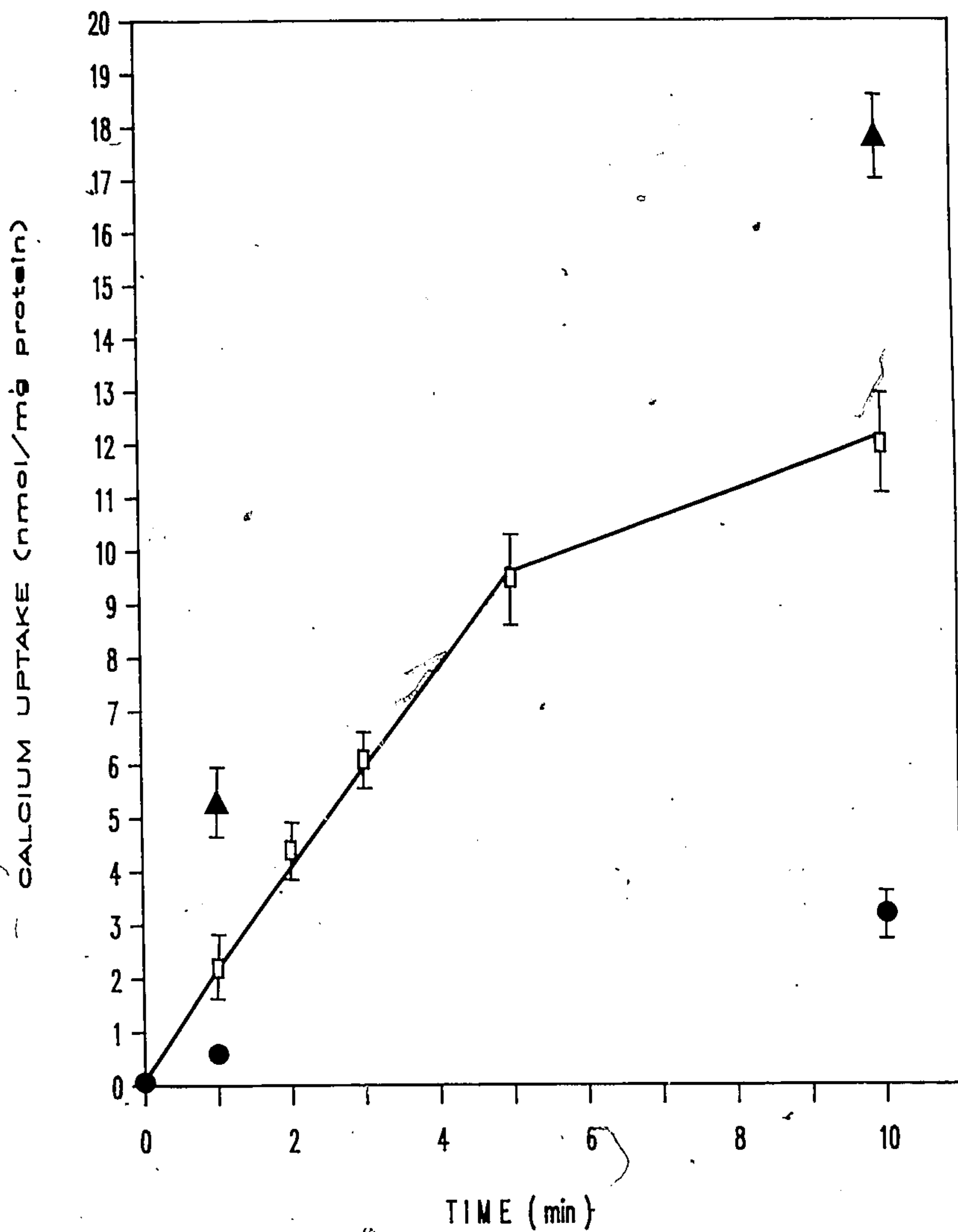

CALCIUM UPTAKE (nmol/mg protein)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
0
2
4
6
8
10
TIME (min)

Figure 2-3

The effect of Ca^{2+} concentration on uptake.

The conditions were as stated in Figure 2-2; however, the temperature of the incubation was 25°C only. The Ca^{2+} concentration was varied as indicated, the incubation time was 5 min except for Ca^{2+} concentrations of 10 and 20 mM, which were measured for 30 s. All values were calculated on a per minute basis. The uptake was terminated by dilution and rapid filtration.

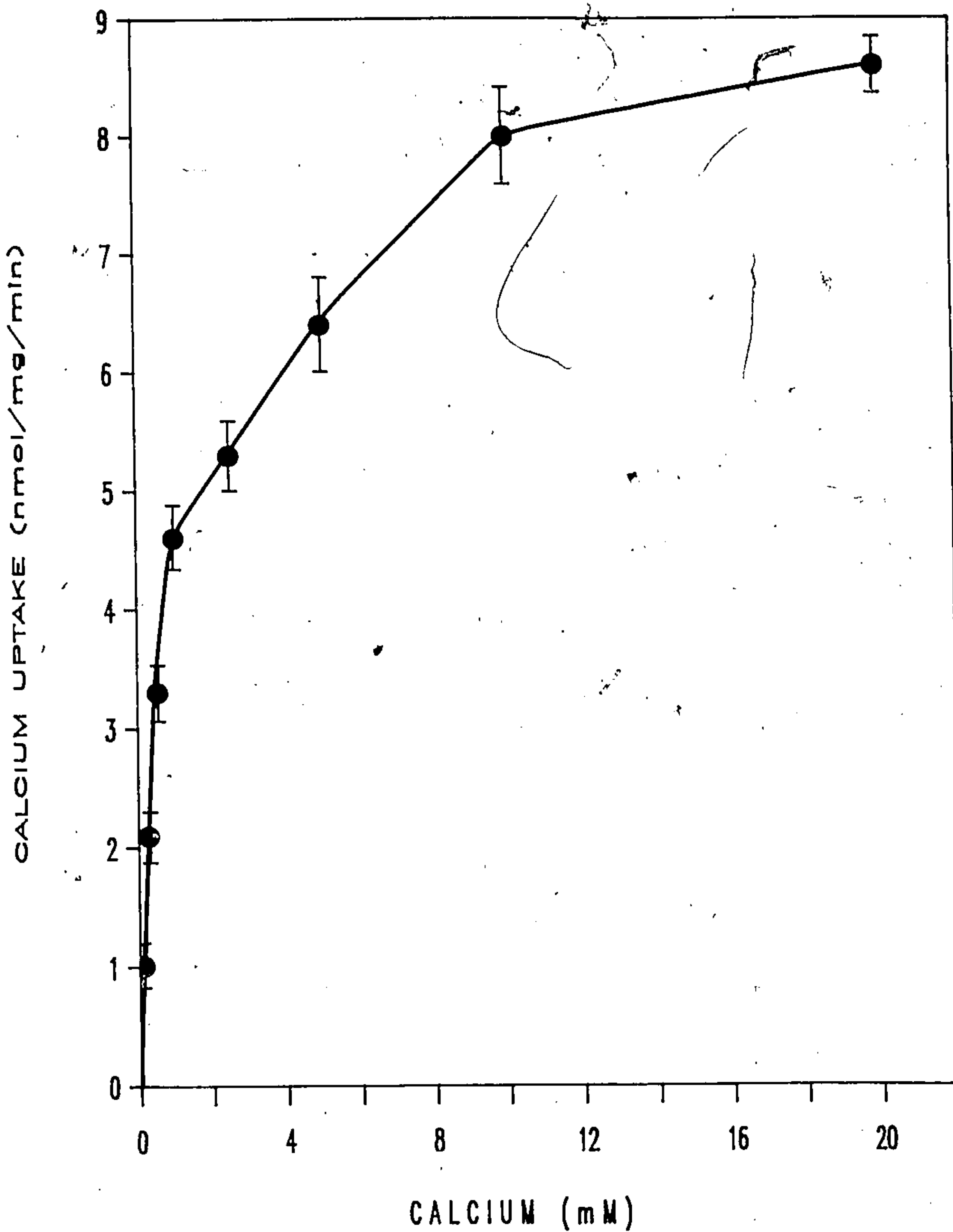
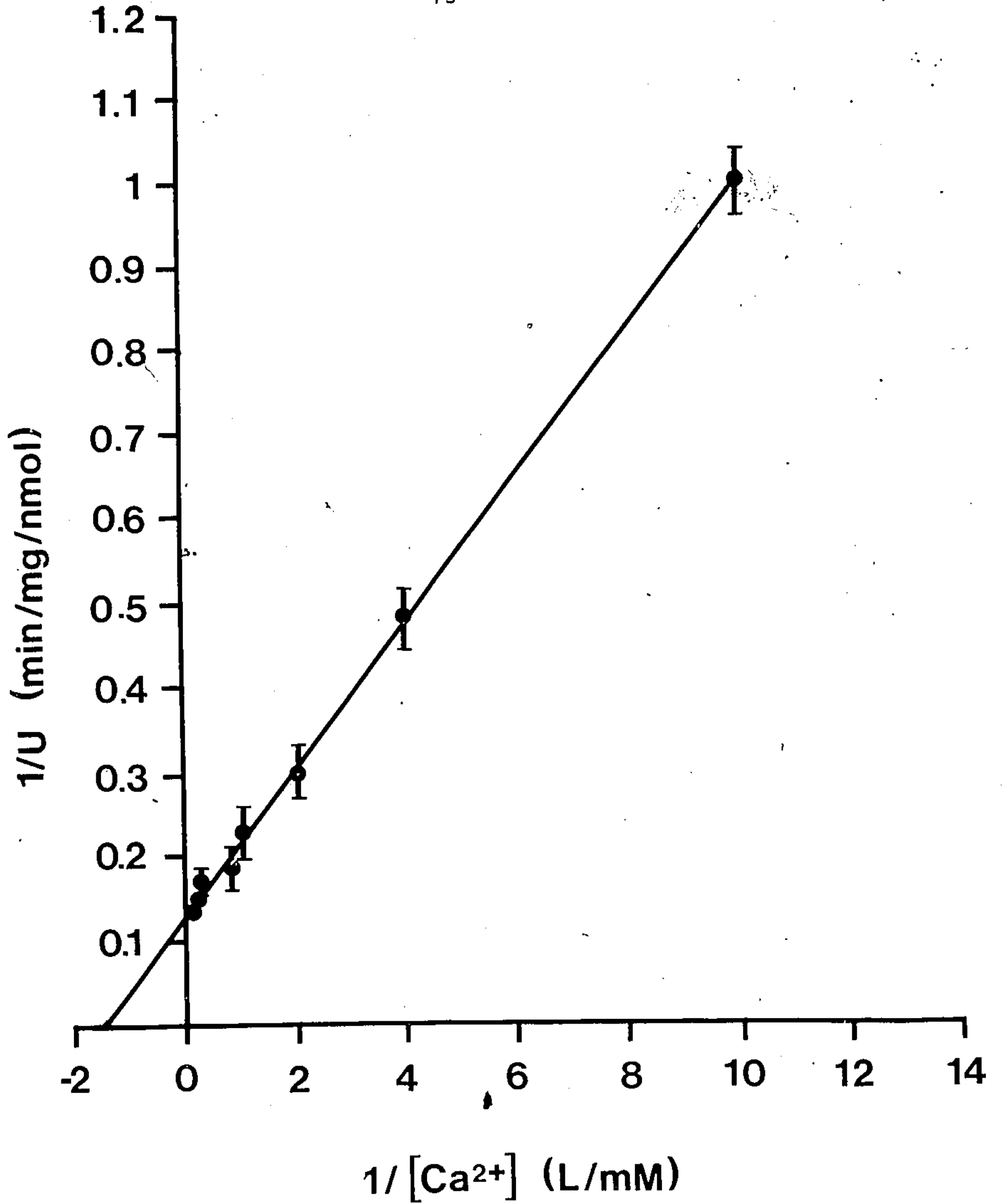

CALCIUM UPTAKE (nmol/mg/min)
0
1
2
3
4
5
6
7
8
9
0
4
8
12
16
20
CALCIUM (mM)

Figure 2-4

Lineweaver-Burke plot of the data plotted in Figure 2-3.

A best-fit line determined by least squares analysis was drawn through the points (correlation coefficient 0.99). The k_M, 0.66 mM, was determined by extrapolation of the line to the abscissa ($-1/k_M$) and V_{max}, 7.6 nmol/min/mg prot., determined from the point of intersection of the best-fit line with the ordinate axis ($1/V_{max}$).


1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1/U (min/mg/nmol)
-2
0
2
4
6
8
10
12
14
1/ [Ca2+] (L/mM)

uptake values, respectively, as measured by a Ca^{2+}-selective electrode (data not shown).

The Ca^{2+} content of the BBMV was measured before and after equilibration with Ca^{2+} by atomic absorption to determine whether the uptake of Ca^{2+} represented a net accumulation or perhaps a simple exchange process. Although not directly comparable, quantitatively, with the results for Ca^{2+} uptake as measured by the isotope method, there was a net increase in Ca^{2+} content of the BBMV after equilibration with the cation (results not shown).

The uptake process exhibited a pH optimum at 7.0-7.5 for the equilibrium value (120 min) and 7.5 for the rate value (5 min; figure 2-5). About one-half of the Ca^{2+} uptake appeared to be pH dependent in the range studied.

The vesicles were sensitive to the osmolarity of the uptake medium; when the osmotic pressure outside the vesicles was several times greater than inside the vesicles the transport of D-glucose was markedly reduced (see figure 2-2). Figure 2-6 displays the effect on Ca^{2+} uptake of increasing osmotic pressure (outside > inside) again with D-cellobiose. When uptake of Ca^{2+} by BBMV was measured at 1 min there was no effect of increasing osmotic pressure (outside > inside), which suggested that this process represented mostly binding of Ca^{2+} to the membrane. This was further supported by the fact that the intravesicular space, could accommodate, in the presence of 0.36 mM Ca^{2+},

Figure 2-5

The effect of pH on calcium ion uptake.

The conditions were as stated for Figure 2-6 except that the pH was varied by using the following buffers prepared in 100 mM mannitol; pH 4.0-6.0, 10 mM Mes-Tris; pH 6.5-9.0, 10 mM Hepes-Tris; pH 9.5-11.0, 10 mM Caps-Hepes and the time of incubation was 5 min (●) or 120 min (■).

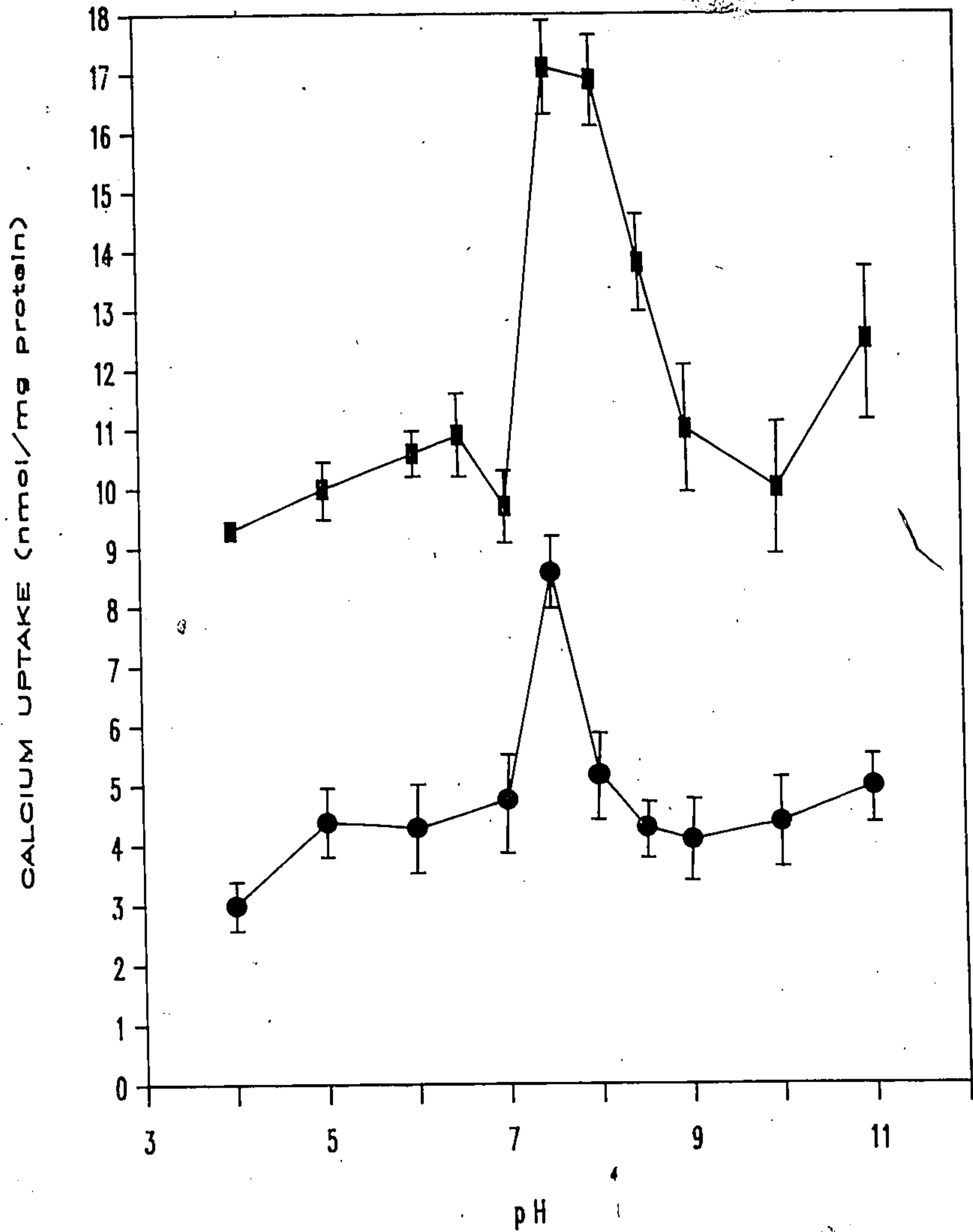
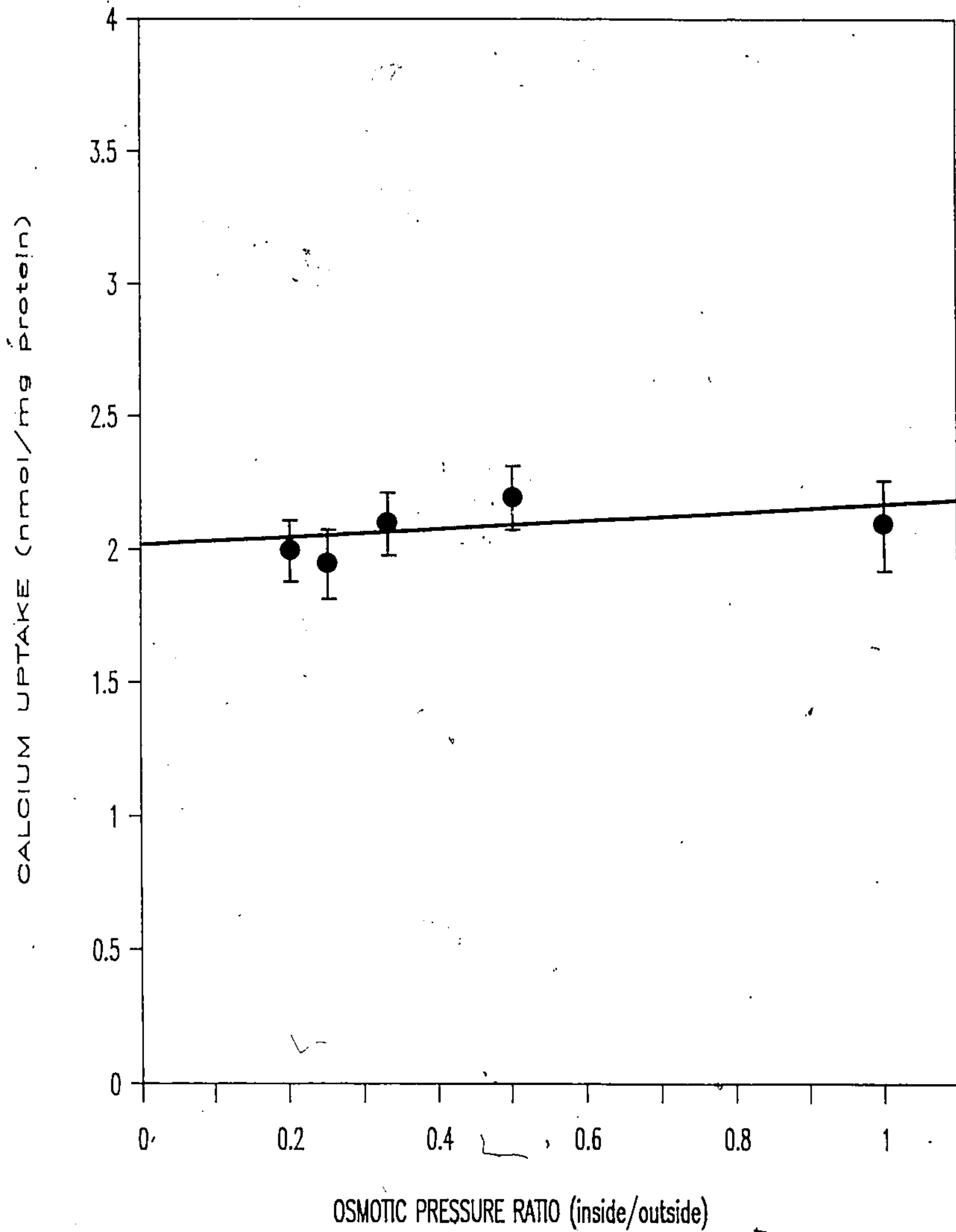

CALCIUM UPTAKE (nmol/mg protein)
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
3
5
7
9
11
pH

Figure 2-6

The effect of osmolarity on uptake.

Brush border membranes were prepared in the presence of 50 mM D-cellobiose to entrap this disaccharide. The Ca^{2+} uptake was then measured at 25°C as stated for Figure 2-6 except that the incubation mixture contained various concentrations of D-cellobiose. The osmotic ratio was taken to be that of the concentration of cellobiose inside/outside. Uptake was measured for 1 min.


CALCIUM UPTAKE (nmol/mg protein)
OSMOTIC PRESSURE RATIO (inside/outside)
0
0.5
1
1.5
2
2.5
3
3.5
4
0
0.2
0.4
0.6
0.8
1

no more than 0.30-0.35 nmol Ca^{2+} per mg protein at equilibrium. From these data it could be seen that a good portion of the Ca^{2+} uptake was binding because the equilibrium uptake value was 18 ± 3 nmol/mg protein, some 50 times greater than would be the case if the process was exclusively due to transport and intravesicular storage in a free state.

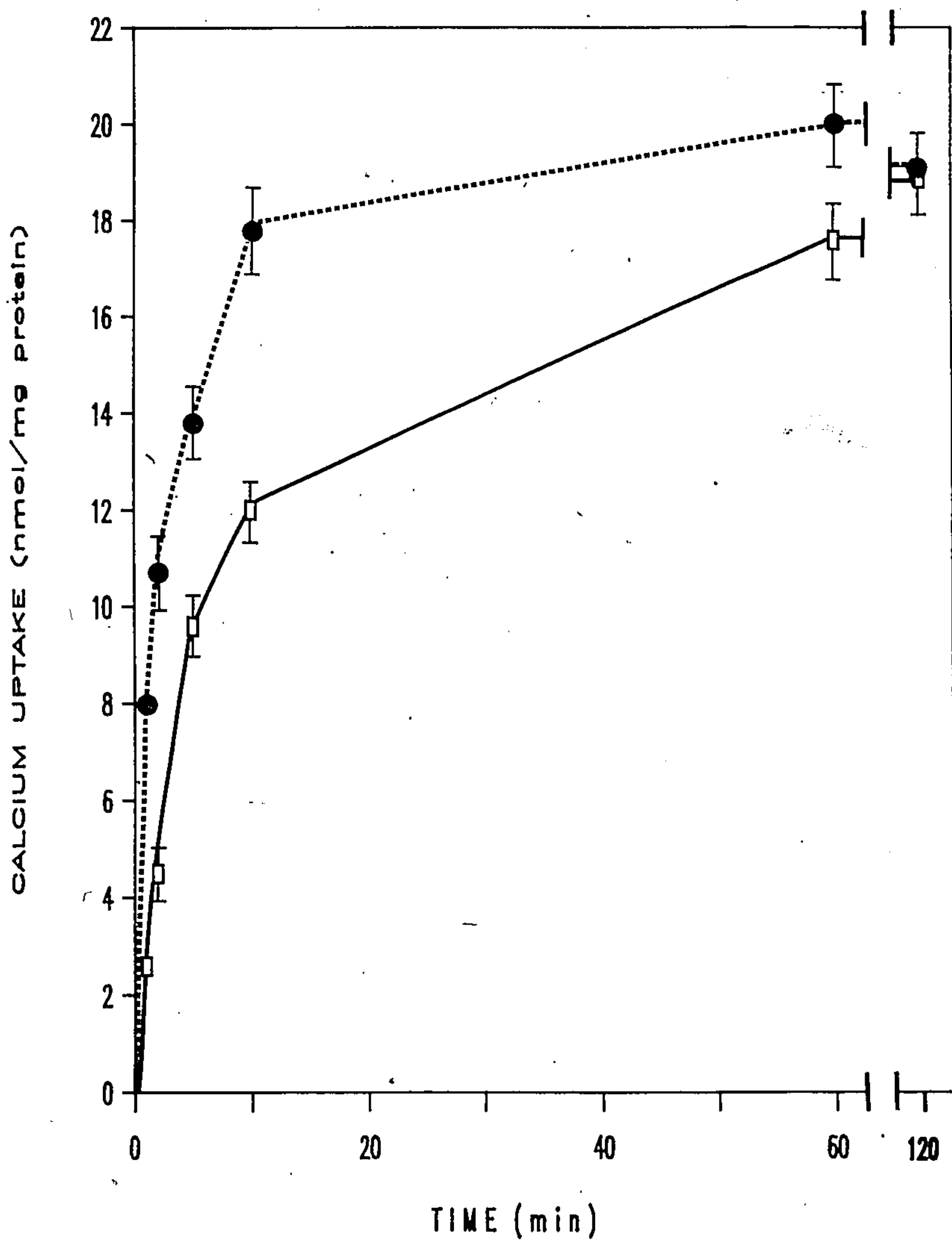
Preliminary results obtained with BBMV prepared in the presence and absence of 5 mM ATP added externally or internally, demonstrated that an added energy source had no effect on Ca^{2+} uptake. However, it was determined that during the course of the experiment ATP was rapidly destroyed, probably due to the activity of the ATPase-alkaline phosphatase complex in this membrane. The experiments were not pursued further with the question of whether Ca^{2+} uptake in the brush border membrane depends, at least partly, on metabolic energy-assisted processes remaining an open one. Further experiments would need to be designed such that membrane background phosphatase activity is completely inhibited or that an ATP-generating system be entrapped inside the vesicles as an energy source for any nucleotide-dependent cation pump that may reside in the vesicles.

Results illustrated in Figure 2-7 show a marked effect of the Ca^{2+} ionophore, A23187, on the rate of Ca^{2+} uptake but not the equilibrium value. These results indicated that

Figure 2-7

Effect of ionophore A23187 on uptake.

Incubations were at 25°C under conditions stated for Figure 2-6. (□) no ionophore added; (●) 15 μM A23187 added.


CALCIUM UPTAKE (nmol/mg protein)
22
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
120
TIME (min)

the membrane acts as a permeability barrier to the movement of Ca^{2+} and that the ionophore was merely circumventing this barrier by facilitating the rapid movement of Ca^{2+} to inner binding sites. Figure 2-8 indicates that efflux from vesicles equilibrated in the presence of 0.36 mM Ca^{2+} and diluted 40-fold with buffer containing EGTA or EGTA + A23187 was markedly enhanced by the presence of the ionophore. Careful examination reveals that after 1 minute an increase of approximately 75% in efflux, due to the ionophore occurred, which corresponds to an increased loss of 13-14 nmol/mg protein. This value obtained for efflux most likely represented Ca^{2+} that was located inside the vesicles since it was a pool not readily accessible by chelating agents and since the effect of the ionophore could only be rationalized on the basis of an increased rate of translocation of Ca^{2+} from an internal pool to the exterior of the vesicle.

The data presented in Table 2-1 reveals that about one-third of the Ca^{2+} that was bound (at equilibrium) after briefly washing with Hepes-Tris buffer could be removed by a rapid wash with EDTA-or EGTA-containing stop solutions. The amount that was removed after a 5 minute incubation of the vesicles with Ca^{2+} was proportionally smaller (10-20%) which indicated that binding to internal sites most likely proceeds at a rate greater than that of nonspecific adsorption to external sites of the vesicles. In other words, it appeared that at least two binding processes could

Figure 2-8

Effect of ionophore A23187 on efflux.

Membrane vesicles (30 µg protein) were preloaded to equilibrium by incubation for 120 min at 25°C with 0.36 mM $CaCl_2$. The membranes were then diluted 40-fold with 10 mM Hepes-Tris buffer (pH 7.5) containing 0.1 mM EGTA (●) or containing 0.1 mM EGTA + 15 µM A23187 (□) and filtered after the times indicated. The control value (100%) represented vesicles that were subjected to a brief (several second) chelator wash as described in the Ca^{2+} uptake assay procedure (see methods section C.)

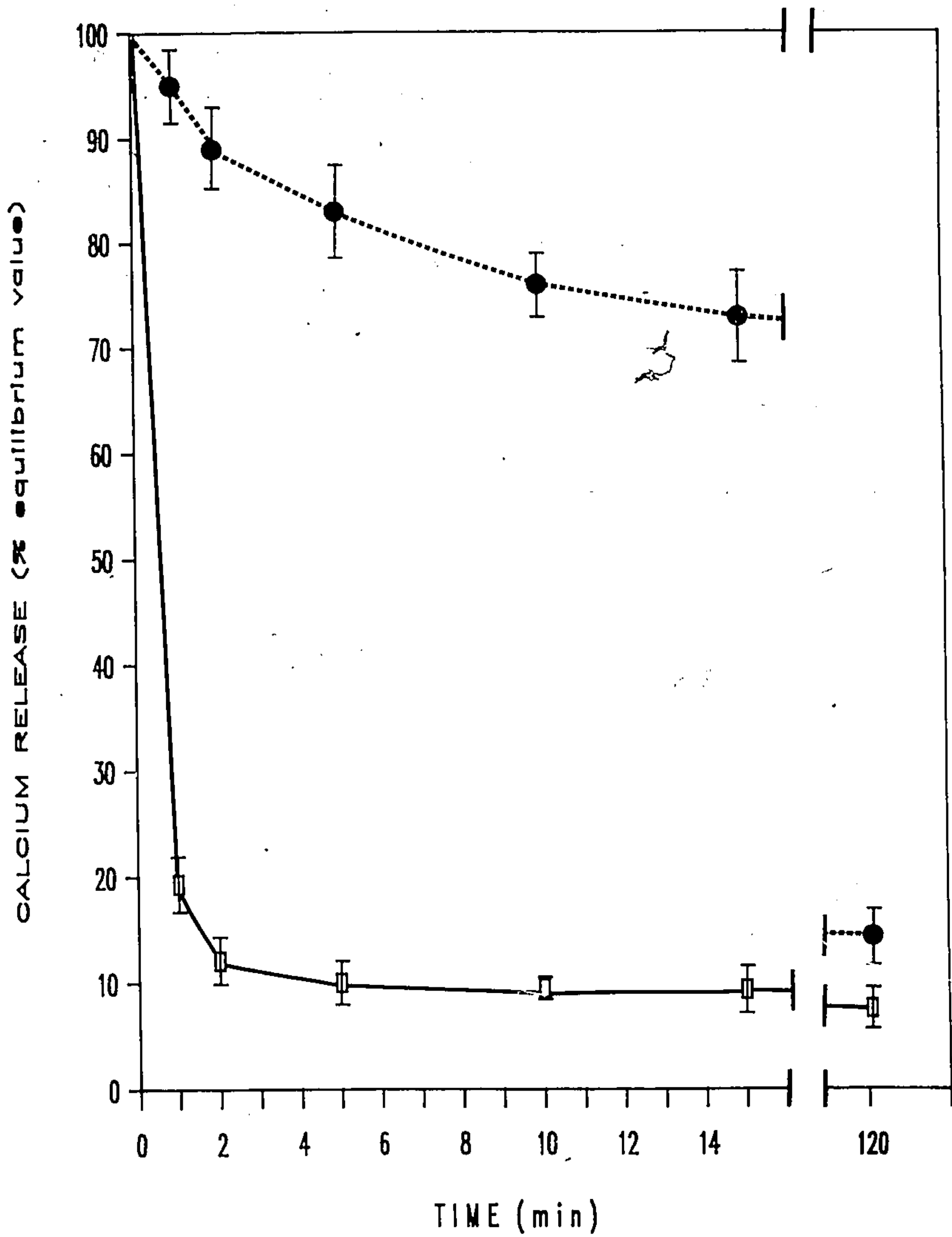

CALCIUM RELEASE (% equilibrium value)
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
120
TIME (min)

TABLE 2-1

The Effect of Washing with Chelating Agents on Ca^{2+} Uptake

Wash conditions	Ca^{2+} Uptake (nmol/mg protein)		
	5 min	60 min	120 min
Buffer	9.13 ± 0.44	22.3 ± 1.6	22.7 ± 0.7
Buffer + EDTA	8.13 ± 0.20	13.6 ±-1.4	15.8 ± 1.8
Buffer + EGTA	7.38 ± 0.37	14.8 ± 1.3	16.8 ± 1.4

The uptake was measured at 25°C for 5, 60 and 120 min under conditions stated for the Ca^{2+} uptake assay in the Methods section. The reaction was stopped by addition of ice-cold 100 mM mannitol, 10 mM Hepes-Tris (pH 7.5) solution containing either no chelating agent, 5 mM EDTA or 5 mM EGTA as indicated. The suspension was then rapidly filtered and collected membranes washed. Results are the means ± S.D. of six samples from two separate membrane preparations.

occur in these vesicles: (1) binding to the exterior of the vesicles and (2) binding to internal (intravesicular) sites after the cation was translocated across the membrane bilayer. Normally, only the second binding process was measured throughout since the processing of the BBMV after uptake included an EDTA wash. It was interesting to note that under equilibrium conditions the pool of internally-bound Ca^{2+}, determined by washing with chelators, was similar in size to the one measured with the use of the ionophore.

Results summarized in Table 2-2 reveal the effect of adding various metal cations on the uptake of $^{45}Ca^{2+}$ by BBMV. The lanthanides, La^{3+} and Tb^{3+}, were the most effective cations in competing with Ca^{2+} for the uptake system. Ca^{2+}, itself, was the next most specific for the uptake system followed by the divalent cations, Mn^{2+}, Mg^{2+}, and Ba^{2+}, respectively. The monovalent cations, Na^{+} and K^{+}, were almost without effect at the concentrations used in this study. The highest concentration, 2.5 mM, of competing cation used represents a ratio of competing cation to Ca^{2+} of 25:1. It had previously been shown by Langer and Frank (1972) that lanthanides, in particular La^{3+}, block Ca^{2+} channels in heart muscle cell plasma membranes. This information, along with the result shown in Table 2-2, prompted a further investigation into the effect of the lanthanides on Ca^{2+} uptake (Figure 2-9). Terbium was

TABLE 2-2

Effect of Metal Cations on Ca^{2+} Uptake

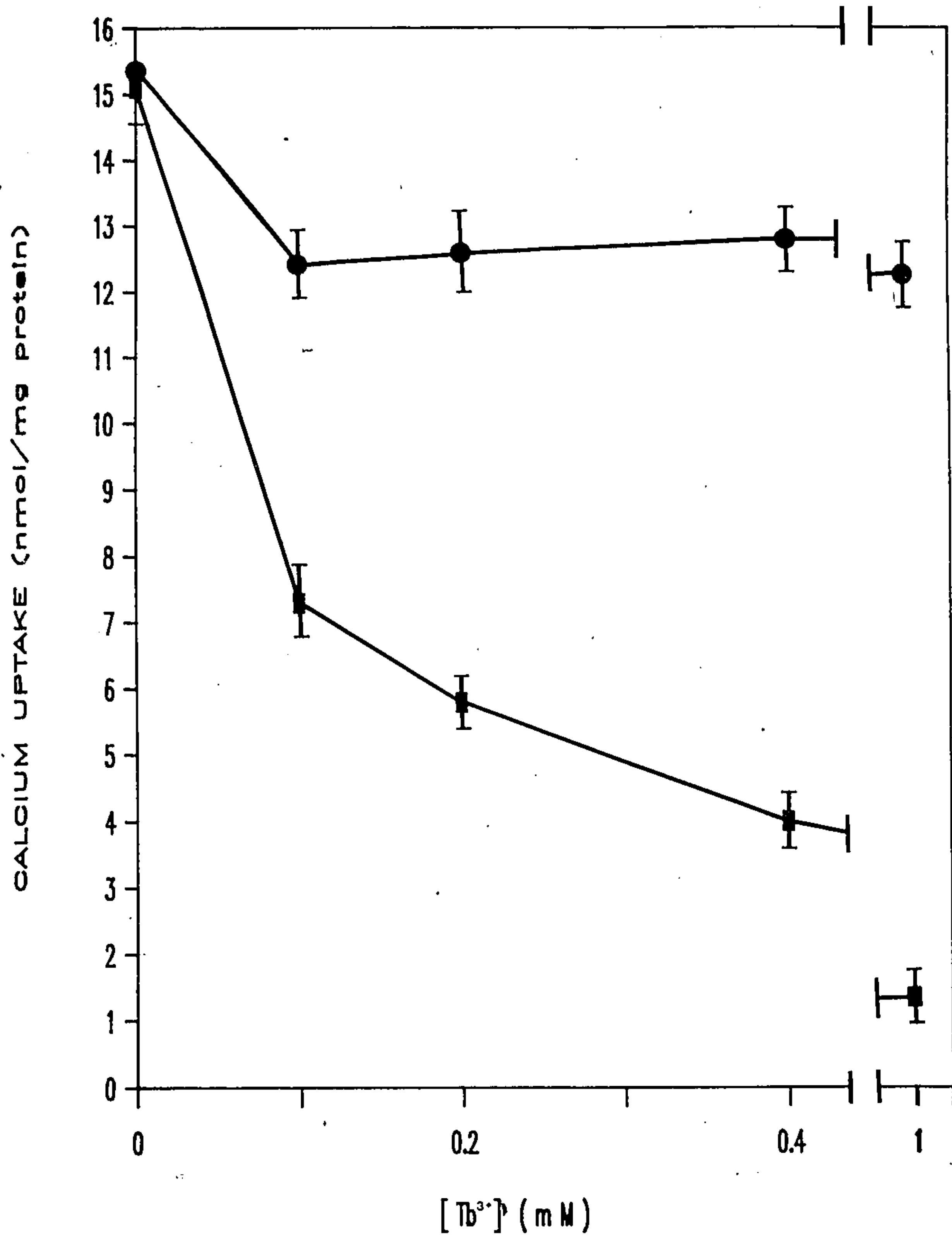
	Rate of Ca^{2+} uptake (nmol/mg for 5 min)		Equilibrium uptake (nmol/mg for 120 min)	
Cation	0.50 mM	2.5 mM	0.50 mM	2.5 mM
La^{3+}	0.3	-	1.7	-
Tb^{3+}	0.7	-	3.0	-
Ca^{2+}	1.2	0.4	4.1	1.4
Mn^{2+}	1.8	0.8	5.6	2.3
Mg^{2+}	2.8	2.2	6.8	4.0
Ba^{2+}	3.1	1.7	6.5	3.6
K^{+}	3.0	3.0	9.7	8.9
Na^{+}	3.2	3.2	11.9	8.5

The incubations were performed for 5 and 120 min at 25°C. The other conditions for incubation were as specified for the Ca^{2+} uptake assay in the Methods except that the incubation mixtures contained 0.10 mM $^{45}CaCl_2$ together with chloride salts (0.5 and 2.5 mM) of other metal ions as indicated. In the case of Ca^{2+}, the labelled Ca^{2+} was diluted with unlabelled Ca^{2+} to give a concentration of 0.6 mM or 2.6 mM. The values represent averages of 4-8 determinations performed with membrane from two or more rabbits. The standard deviations were within 12% of the mean. Control values in the absence of any competing cations were 3.2 and 12.1 nmol/mg protein for the rate of Ca^{2+} uptake and the equilibrium value, respectively.

Figure 2-9

Effect of Tb^{3+} on Ca^{2+} uptake.

Membranes were incubated at 25°C for 120 min with 0.36 mM $^{45}CaCl_2$ as stated for Figure 2-6 after which time various concentrations of Tb^{3+} were added and incubations were pursued for another 120 min (●). Membranes were incubated for 120 min at 25°C with various concentrations of $TbCl_3$ after which time 0.36 mM $^{45}CaCl_2$ was added and incubations were pursued for another 120 min (◘).


CALCIUM UPTAKE (nmol/mg protein)
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0
0.2
0.4
1
[Tb³⁺] (mM)

selected for study because its effects had not been examined in detail, and because this particular lanthanide possesses useful spectroscopic properties (Richardson, 1982) which can be exploited to study Ca^{2+}-binding proteins.

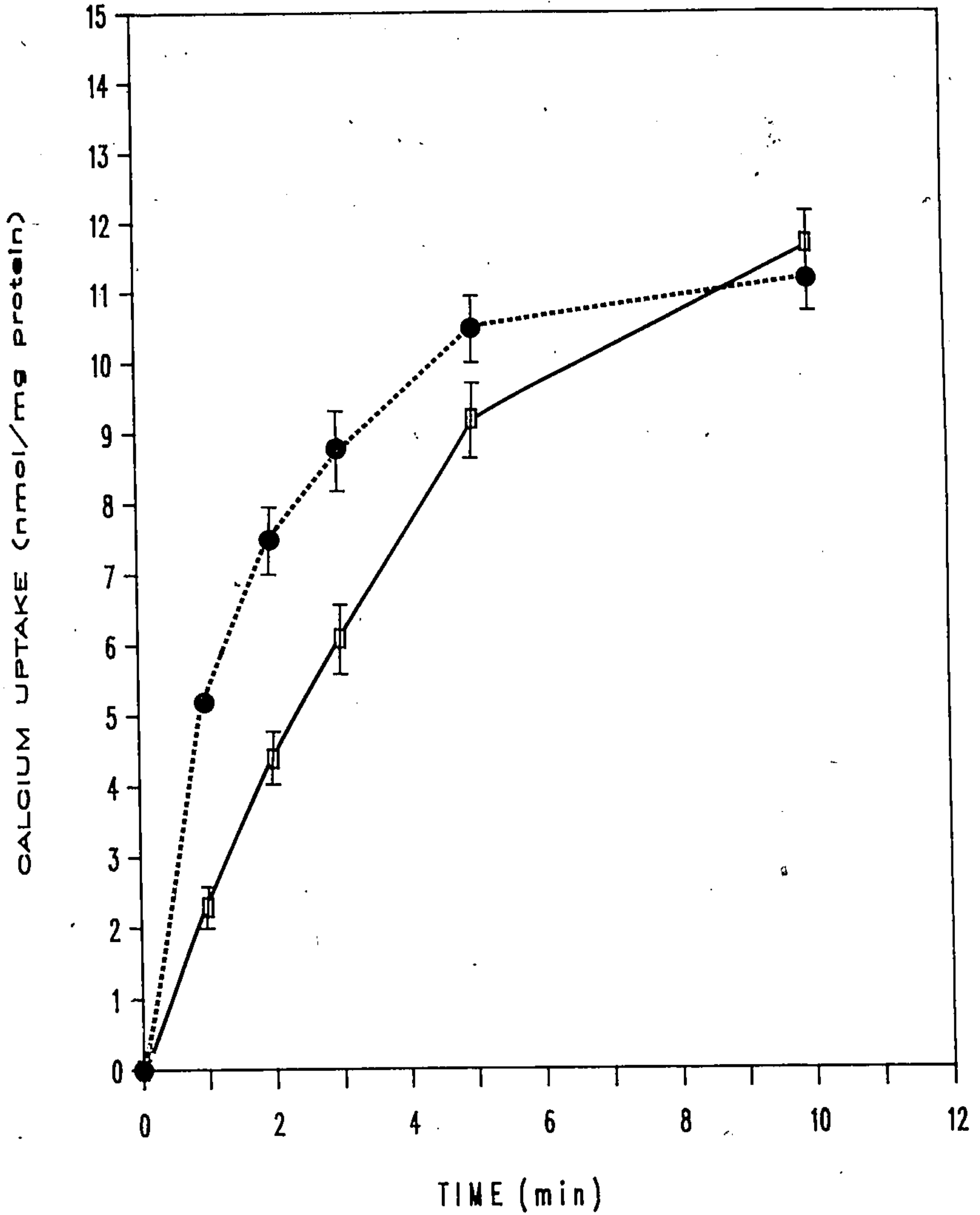
Results presented in Figure 2-9 suggest that when BBMV were incubated with Ca^{2+}, washed with stop solution to eliminate externally bound Ca^{2+}, and then incubated with increasing concentrations of Tb^{3+}, there was relatively little loss of Ca^{2+} from the vesicles. However, when the vesicles were preincubated with Tb^{3+} first, followed by incubation with Ca^{2+}, the Tb^{3+} blocked the uptake of Ca^{2+} in a concentration dependent manner. From this data it appeared that Tb^{3+} was competing with Ca^{2+} for an external binding site involved in the translocation of Ca^{2+} across the vesicle bilayer. Terbium was not transported into the vesicles however, and consequently was not able to displace Ca^{2+} from its internal binding sites.

The effect of generating a membrane potential on Ca^{2+} uptake is presented in Figure 2-10. The samples having an imposed negative-inside potential gradient showed an initial enhancement in the rate of Ca^{2+} uptake compared with the control samples. At later time points, 10-120 min, there was no significant difference in the uptake values between treated and control samples, this likely being due to the slow dissipation of the gradient imposed during these relatively longer times. The differences in the initial

Figure 2-10

The effects on Ca^{2+} uptake of a valinomycin-induced electrochemical potential (inside negative).

Brush border membrane vesicles were prepared in the presence of 50 mM K_2SO_4 to entrap this salt. Control brush border membranes were prepared in the same manner but in the absence of K_2SO_4. 10 µl of control and experimental brush border membranes (100 µg protein) were diluted 20-fold at time 0 with 190 µl of a solution containing $^{45}CaCl_2$ (0.36 mM), mannitol (100 mM) and Hepes-Tris buffer (10 mM, pH 7.5). The uptake was pursued for up to 120 min at 25°C and terminated with the addition of 2 ml ice-cold stop solution. (●) K_2SO_4; (□) no K_2SO_4.


CALCIUM UPTAKE (nmol/mg protein)
TIME (min)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

uptake rates between treated samples and control were not likely due to any decrease in free Ca^{2+} concentration inside the vesicles resulting from interaction between SO_4^{2-} and Ca^{2+} because the product for the concentrations of these ions was lower (1.80×10^{-5}) than the solubility product of Ca_2SO_4 at 25°C (2.45×10^{-5}). In the light of the known function of valinomycin to transport K^+ down a concentration gradient (ionophoric action), while the flux of SO_4^{2-} remains very slow, these results indicated that Ca^{2+} movement into rabbit BBMV was accelerated by a membrane potential (inside negative). This further suggested that Ca^{2+} was moving across the brush border membrane and therefore must be binding to sites inside the vesicles.

V. DISCUSSION

The movement of Ca^{2+} from the luminal contents of the small intestine into the mucosal cell across the brush border membrane, followed by the transcellular shuttling and pumping of the cation across the basolateral membrane of the cell is a composite and very complex process. This study focused on one aspect of the overall mechanism and revealed some of the properties of the process involved in the flux of Ca^{2+} across the brush border membrane *in vitro*.

The data shown in Figure 2-1 established the validity of the BBMV preparation for uptake studies. The ability of the vesicles to briefly sustain a sodium ion gradient which

promotes enhanced entry of D-glucose, as evidenced by the overshoot phenomenon, attests to the integrity of this vesicle suspension. It should be mentioned as well that examination of these BBMV by electron microscopy had previously indicated to investigators in this laboratory (Proulx et al., 1982) that the preparation consisted of closed membranous structures.

The calculated K_m for Ca^{2+} uptake into these vesicles was 0.66 mM (Figure 2-4) a value that agrees quite well with the values calculated by van Os (1987) from the data of several investigators. It was interesting that van Os found that the K_m values for Ca^{2+} uptake into intact intestine using either an Ussing chamber, isolated cells or BBMV were all in the same range. It should be noted that the K_m value for Ca^{2+} uptake by BBMV was similar to the gut concentration of free Ca^{2+}, i.e., in the mM range.

The results in Figure 2-2 show that the uptake of Ca^{2+} into rabbit BBMV was similar in certain respects to that which occurred in other species such as rat and chicken, in its saturability, as well as in its time and temperature dependency. It has been well documented that vitamin D regulates Ca^{2+} uptake by intestinal cells and that this regulation occurs at the level of the brush border membrane (Bikle and Munson, 1985; Wilson and Lawson, 1980). The vitamin D-stimulated component of brush border membrane Ca^{2+} uptake has been shown to be a saturable one (Bronner 1982;

1985; Bronner et al., 1986), contrasted with simple diffusion which is independent of vitamin D regulation.

It was interesting that the value for the amount of endogenous Ca^{2+} found in these rabbit BBMV (140 nmol/mg protein, data not shown) compares favorably with the value of 120-130 nmol/mg protein found in rat BBMV by Kessler et al. (1978), who also used atomic absorption analysis for this determination.

The uptake of Ca^{2+} by rabbit BBMV as shown in Figure 2-6 indicated a measure of pH dependency but it was not clear as to why almost one-half of the uptake was not sensitive to pH over the relatively-wide range studied. Many factors could be interacting to give this result, such as the pK_a of certain amino acid residues on proteins, denaturation of proteins, breakdown of vesicle integrity at extremes of pH, and the charge of lipid head groups. It was noteworthy, however, that the pH optimum for this process was in the physiological range for both the uptake rate and the equilibrium value.

One noteworthy difference between the Ca^{2+} uptake in rabbit BBMV and that occurring in chicken and rat vesicles was illustrated in Figure 2-6. The uptake of Ca^{2+} by rabbit BBMV showed no sensitivity to the osmolarity of the extravesicular medium for the rate value. Rasmussen et al. (1979), using chicken BBMV, found that up to as much as two-thirds of the uptake of Ca^{2+} by BBMV was sensitive to the

osmotic pressure ratio, outside greater than inside. Likewise, although not to the same degree, Miller and Bronner (1981) found that Ca^{2+} uptake in rat BBMV was affected by an osmotic pressure gradient. The data in Figure 2-6 suggested that the Ca^{2+} uptake process in the rabbit involved mostly binding of the cation to the membrane and little or no intravesicular storage in the free state. Subsequent experiments designed to further characterize this uptake process suggested that translocation of Ca^{2+} occurred coupled with binding to internal sites of the membrane. This conclusion was based on the fact that chelating agents such EDTA and EGTA, employed during the stop and wash steps of the uptake procedure, removed no more than one-third of the Ca^{2+} taken up at equilibrium conditions in the absence of chelator (Table 2-1).

Also, the data in Figures 2-7 and 2-8 demonstrated that both the influx and efflux of Ca^{2+} were enhanced by the Ca^{2+} ionophore, A23187, and that the efflux enhancement involved a pool which was similar in size to the one resistant to the action of chelating agents added externally. It was likely, then, that this process was one of a translocation of Ca^{2+} across the vesicular membrane followed by a binding of the cation to sites located inside the vesicles with this reservoir of sites being very large compared to the aqueous capacity of the vesicles for Ca^{2+} (see Table A-1, Appendix).

Another piece of evidence - in favor of this proposed mechanism for Ca^{2+} was the sensitivity of the uptake process to the membrane potential (inside negative) as shown in Figure 2-10. This was again indicative of the movement of Ca^{2+} from the incubation medium across the membrane and inside the vesicle. If the Ca^{2+} was binding to the outside of the vesicle or just in the membrane bilayer itself then one would not expect this process to be subject to any membrane potential effect. A series of preliminary studies (data not shown) employing various counter ions to Ca^{2+} in the uptake process indicated that the hydrophobicity of the counter ion affected the rate of uptake but not the equilibrium value, indicating that Ca^{2+} translocation across the vesicle bilayer was potential sensitive (Gamble and Lehninger, 1973). Again, this demonstrated that the Ca^{2+} was moving across the vesicle membrane.

It appeared from preliminary investigations that the addition of high energy nucleotides did not affect the uptake of Ca^{2+} by rabbit BBMV. It seemed that this process was an energy independent process. However, to fully confirm this, further studies need to be carried out using non-hydrolyzable ATP analogues, an ATP-generating system, or specific ATPase inhibitors.

The specificity of the uptake process for Ca^{2+} was indicated from the results of Table 2-2. The process showed good specificity for Ca^{2+} when compared with the other

physiological divalent cations, Mg_{2+}, Mn_{2+}, and Ba_{2+}. The higher affinity of the lanthanides, Tb^{3+} and La^{3}_{+}, for the uptake system was not surprising in view of previous findings that such trivalent cations exhibit as much as an hundred fold greater affinity for Ca^{2+}-binding proteins (Leavis et al., 1980; Dockter 1983). This information prompted further study on the ability of Tb^{3+} to block the uptake of Ca^{2+} into the BBMV as illustrated in Figure 2-9. The finding that Tb^{3+} blocked Ca^{2+} uptake in a concentration-dependent manner indicated that the lanthanide was binding possibly to the entity responsible for the carrier mechanism in the brush border membrane. However, it appeared as though the lanthanide was not being transported because of its inability to displace the Ca^{2+} once inside the vesicle. This result may be taken as further evidence that Ca^{2+} was internalized inside the vesicles presumably bound to acidic sites located on the inner surface of the vesicles.

At the lower concentration (0.5 mM) the divalent cations, Mg^{2+}, Mn^{2+}, and Ba^{2+} inhibited the equilibrium uptake of Ca^{2+} whereas at the higher concentration (2.5 mM) both the rate and equilibrium uptake values were affected. Assuming that the translocation rate was the rate-limiting step in the uptake process, one could suggest from this that the internal binding sites were not the same as those used for the translocation of Ca^{2+} and that the interior sites

may have a lower affinity for Ca^{2+}. This would be a rational explanation if the number of internal sites was large in relation to the number of translocating sites so that the Ca^{2+} could be transferred from the one set to the other by simple mass-action. As well, the binding of Ca^{2+} inside the vesicles would keep the free Ca^{2+} concentration inside always very low compared to outside. This situation would favor a net entry by a facilitated diffusion process. When internal binding sites were occupied by other cations, the free Ca^{2+} concentration inside would accumulate and this inhibited the rate of translocation because the Ca^{2+} gradient was reduced.

Chapter Three

EFFECT OF EXOGENOUS FATTY ACIDS AND METHYL OLEATE ON Ca^{2+} UPTAKE IN BBMV

I. INTRODUCTION

The brush border membrane of the small intestinal epithelial cell is highly specialized for performing the functions of nutrient absorption of many constituents of the diet including minerals such as calcium and lipid digestion products such as fatty acids. These cells are constantly exposed to exogenous lipids derived from the diet. Schachter (1985) estimated that in a 200 g rat the brush border membrane involved in lipid absorption (i.e., the proximal 2/3 of the intestine) contained approximately 3 x 10^{18} acyl chains, whereas the triglyceride ingested and absorbed per day (taken as approx. 0.5 g) contains roughly 10^{21} acyl chains. In other words, the daily flow of dietary acyl chains greatly exceeds, by as much as three hundred fold or more, the number of acyl chains in the membrane itself. Such lipids when taken up by the luminal membrane have the capacity to modify its composition. Subsequent changes in the membrane composition may play an important role in altering and modulating membrane structure and functions found in the brush border.

It has been demonstrated that various enzyme activities are modulated by the presence in the membrane of amphipathic substances of exogenous origin which modify the motional

parameters of the lipids (Gordon et al., 1980). Thus, for example, the activity of erythrocyte (Na^+ + K^+)-ATPase can be modified by various amphipathic compounds such as aliphatic alcohols and detergents (Roelofsen et al., 1971). As well, physical manipulation of the lipid environment in cell membranes has been reported to affect activities including (Ca^{2+} + Mg^{2+})-ATPase (Galo et al., 1981), adenylate and guanylate cyclase (Brasitus and Schachter, 1981). Amphipathic molecules such as fatty acids could be expected to partition into the lipid component of the membrane but they could also bind to hydrophobic areas of membrane proteins. In addition, their carboxyl ends would interact with water or with the phospholipid head groups of the membrane bilayer or possibly with charged amino acid residues on proteins therein. These possible interactions of fatty acids with membrane bilayers as absorption proceeds could be responsible for modification of membrane fluidity and enzyme or carrier protein function.

It has been well documented that vitamin D ($1,25(OH)_2$-D_3) affects both the fatty acid and phospholipid composition of the intestinal brush border membrane. These changes, which were found to increase fluidity of the brush border membrane, are effected through the phospholipid acylation-deacylation cycle presumably found in the endoplasmic reticulum (Fontaine et al., 1981). In response to this hormone, there is an increase in phosphatidylcholine and in

the proportion of its linoleic and arachidonic acid constituents. These changes have been correlated with alterations in Ca^{2+} transport (Matsumoto et al., 1981).

Also, Bikle and Munson (1984) reported that cis and trans vaccenic acid, when incorporated into chicken BBMV in vitro affected the rate of Ca^{2+} uptake by these vesicles. This work as mentioned in greater detail in the General Introduction was not in accord with an earlier finding of Fontaine et al. (1981), who likewise studied the effect of these fatty acid isomers on the Ca^{2+} uptake process in chicken BBMV. Earlier work, in our laboratory, had been completed which indicated that free fatty acids were taken up by rabbit BBMV and that the amounts incorporated were dependent upon the chain lengths of the acids (Proulx et al., 1985). However, no attempt at that time was made to correlate the uptake of these lipids with alterations in the fluidity of the membrane or to link their incorporation with an effect on a membrane bound enzyme/carrier activity. With this knowledge at hand, it became feasible to study in a more detailed manner the effect of fatty acid incorporation on Ca^{2+} uptake in rabbit BBMV in vitro .

II. MATERIALS

[^{14}C]-labelled fatty acids and tri-[1-^{14}C]-oleoyl glycerol were obtained from New England Nuclear Corp. (Boston, MA) and diluted to the required specific activity

with unlabelled analogues. The unlabelled fatty acids and the methyl ester of oleic acid (> 99% pure) were obtained from Sigma Chemical Co. (St. Louis, MO.) and were used without further purification.

III. METHODS

A. Preparation of methyl oleate

Methyl [1-^{14}C]oleate was formed by mild alkaline methanolysis of tri[^{14}C]-oleoylglycerol as described by Marshall and Kates (1972) and purified by thin-layer chromatography according to Mangold and Malins (1960).

B. Preparation of fatty acid and methyl oleate solutions

Various fatty acids and methyl oleate, [1-^{14}C]-labelled and unlabelled, were dissolved together with sodium taurocholate in 2 ml of chloroform/ethanol (1:1, v/v). The resulting clear solutions were dried by rotoevaporation (under N_2 in the case of unsaturated fatty acids) and the residues were suspended in 10 mM Hepes-Tris buffer, pH 7.5 containing 100 mM mannitol. The suspensions were shaken at room temperature for 15 min and then sonicated twice for 2 min with the small probe of an Ultrasonics sonicator (Model A350, Ultrasonics Ltd., New York, NY.) at a setting of 5. Solutions were clarified by centrifugation at 1 x 10^7 g-min. The clear fraction of the micellized lipid-bile salt solutions was drawn off with a Pasteur pipet and the

concentrations of the suspensions were determined by counting a 200 µl aliquot.

C. Uptake of fatty acids and methyl oleate by BBMV

Membrane vesicles (300 µg protein) and various concentrations of micellized fatty acids or methyl oleate were incubated at 25°C in a water bath for 10 min (fatty acids) and up to 4 h (in the case of methyl oleate) in 1 ml of 100 mM mannitol and 10 mM Hepes-Tris buffer, pH 7.5. For all fatty acids and for methyl oleate the final concentration of taurochclate required to obtain clear micellar suspensions was 7.2 mM except for palmitic acid which required 10 mM bile salt. After incubation, the membranes were pelleted by centrifuging the incubation mixture at 40,000 x g for 30 min followed by a wash with ice-cold incubation buffer. The pellets were resuspended in a small volume of distilled water for counting as previously described (Proulx et al., 1984), for extraction of lipids (Bligh and Dyer, 1959), or for Ca^{2+} uptake studies.

D. Analytical procedures

For gas chromatography, lipids were extracted from BBMV and their acyl chains converted to the methyl esters for gas chromatography as described in Chapter One.

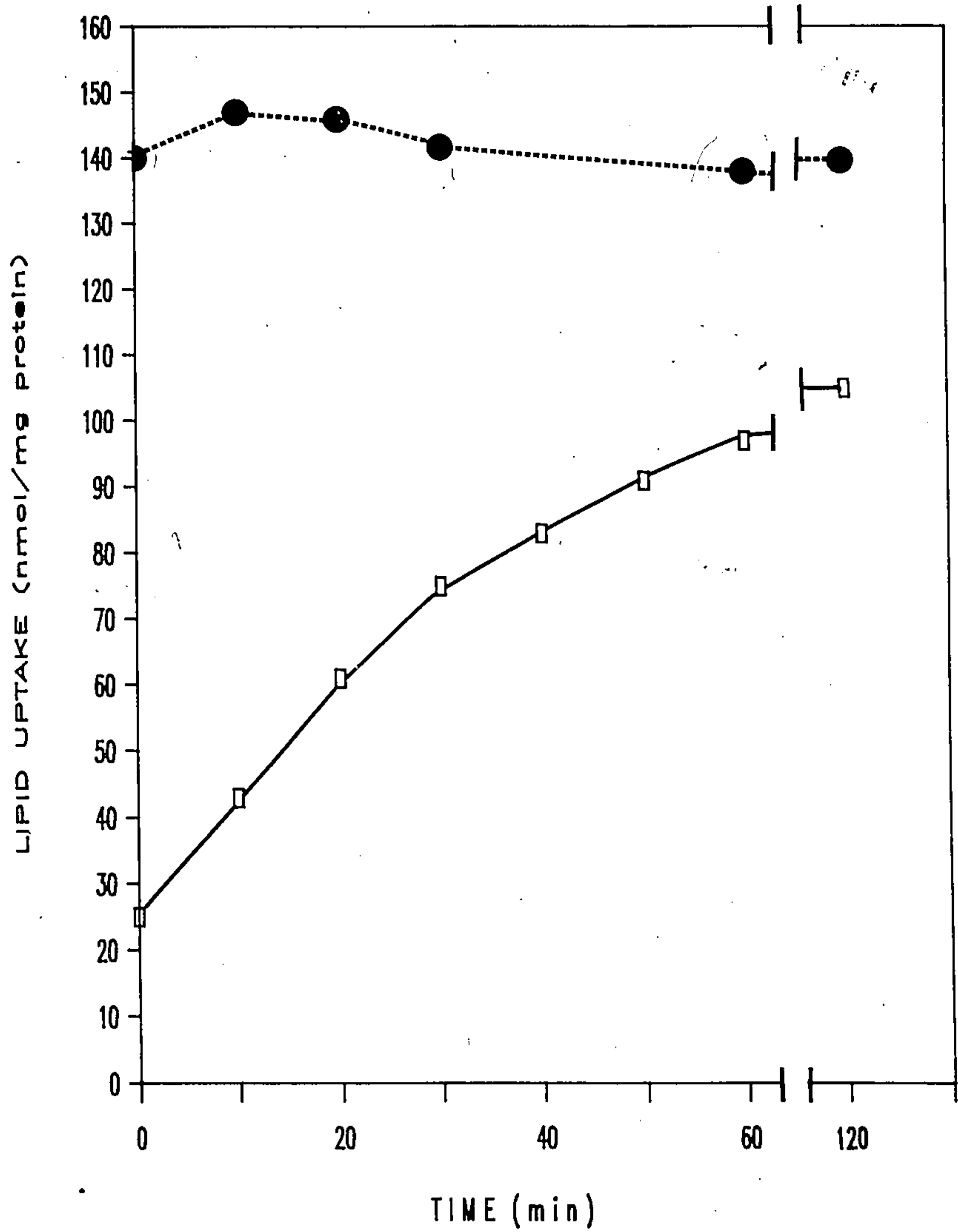
IV. RESULTS

The results in Figure 3-1 display the time course for the uptake of oleic acid compared with that for methyl

Figure 3-1

Time course of oleic acid and methyl oleate incorporation.

Time course of oleic acid (●) and methyl oleate (□) incorporation into brush border membranes. The incubation mixture contained 1 ml, 0.3 mg membrane protein, 7.2 mM taurocholate, 0.10 mM oleic acid and 0.09 mM methyl oleate, 100 mM mannitol and 10 mM Hepes/Tris buffer (pH 7.5). Incubations were maintained at 25°C for the times indicated. The values are averages of 3-6 determinations giving variation of less than 7% and are representative of several other experiments performed under similar conditions with different animals.


LIPID UPTAKE (nmol/mg protein)
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
120
TIME (min)

oleate. The incorporation of oleic acid into BBMV was very rapid, equilibrium being attained almost instantaneously. Prior attempts to reduce this rate by modifying the conditions for sample incubation and processing, in the hope of defining the kinetics of oleic acid uptake, were unsuccessful (Proulx et al., 1985). On the other hand, the incorporation of methyl oleate proceeded at a much slower rate, equilibrium being attained after 4 h at 25°C. Under the conditions of incubation and processing, even for methyl oleate, there was some incorporation corresponding to zero time. This occurred because of the 30 minute period required to pellet the membranes by centrifugation at 4°C.

Results in Table 3-1 reveal the extent of binding of various $[^{14}]$C-labelled fatty acids to brush border membranes. This binding was assessed by the amount of radioactivity found to be associated with the membranes after incubation with labelled fatty acid suspensions followed by washing with buffer of low ionic strength i.e., the incubation buffer. It was clear that the extent of fatty acid uptake increased in concert with the acyl chain length (Table 3-1, top to bottom), octanoic acid (C8:0) having the lowest level of incorporation.

To determine in more detail the nature of the incorporation of these fatty acids into BBMV, the vesicles, upon incubation with oleic acid were washed with high ionic strength buffer and the results are summarized in Table 3-2.

TABLE 3-1

The Effects of Exogenous Fatty Acid on Ca^{2+} Uptake by Brush-Border Membrane Vesicles

Fatty acid added	Concn. (mM)	Fatty acid uptake (nmol/mg protein)	Rate of Ca^{2+} uptake (percent of control)[a]
None	-		100
Caprylic	0.100	3.4 ± 0.1	113 ± 4
	1.000	13.0 ± 0.8	127 ± 3
	3.000	37.3 ± 3.1	161 ± 9
Lauric	0.100	9.6 ± 0.2	102 ± 5
	1.000	270.7 ± 11.2	106 ± 7
Palmitic	0.060	58.6 ± 3.0	85 ± 8
	0.200	153.4 ± 2.9	85 ± 1
	0.380	274.5 ± 4.7	84 ± 7
Oleic	0.010	18.2 ± 0.8	113 ± 10
	0.025	47.8 ± 0.3	117 ± 7
	0.050	88.8 ± 3.7	137 ± 11
	0.100	147.9 ± 3.3	65 ± 8
	0.200	341.8 ± 16.6	48 ± 8
Linoleic	0.050	50.2 ± 3.2	143 ± 7
	0.600	1075.1 ± 36.6	6 ± 1

[a]The control uptake ranged from 1.5 - 2.0 nmol/mg protein per min

Fatty acid uptake was estimated from the radioactivity recovered in the membrane pellets incubated with labelled fatty acids. Ca^{2+} uptake was measured with membranes that had been treated with similar amounts of unlabelled fatty acid.

TABLE 3-2

Effect of Washing with CsBr on the Uptake of Oleic Acid by Brush-Border Membrane Vesicles

Oleic acid concentration (mM)	Wash solution	Fatty acid uptake (nmol/mg protein)
0.05	buffer	93 ± 7
0.05	buffer + CsBr	104 ± 4
0.20	buffer	273 ± 21
0.20	buffer + CsBr	293 ± 23

Where indicated 0.25 M CsBr was present in the wash buffer together with the other usual constituents, 100 mM mannitol and 10 mM Hepes-Tris (pH 7.5)

These results indicated that the association of oleic acid with the membrane was likely a hydrophobic one because washing with CsBr (250 mM), known to dissociate any membrane surface, ionically-bound lipid (Lyte and Shinitzky, 1985), had no significant effect on the uptake of this fatty acid at either concentration studied.

As well, Table 3-1 shows the effect of the incorporation of various fatty acids on the uptake of Ca^{2+} by BBMV at 25°C. The uptake of unsaturated and shorter chain fatty acids (corresponding to incorporation values of 89 nmol/mg protein or less) resulted in a modest stimulation of Ca^{2+} uptake in BBMV ranging from 113 to 161 percent of the control. The same effect was not seen upon incorporation of saturated fatty acids even when the amounts of incorporation were comparable (cf. palmitic acid 58.6 nmol/mg and oleic acid 47.8 nmol/mg). However, incorporation of larger amounts of oleic and linoleic acid (150 nmol/mg protein for oleic acid) resulted in a very substantial decrease in the rate of Ca^{2+} uptake (Table 3-1). The short chain fatty acid, octanoic acid, although not present in the BBMV in very high amounts caused only a stimulation of the rate of Ca^{2+} uptake.

Table 3-3 shows the incorporation of methyl oleate into BBMV and the effect of increasing concentrations of the ester on the rate of uptake of Ca^{2+} by these membrane vesicles. The most striking result that could be noted from

TABLE 3-3

Effect of Methyl oleate on Ca^{2+} Uptake

Methyl oleate Concentration	Lipid uptake (nmol/mg protein)	Rate of Ca^{2+} uptake (percent of control)[a]
0.010 mM	14 ± 1	118 ± 7
0.050 mM	68 ± 4	124 ± 8
0.090 mM	101 ± 4	133 ± 9
0.120 mM	119 ± 8	143 ± 7
0.180 mM	182 ± 10	156 ± 10
0.180 mM[b]	220 ± 21	207 ± 29

[a]The control uptake was 7.5 ± 1.5 nmol/mg protein per 5 min. All methyloleate values represent incubations of 1 h with membranes except for (b) in which case incubations were for 4 h.

The results represent the mean ± S.E. from 6 to 20 determinations with membranes from 3 to 5 rabbits.

this table was that methyl oleate caused only a stimulation of Ca^{2+} uptake into BBMV. This was in contrast with the dual effects exerted by oleic acid (Table 3-1).

Calcium transport data (initial rate of uptake), presented in Figure 3-2 indicate that higher levels of incorporation of oleic acid inhibit not only the intrinsic Ca^{2+} uptake mechanism of the brush border membrane, but the ionophore, A23187-catalyzed system as well. This result can be best explained by a decrease in Ca^{2+} binding sites produced at the higher levels of oleic acid incorporation. This was substantiated by the :catchard plot data in Figure 3-3. This plot compared the equilibrium binding of control, a stimulatory oleic acid concentration (88.8 nmol/mg protein), and an inhibitory oleic acid concentration (341.8 nmol/mg protein). From this examination it was apparent that the high level of incorporated oleic acid resulted in a substantial change in the Ca^{2+} binding characteristics of the BBMV. On the other hand, incorporation of lower levels of oleic acid into BBMV, resulting in a stimulation of the Ca^{2+} uptake rate, had no effect on the equilibrium value for the cation uptake process, (Figure 3-3).

The results in Table 3-4 indicate that as the oleic acid concentration in the medium increased so did the proportion of oleic acid in the total membrane lipids. This increase was accompanied by a decrease in the proportion of all other fatty acids, as determined by gas chromatography

Figure 3-2

The effect of oleic acid on Ca^{2+} uptake.

The effect of oleic acid on the rate of Ca^{2+} uptake by brush border membrane vesicles (●) with 15 μM A23187 and (□) without ionophore. The uptake data are for 2 min incubations.

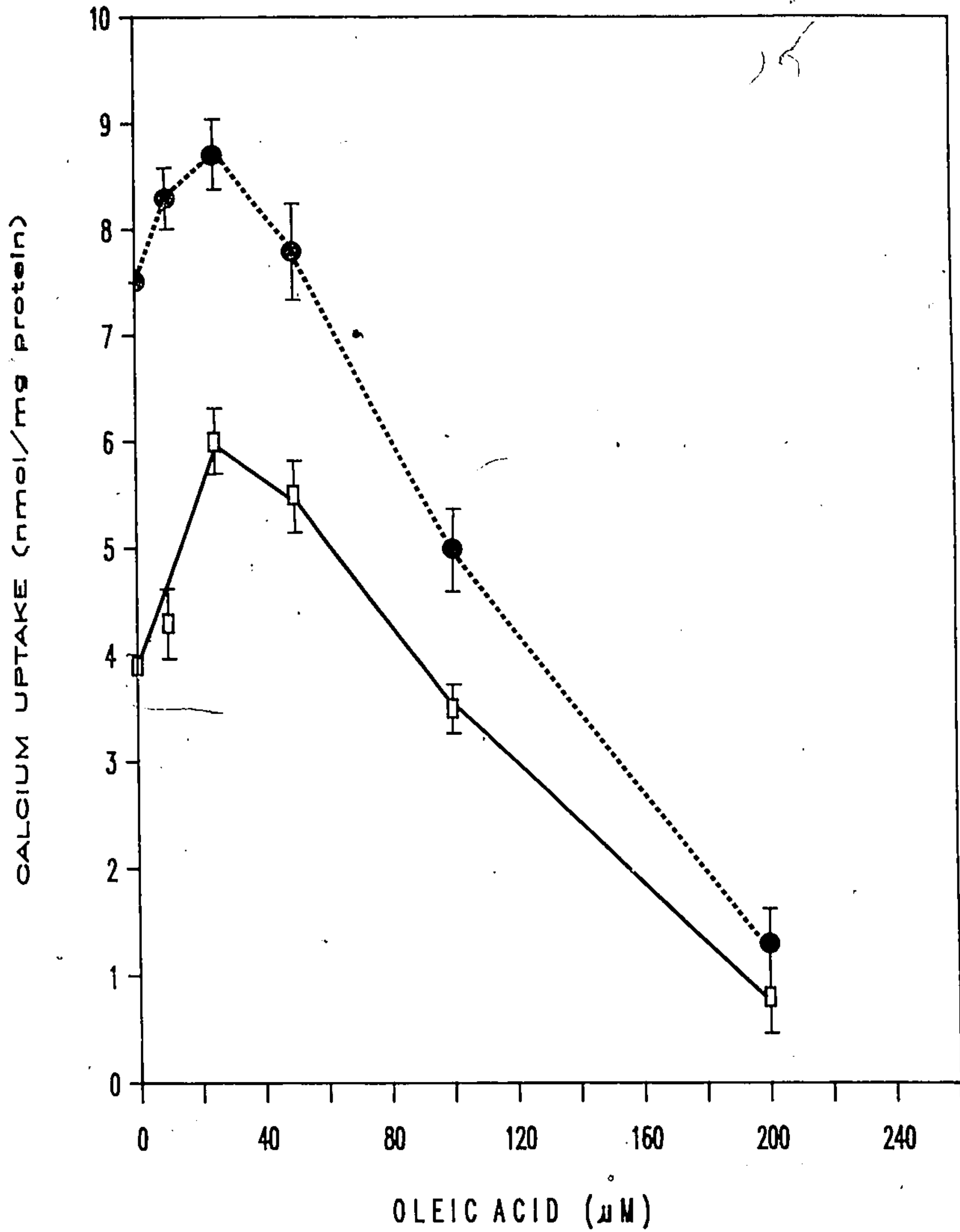

CALCIUM UPTAKE (nmol/mg protein)
OLEIC ACID (μM)
0
1
2
3
4
5
6
7
8
9
10
0
40
80
120
160
200
240

Figure 3-3

Scatchard plot of Ca^{2+} binding to BBMV

Scatchard plot for Ca^{2+} binding obtained with oleic acid-treated membranes and with control membranes (●) no oleic acid; (□) 89 nmol oleic acid incorporated/mg protein; (■) 342 nmol oleic acid incorporated/mg protein. Uptakes were measured after 120 min of incubation to obtain equilibrium values. The results are expressed as the average of four determinations with membranes from a single rabbit and are representative of several experiments yielding similar data.

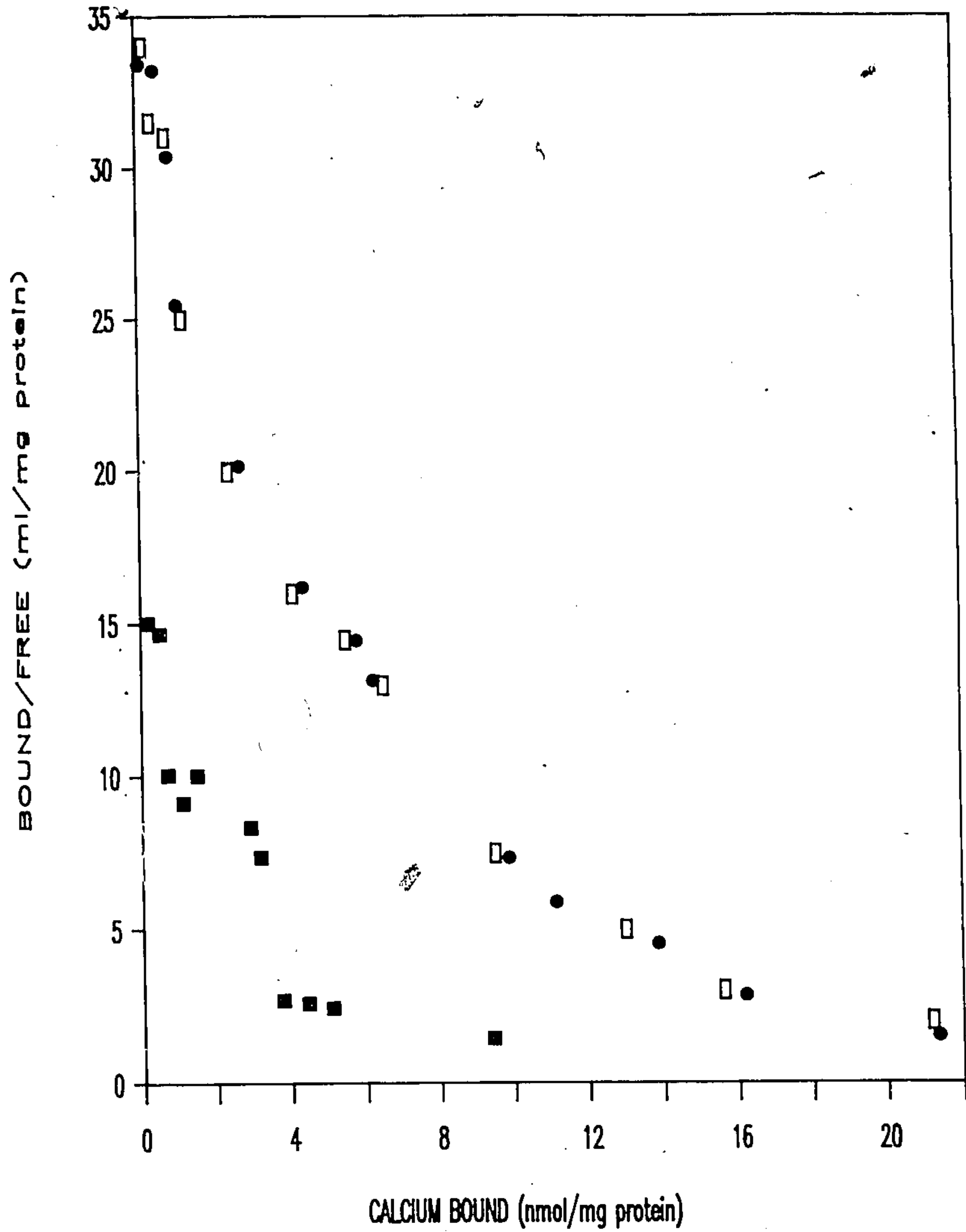

BOUND/FREE (ml/mg protein)
35
30
25
20
15
10
5
0
0
4
8
12
16
20
CALCIUM BOUND (nmol/mg protein)

TABLE 3-4

Fatty Acid Composition of Brush-Border Membranes Before and After Exogenous Fatty Acid Uptake

Fatty acid	Concn. (mM) acid added	Fatty acid composition (percent of total)							Increase in oleate (nmol/mg protein)
		16:0	18:0	18:1	18:2	18:3	20:3 + 20:4	others	
None		12.6	26.3	11.5	34.8	4.0	1.0	9.0	-
Oleic	0.01	11.9	23.5	17.6	30.4	4.0	1.9	10.7	18.3
	0.05	8.3	17.9	36.0	25.6	3.6	1.5	7.1	144.3
	0.20	5.2	11.5	62.0	15.9	2.4	0.8	2.2	404.6
									Increase in palmitate (nmol/mg protein)
Palmitic	0.20	36.0	17.3	9.8	24.2	2.9	1.2	8.6	137.9

The values are averages of duplicate determinations for a single rabbit and are representative of several experiments yielding similar results. Membranes from a different rabbit were used for each oleic acid treatment and compared with untreated membranes of the same rabbit.

analysis of the lipids of the BBMV. From careful examination, it can be appreciated that after exposure of the membranes to 0.2 mM oleic acid and palmitic acid, these exogenous lipid components accounted for 62 and 36 percent of the total fatty acids, respectively. These uptakes were similar to those calculated from the radioactivity incorporated when ^{14}C-labelled fatty acids were used and the increase in the amount of total fatty acids was entirely accountable by the fatty acid added to the medium. Therefore, the incorporation did not involve an acyl exchange process with the medium and from this it can be concluded that a net incorporation of fatty acid into the BBMV occurred.

Likewise, the results expressed in Table 3-5 indicate the absence of any exchange process involving exogenous fatty acid and endogenous cholesterol. Any loss of this sterol during the fatty acid uptake process was entirely accountable by lysis of the membrane. Some membrane lysis was concluded on the basis of comparable losses of pelletable protein and turbidity of vesicular suspensions treated with 0.2 mM oleic acid (turbidity being estimated by the absorbance of vesicle suspensions at 660 nm). Notably, lysis was not detected under other conditions.

TABLE 3-5

Effect of Fatty Acid Uptake on Cholesterol Content of Brush-Border Vesicles

Fatty acid added	Concn. (mM)	Protein loss (% of control)	Decrease in turbidity of vesicles (% of control)	Cholesterol content (μg/mg protein)
None (control)		-	-	76 ± 6
Oleic	0.05	10		78 ± 6
	0.20	26	25	86 ± 2
Palmitic	0.38	0	-	76 ± 4
Caprylic	3.00	2	-	78 ± 6

The values given are representative of several experiments yielding similar results. In the case of cholesterol content, the data are averages ± S.D. of four determinations performed on membranes from a single rabbit and are corrected for protein losses. Values indicating protein and turbidity losses are averages of two or more determinations performed on membranes from a single rabbit.

V. DISCUSSION

The results indicate that uptake of fatty acids by BBMV involved intercalation within the hydrophobic core (lipid bilayer) structure of the membrane. This can be inferred on the basis of increased binding of fatty acid with an increase in acyl chain length (Table 3-1) and from the fact that fatty acid could not be dissociated from the membrane with a high ionic strength wash (Table 3-2). The association must consequently be hydrophobic. Also, less than 2% of the bound lipid would represent contamination from the lipid bulk phase since the calculated 'inulin space' in the pelleted membranes was no more than 2 µl/mg protein (Proulx et al., 1985). The wash following uptake should reduce this contamination even further. In accord with these findings Lyte and Shinitzky (1985) found that the association of phospholipid with artificial membrane vesicles was hydrophobic and could not be reduced with a high ionic strength wash.

At the present time it is not certain as to why there is such a large difference between the rates of uptake of oleic acid and its methyl ester. This difference could be related to the high negative charge density that must exist in oleic acid micelles or to the size of the lipid-bile salt micelles both of which could affect the interactions of these aggregates with the membrane. As well, the observed difference could be due to the more limited solubility of

methyl oleate in the monomer phase. A valid explanation awaits the further elucidation of the mechanism involved in the incorporation of both these lipids into the brush border membrane.

The results, (Table 3-1 and Figure 3-2), indicated that oleic acid at low concentration acted as a Ca^{2+} ionophore. This could be inferred from the data showing that the rate of Ca^{2+} uptake was enhanced without the concomitant increase in equilibrium Ca^{2+} uptake. Ionophoric activity has been attributed to ricinoleic acid on the basis of similar results obtained in porcine jejunal BBMV exposed to low and high concentrations of this hydroxy fatty acid (Maenz and Forsyth, 1982). Interestingly, higher concentrations of ricinoleic acid had an inhibitory effect on the uptake of Ca^{2+} similar to the effect observed in the present study. These opposing effects of low and high concentrations of unsaturated fatty acid have also been seen in the case of Ca^{2+}ATPase activity of human erythrocyte membranes (Wetzker et al., 1983). In this study it was found that increasing concentrations of oleic acid progressively stimulated Ca^{2+}-ATPase activity several fold up to a maximum of 750 nmol oleic acid/mg membrane protein. Incorporated oleic acid, above 750 nmol/mg protein, inhibited the enzyme activity, which approached zero above 2000 nmol oleic acid/mg protein.

Absorption of large amounts of unsaturated fatty acid destabilized the membrane structure, as was evidenced by the

lysis (about 20%) that occurred when membranes were exposed to concentrations of unsaturated fatty acids greater than 0.2 mM. The possibility exists that certain protein-mediated processes may be sensitive to these changes such as those believed to be involved in Ca^{2+} uptake (Fontaine, et al., 1981). Now, whether the effect is one of stimulation of Ca^{2+} uptake or inhibition could depend on the extent of fluidization caused by exogenous lipid. Such effects could possibly be due to changes in conformation of ion-binding proteins or in the structure of the ion channels involved. Another explanation is possible, at least for the inhibitory effect seen with higher concentrations of oleic acid. Selective release of proteins that may be part of the Ca^{2+} uptake mechanism may occur. However, protein losses were not selective since they were found to parallel closely the extent of lysis when an inhibitory concentration of oleic acid was used.

It was found in preliminary experiments performed with lipid-treated BBMV that D-glucose and D-fructose uptake were not affected by oleic acid incorporation into the membrane, at concentrations which inhibit Ca^{2+} uptake. Similar negative results were obtained by Fontaine et al. (1981) in studies involving D-glucose transport into chick BBMV. It is tempting to speculate and define the effects of unsaturated fatty acids on membrane function as being very selective in nature, but this specificity could merely

reflect individual susceptibility of proteins to alteration in fluidity of the membrane. Further investigations were necessary to fully elucidate these findings.

In a series of investigations, Klausner et al. (1980) suggested that immiscible lipid domains were an important structural feature of plasma membranes. By preferentially partitioning into a particular domain, free fatty acids were found to alter lipid structure differently depending upon whether they were cis-unsaturated or either trans-unsaturated or saturated. These results, together with observed variable effects of free fatty acids on membrane functions such as surface receptor capping (Klausner et al., 1980) or D-glucose transport activity and bilayer fluidity in plasma membranes derived from control and insulin-treated adipocytes (Pilch et al., 1980), suggest that free fatty acids may, by appropriate interactions, alter the structure of proteins in particular lipid domains. It is interesting to note, however, the finding in our laboratory that changes in fluidity engendered by fatty acid uptake in BBMV had no effect on D-glucose transport. The reasons for this, at present, are unclear, though Kinne et al. (1983), in rat BBMV, also found that glucose transport was not sensitive to alterations of its lipid environment.

The results presented in this study agree, in part, with those of previous studies showing the stimulatory effect of unsaturated methyl esters of vaccenic acid on Ca^{2+}

transport in isolated chick BBMV (Fontaine et al., 1981; Bikle et al., 1984). However, it is not clear from these reports as to whether it is the cis or the trans isomer that is the most effective. In the study by Fontaine et al. (1981), no attempt was made to establish the amount of vaccenic acid ester that entered the membrane, and whether or not essential components such as cholesterol and other lipids were lost during the various incubations. In addition, the effects of higher concentrations were not assessed.

A study by Kruetter et al. (1984) demonstrated the effect of fatty acids, incorporated into the brush border membrane phospholipids as their CoA derivatives, on the Ca^{2+} transport properties of chicken intestinal BBMV. These investigators found that the initial rate of Ca^{2+} uptake by BBMV was stimulated by 1.6 fold upon incubation with 50 μM linoleoyl CoA, which was the most effective CoA ester examined. Oleoyl CoA also caused a stimulation of Ca^{2+} uptake but stearyol CoA, palmitoyl CoA and arachidonyl CoA displayed no activity. It was established that the effect of linoleoyl CoA was specific for Ca^{2+} transport; sodium dependent phosphate was slightly inhibited and the activity of alkaline phosphatase, was unaffected. The study with free fatty acids, presented in this chapter extends such findings to rabbit BBMV and defines the binding process more precisely in terms of whether or not a net uptake is involved and relates effects of Ca^{2+} transport not only to

the type of fatty acid incorporated by rabbit BBMV but quantifies this uptake as well.

In must be remembered that this study involves the uptake of fatty acids by BBMV *in vitro* only and whether or not the unsaturated fatty acid content of brush border membrane can vary as extensively *in vivo* remains to be established and correlated with the *in vitro* results. Analysis of brush border membrane lipids as reported in Chapter One have shown that the content of free fatty acids is relatively high in BBMV even when the characterization is performed in Ca^{2+}-free conditions. It is readily conceivable that such a large pool of free fatty acids may arise from the absorption of digestion products and consequently would have a composition fluctuating rapidly in conjunction with the fatty acid content and composition of the diet. It is highly possible, therefore, that the absorption of minerals such as Ca^{2+} would be affected to a large extent by the amount and types of fatty acids, present as products of digestion, through events occurring at the level of the brush border membrane.

Chapter Four

RELATION BETWEEN CALCIUM UPTAKE AND FLUIDITY IN RABBIT BBMV

I. INTRODUCTION

It appeared evident from the studies described in the previous chapter that Ca^{2+} uptake by rabbit BBMV was affected by the incorporation of free fatty acids and methyl oleate into these membranes. There were different effects seen upon fatty acid incorporation. Low concentrations of unsaturated fatty acids caused a stimulation of Ca^{2+} uptake while saturated fatty acids, at various concentrations, were without effect or slightly inhibitory. On the other hand, high levels of unsaturated fatty acids were found to be inhibitory to the Ca^{2+} uptake process. Methyl oleate, however, had only a stimulatory effect on Ca^{2+} uptake in BBMV. One plausible explanation for these observations is that the uptake was largely influenced by changes in fluidity[1] brought about by the added fatty acids. These changes in membrane structure and function would be related to the type and amount of fatty acid taken up by the BBMV.

A common approach to the study of changes in membrane composition and fluidity has involved fluorescence polarization or anisotropy methods (Shinitzky and Inbar,

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[1]For a complete explanation of what is meant by the term "fluidity" as used in this context refer to the description given in footnote number 1 in the General Introduction.

1976; Vanderkooi et al., 1974; Fuchs et al., 1975). The particular usefulness of these methods applied to the study of the fluidity of biological membranes has been adequately addressed in the Introduction. The hydrocarbon fluorophore, 1,6-diphenyl-1-3-5-hexatriene (DPH), the structure of which is depicted in Figure A-1 (Appendix), locates in the acyl chain, hydrophobic regions of membranes and has been used as a probe to measure the degree of fluidity of membranes.

The approach taken in the present study involved the use of DPH as a probe to determine whether there was any relationship between enhanced Ca^{2+} uptake and fluidity of the BBMV.

II. MATERIALS

Tetrahydrofuran (ultraviolet cutoff 215 nm) and spectroscopic grade methanol, used to clean quartz fluorescence cells, were obtained from Fischer Scientific Co. Ltd. (Whitby, Ont.). The fluorescent probe, DPH, was supplied by Molecular Probes Inc. (Los Angeles, CA.).

III. METHODS

A. Fluorescence measurements

To 2.5 ml aliquots of BBMV (0.16 mg/ml) 10 µl of 1 $\cdot 10^{-4}$ M DPH in tetrahydrofuran (spectroscopic grade) was added to give a probe:phosphoacylglycerol ratio of 1:100. After thorough mixing with a Vortex shaker and incubation at

room temperature for 30 min, the suspension was transferred to a 1 cm quartz cell in which mixing was continued by means of a small teflon stir bar. Steady state anisotropy measurements were made with a Perkin-Elmer spectrofluorimeter, model MPF-44A equipped with a DCSU-2 corrected spectra unit interfaced with an analog-to-digital converter and a Commodore PET computer. The sample temperature was increased from 0°C at a rate of approximately 1°C/min. Excitation and emission wavelengths were 350 and 430 nm and excitation and emission bandpasses were 5 nm and 10 nm, respectively. Corrections for background fluorescence and light scattering were made with blanks containing BBMV and no probe.

Anisotropy (r) was calculated from the fluorescence intensities measured parallel and perpendicular to the plane of the light of excitation according to the following equation:

$$r = \frac{I_{\parallel} - I_{\perp}(G)}{I_{\parallel} + 2I_{\perp}(G)}$$

where G = HV/HH, a correction factor for the instrument (Parker, 1978).

B. Absorbance measurements

Absorbance determinations of the various samples were made with a Varian, model Cary-219 double-beam spectrophotometer equipped with thermostatted bath.

C. Extraction and reconstitution of total BBMV lipids

Total lipids were extracted from rabbit BBMV by the method of Bligh and Dyer (1959). For the preparation of the sonicated dispersions of lipids (liposomes), the dried, extracted lipid was suspended in 100 mM mannitol, 10 mM Hepes-Tris buffer, pH 7.5, to a final concentration corresponding to that used in studies with intact vesicles. The mixture was sonicated twice with the small-tip probe of an Ultrasonics sonicator at a setting of 5 for 2 min under N_2 at 25°C. The resulting solutions were clear and were used as such for anisotropy studies.

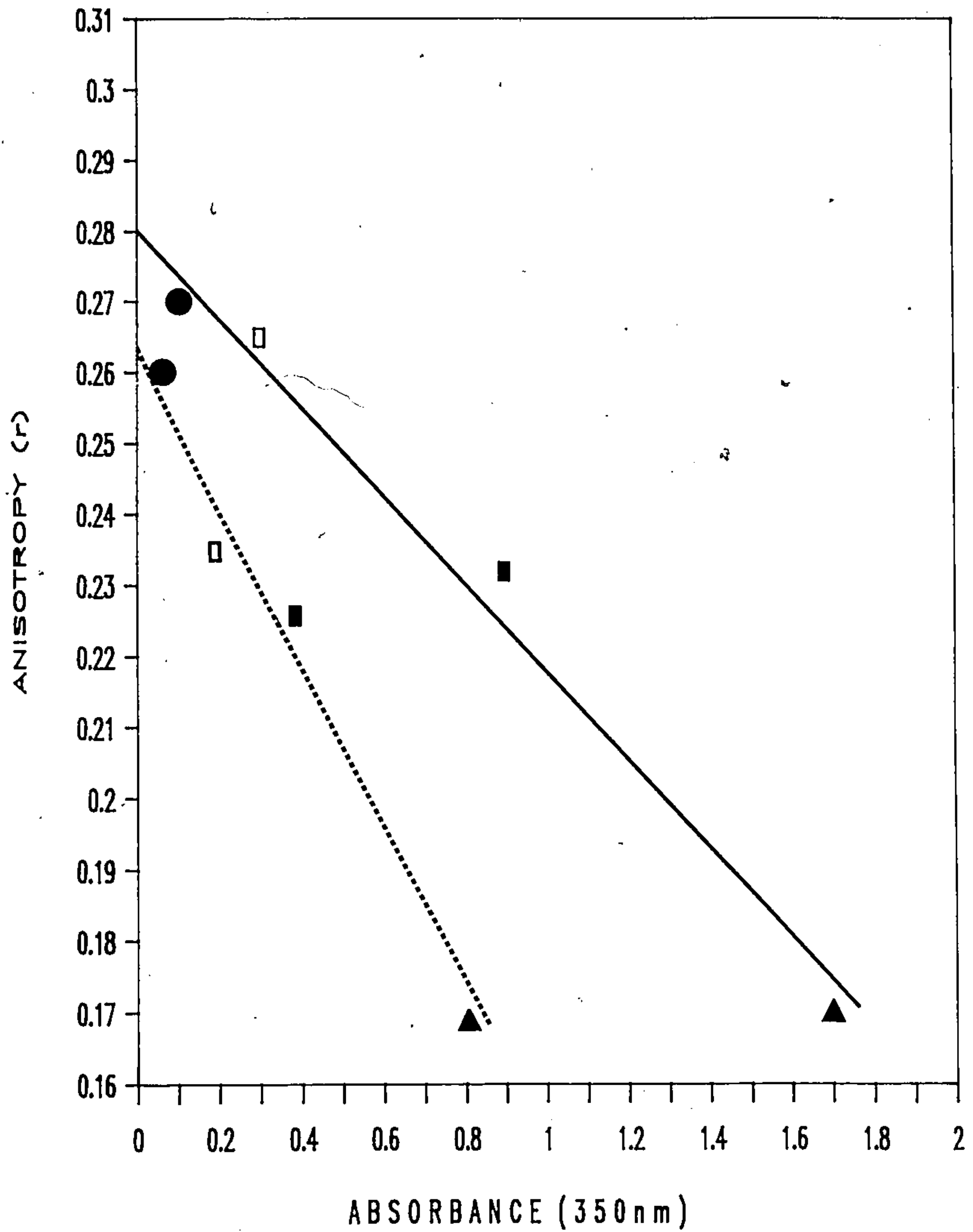
IV. RESULTS

It has been reported that the anisotropy of DPH in membrane preparations was dependent on the membrane concentration (Teale, 1969; Lentz et al., 1979). This dependence of DPH fluorescence anisotropy was due to a depolarization of the light caused by light scattering in such suspensions. In order to avoid this artifact, the fluorescence anisotropy of both the control and treated vesicles, at various vesicle concentrations, was monitored as suggested by Teale (1969) and Lentz et al. (1979). The results shown in Figure 4-1 indicated that a depolarization due to light scattering occurred as the concentration of BBMV increased from 0.08 to 0.96 mg/ml. It was found that a

Figure 4-1

Fluorescence anisotropy versus absorbance.

Various concentrations of BBMV for control (——) and oleic acid-treated (········) samples were assessed for the amount of light absorbed at 350 nm and the steady state fluorescence anisotropy was determined using DPH as the molecular probe. The concentrations of membrane protein studied were: (●) 0.08 mg/ml; (□) 0.16 mg/ml; (■) 0.48 mg/ml; (▲) 0.96 mg/ml. The temperature for both absorbance and anisotropy determinations was 30°C.


0.31
0.3
0.29
0.28
0.27
0.26
0.25
0.24
0.23
0.22
0.21
0.2
0.19
0.18
0.17
0.16
ANISOTROPY (r)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
ABSORBANCE (350nm)

vesicle concentration corresponding to 0.16 mg/ml gave a fluorescence anisotropy value close to the value at infinite dilution and hence this was the optimum concentration used in the fluorescence anisotropy experiments. It should be noted that in such experiments, although the lower the brush border membrane protein concentration the greater the reduction in depolarization due to light scattering, a compromise must be made so that a reasonably intense probe signal was obtained while still maintaining a high lipid:probe ratio.

The fluorescence anisotropy versus temperature curves shown in Figure 4-2 compare the fluorescence anisotropy of membranes treated with low and higher concentrations of oleic acid. It can be seen that oleic acid decreased the anisotropy in a dose-related manner and these differences became more apparent above 15°C.

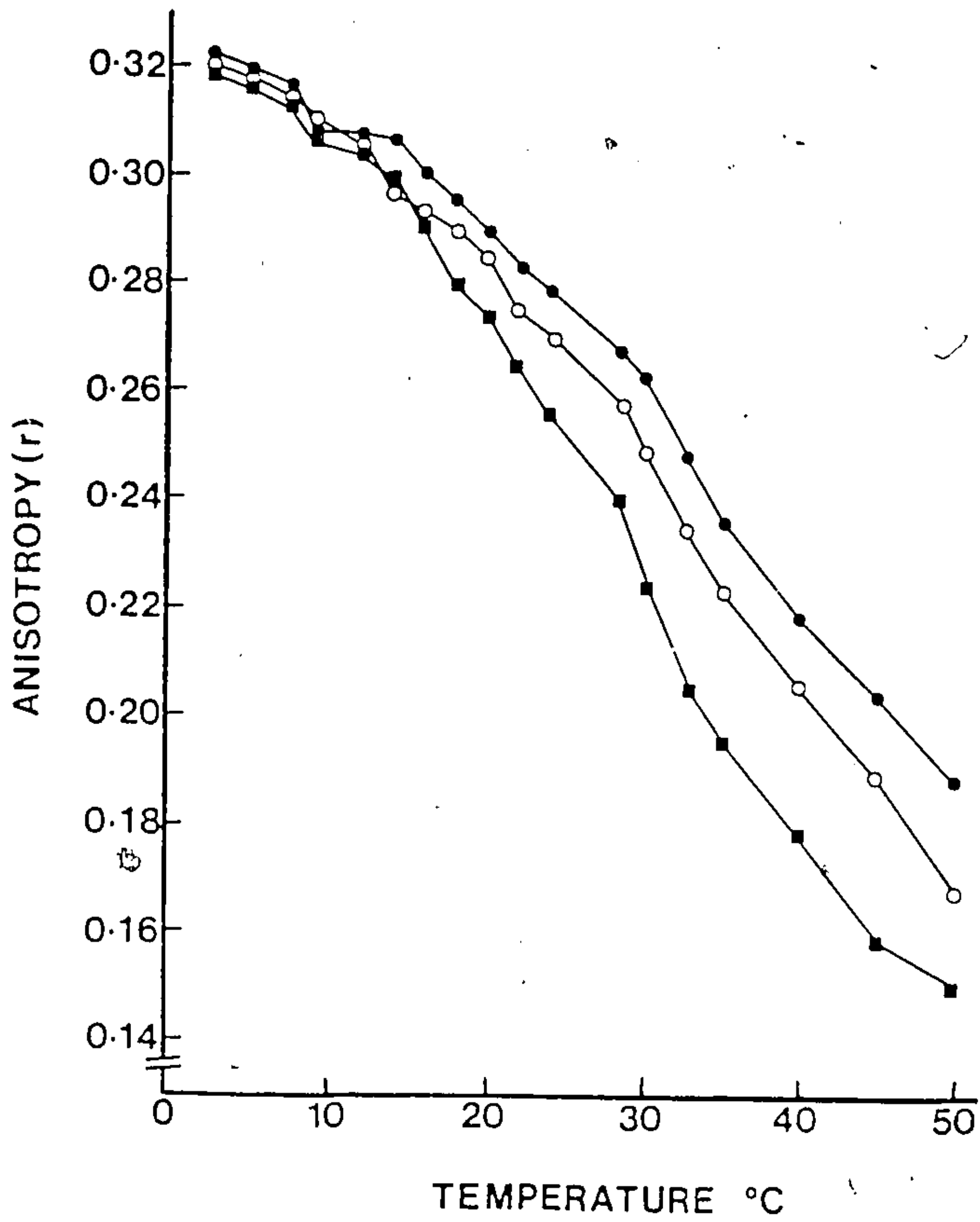
The results in Figure 4-3 show the effect of linoleic acid on the DPH-reportable fluidity of the BBMV. These results indicated that for low incorporation of linoleic acid there was a slight decrease in the anisotropy while for the high level of incorporation (1074 nmol/mg protein) the fluorescence anisotropy was markedly reduced.

Since oleic and linoleic acids at low and high concentrations caused stimulation and inhibition of Ca^{2+} uptake, respectively, no simple relationship between fluidization and activation of uptake could be found. It

Figure 4-2

The effect of oleic acid on anisotropy

The effect of oleic acid on fluorescence anisotropy was studied: (●) control, no oleic acid incorporated; (○) 89 nmol (■) 342 nmol oleic acid incorporated/mg protein. The samples contained 0.16 mg of brush border membrane protein.

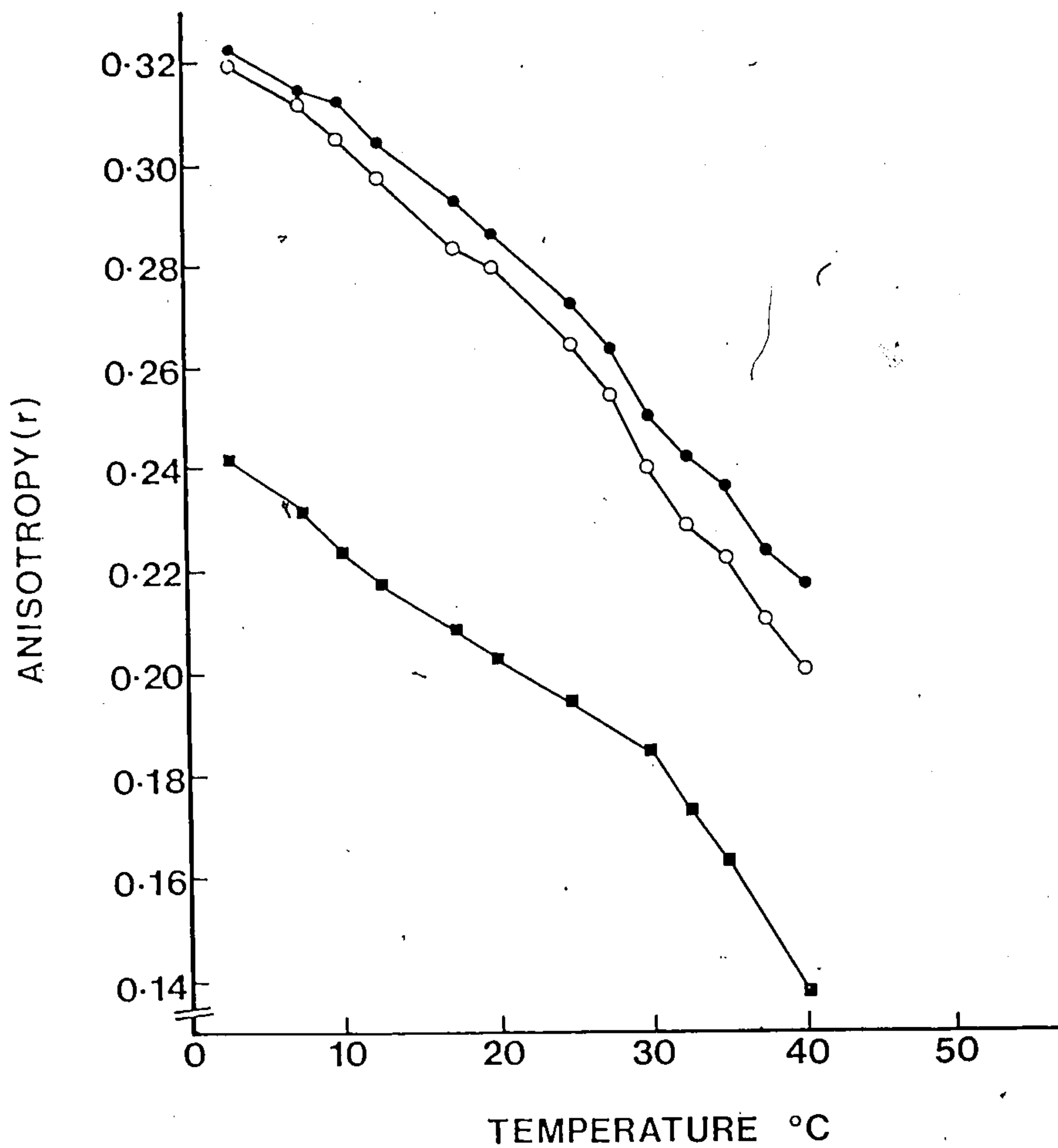


ANISOTROPY (r)
0·32
0·30
0·28
0·26
0·24
0·22
0·20
0·18
0·16
0·14
0
10
20
30
40
50
TEMPERATURE °C

Figure 4-3

The effect of linoleic acid on anisotropy

The following samples were studied: (●) control, no linoleic acid incorporated (○) 50 nmol linoleic acid incorporated/mg protein and (■) 1074 nmol linoleic acid incorporated/mg protein. The samples contained 0.16 mg of brush border membrane protein.



0·32
0·30
0·28
0·26
0·24
0·22
0·20
0·18
0·16
0·14
ANISOTROPY (r)
0
10
20
30
40
50
TEMPERATURE °C

was suggested previously that the inhibition seen at high levels of cis-unsaturated fatty acid might result from an excess of fluidity which might lead to disorganization of the uptake process (Chapter 3). However, results in Figure 4-4 do not support such a conclusion. All concentrations of methyl oleate incorporated into BBMV caused a decrease in the anisotropy but this lipid had only a stimulatory effect on Ca^{2+} uptake. By comparison, incorporations of 342 nmol/mg protein of oleic acid inhibited Ca^{2+} uptake (Table 3-1, Chapter 3) while the fluorescence anisotropy of DPH for these oleic acid-treated vesicles was greater than that found when 220 nmol/mg protein of methyl oleate was incorporated.

It was evident that the effect of oleic acid on the fluorescence anisotropy of DPH in BBMV was markedly different from that of methyl oleate in the same concentration range. As successive amounts of methyl ester were incorporated into the vesicles the entire anisotropy-temperature plot was shifted to lower values. For example, when 220 nmol/mg protein of methyl oleate was incorporated the anisotropy at 25°C was 0.17 compared with the value of the control sample which was 0.27. When 342 nmol/mg protein of oleic acid was taken up, the anisotropy value was 0.255 at 25°C. The effect on the fluorescence anisotropy was greater for methyl oleate even though less was incorporated than for the oleic acid-treated samples.

Figure 4-4

The effect of methyl oleate on anisotropy

The effect of methyl oleate on fluorescence anisotropy was determined: (●) control, no methyl oleaete incorporated; (■) 68 nmol, (▲) 101 nmol, (○) 220 nmol methyl oleate incorporated/mg protein. The samples contained 0.16 mg of brush border membrane protein.

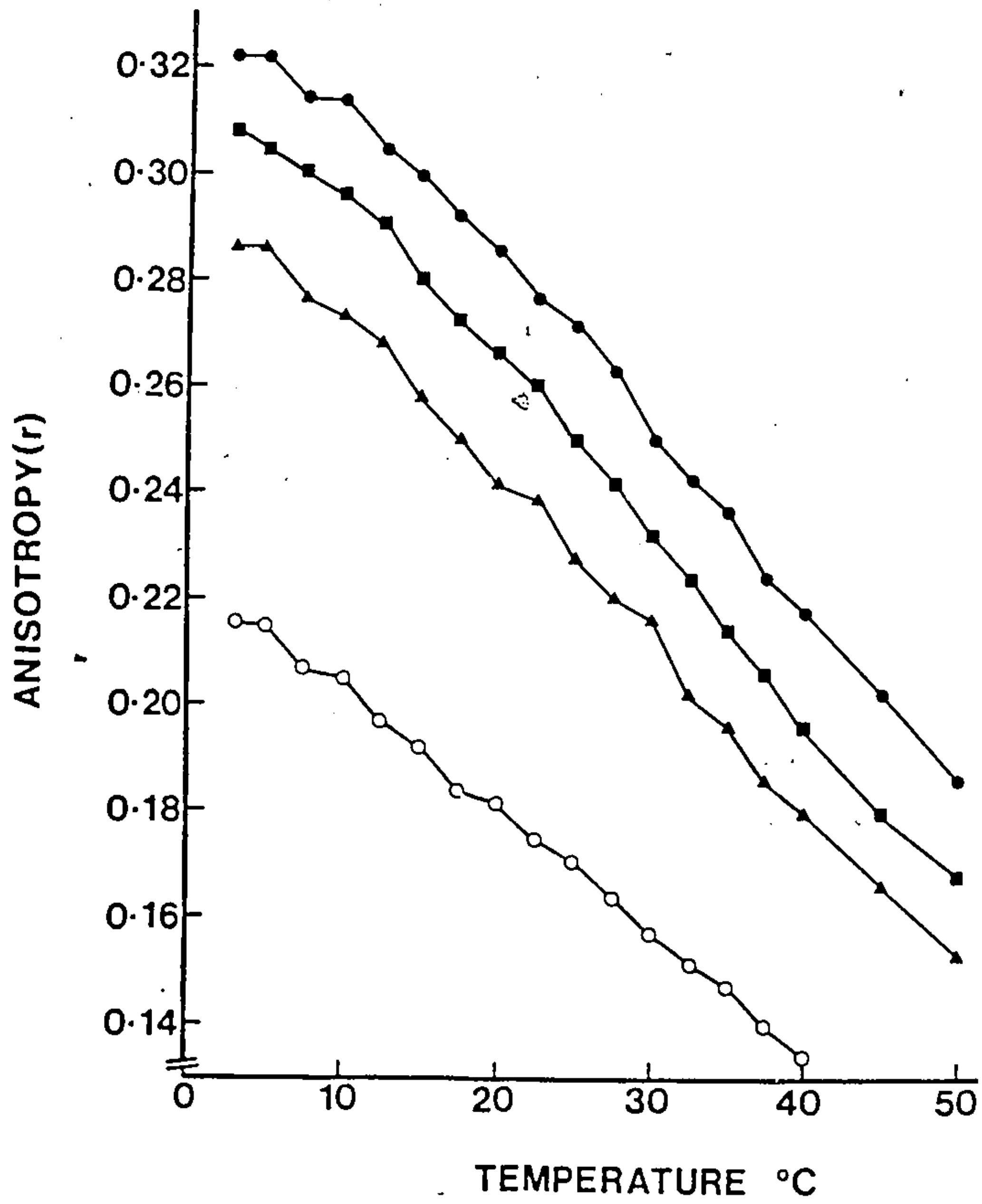

0·32
0·30
0·28
0·26
0·24
0·22
0·20
0·18
0·16
0·14
ANISOTROPY(r)
0
10
20
30
40
50
TEMPERATURE °C

Figure 4-5

Effect of palmitic acid on anisotropy

The following samples were studied: (●) control, no palmitic acid incorporated. (○) 153 nmol, (■) 275 nmol palmitic acid incorporated/mg protein. The samples contained 0.16 mg of brush border membrane protein.

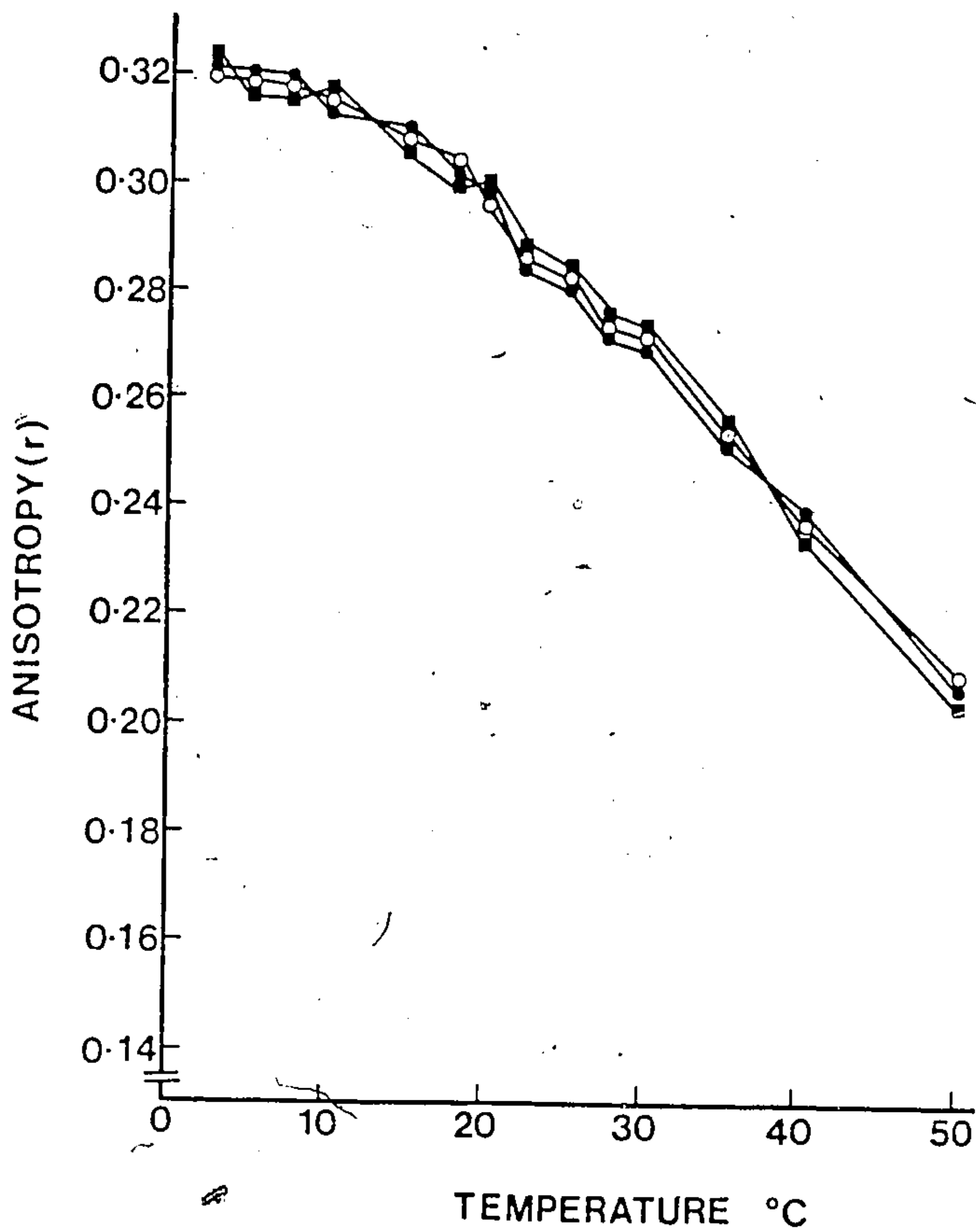

0·32
0·30
0·28
0·26
0·24
0·22
0·20
0·18
0·16
0·14
ANISOTROPY (r)
0
10
20
30
40
50
TEMPERATURE °C

Figure 4-5 illustrates that palmitic acid had no significant effect on the fluorescence anisotropy of the BBMV even though levels of incorporation were similar to those for oleic acid which, in the case of oleic acid, caused a decrease in the fluorescence anisotropy.

A notable characteristic of the anisotropy versus temperature plots was that the decrease in anisotropy as a function of temperature indicated gradual melting and no sharp transition temperatures in all cases.

There was a difference in the behavior of the extracted lipids from oleic acid-treated vesicles compared to that observed for the corresponding untreated vesicles (Figure 4-6). The curve was shifted to lower anisotropy values over the entire temperature range in the lipid extracts. This contrasted with the intact BBMV where differences in the fluorescence anisotropy of the oleic acid-treated vesicles were readily observed only at temperatures greater than 15°C. Overall, the anisotropy values were lower for the lipid extracts compared to the intact membrane vesicles at temperatures below 30°C.

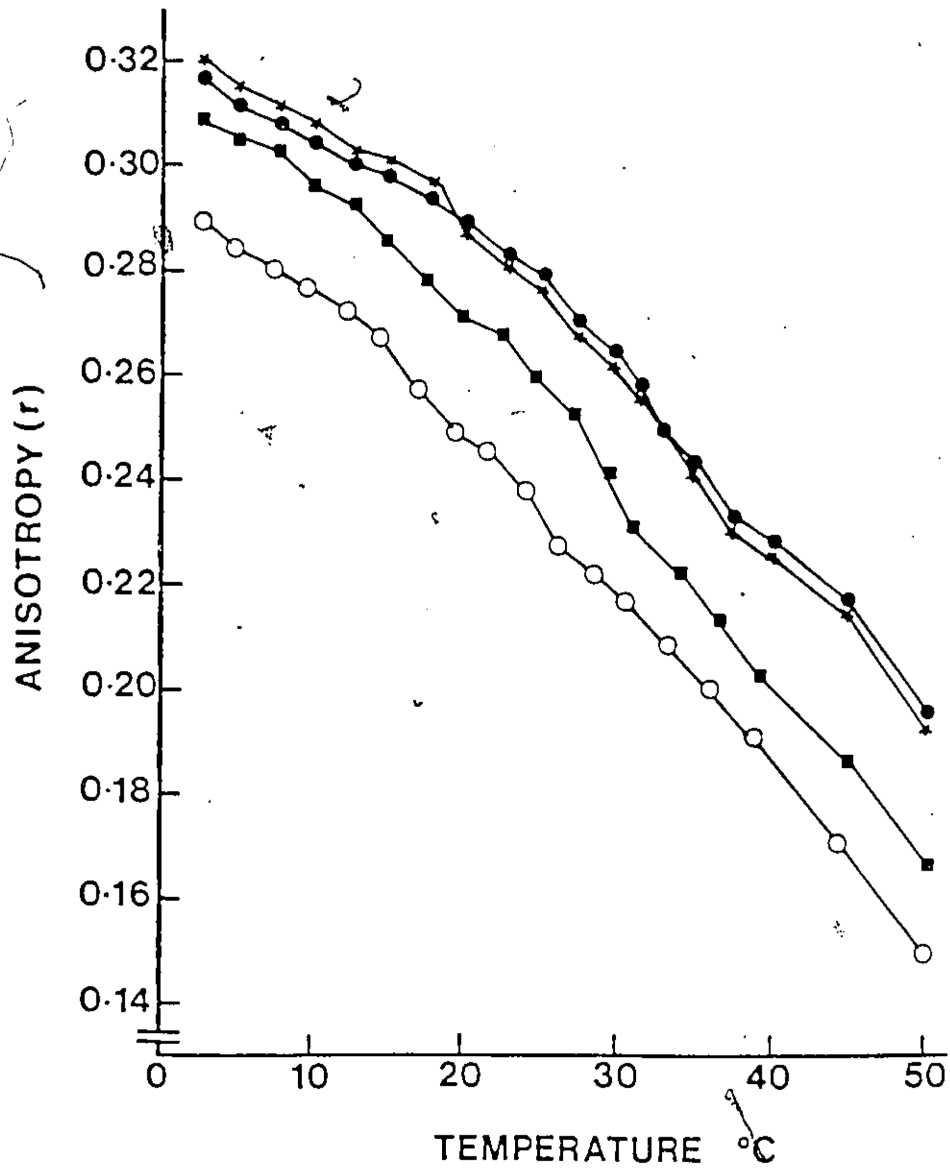
V. DISCUSSION

It is not possible at the present time to explain the differences in the anisotropy results observed for oleic acid and methyl oleate-treated membranes. The presence of the carboxyl group in the cis-unsaturated fatty acids could

Figure 4-6

The lipid extracts of fatty acid-treated brush-border membrane vesicles.

The fluorescence anisotropy of the lipid extracts of fatty acid-treated BBMV was determined: (●) control; (*) 153 nmol palmitic acid; (■) 89 nmol oleic acid; (○) 342 nmol oleic acid. The lipid concentrations of the extracts are identical to those used for intact brush border membrane vesicles.



0·32
0·30
0·28
0·26
0·24
0·22
0·20
0·18
0·16
0·14
ANISOTROPY (r)
0
10
20
30
40
50
TEMPERATURE °C

favor interactions with endogenous membrane constituents such as protein, for example, which would not occur with the methyl ester derivatives of these fatty acids. Such interactions could restrict the extent of mixing with endogenous lipids and limit the effect of acyl chain unsaturation. However, the effect of unsaturation would become apparent when relatively high levels of incorporation were reached or when the temperature of the membrane became sufficiently elevated.

The results obtained with the lipid extracts of the BBMV suggest that the proteins can modify membrane fluidity as has been demonstrated by other investigators (Brasitus et al., 1985; Brasitus et al., 1979). This effect of brush border membrane proteins on membrane fluidity can readily be seen in the case of the oleic acid-treated membrane extracts. These extracts display decreased anisotropy throughout the lower temperature range. However, these effects of proteins are most evident at temperatures well below physiological values so the relevance of this finding *in vivo* must be further determined.

The present study demonstrated that while cis-unsaturated fatty acids affected the fluidity of brush border membranes, as reported by DPH fluorescence anisotropy, saturated fatty acids apparently had no effect. These findings agreed with those of Klausner et al. (1980), who showed that the membranes of mouse lymphocytes and baby

hamster kidney cells possessed lipid domains of different fluidities. Saturated and trans-unsaturated fatty acids were taken up by the less fluid domains without an apparent change in the order of the interior apolar regions as reported by DPH fluorescence anisotropy measurements. On the other hand, cis-unsaturated fatty acids were taken up by the more fluid domains where they caused further disruption of acyl chain packing and a lowering of the DPH fluorescence anisotropy.

The Ca^{2+} transport channels/binding proteins must interact with the fluid domains in the brush border membrane to some extent since Ca^{2+} uptake responds to the incorporation of cis-unsaturated fatty acids as well as to their CoA and methyl ester derivatives (Fontaine, et al., 1981; Kreutter, et al., 1984). However, excess fluidization of the brush border membrane, in response to large incorporations of cis-unsaturated fatty acid, cannot be in itself, the cause of inhibition of Ca^{2+} uptake since it would have been expected that higher levels of methyl oleate incorporation would have exerted a similar inhibitory effect. It is suggested that the accumulation of a large number of free carboxyl groups in the vicinity of the Ca^{2+} channels is in some manner responsible for the inhibitory effects of oleic and linoleic acids at higher levels of incorporation. The binding of Ca^{2+} to these fatty acid carboxyl groups at the external surface of the membrane

bilayer would compete with Ca^{2+} uptake through the channels. This pool of bound cation would be removed by the wash procedures used in the Ca^{2+} binding assay procedure and consequently decreased Ca^{2+} uptake values would be seen. The general lack of effect of saturated fatty acid incorporation into BBMV on Ca^{2+} uptake could be explained on the basis of their being incorporated into less fluid domains of the membrane relatively remote from the sites of the Ca^{2+} channels/binding proteins.

In addition, it is also possible that the presence of free carboxyl groups could effect conformational changes in the channel/binding proteins or modify charged group relationships across the channel which, in turn, would alter the Ca^{2+} translocation rate. The stimulatory effect of lower levels of cis-unsaturated fatty acids could be explained on the basis of enhanced fluidity at the assay temperature (25°C) since methyl oleate causes the same enhancement of Ca^{2+} uptake as does lower concentrations of oleic acid. It could be argued also that the stimulation observed at low levels of unsaturated fatty acid is due to some extent to an increase in free calcium at the site of Ca^{2+} translocation across the membrane resulting from the Ca^{2+} buffering action of carboxyl groups. Further tests involving other unsaturated lipids such as dioleoylphosphatidylcholine, dioleoylphosphatidic acid, alkenylamines and alkenols could help to clarify this point

and possibly help supply answers to the question of how Ca^{2+} is moved across the brush border membrane.

It is evident from this study that the mechanism for Ca^{2+} uptake in BBMV is complex and is related, not only to general physical effects on the membrane, but also to specific differences in the molecular structure of the incorporated lipids. Further knowledge concerning the Ca^{2+} uptake process can most likely be obtained by defining more clearly the entities responsible for this system. Such an approach would undoubtedly lead to the eventual purification of these carrier/binding proteins and their reconstitution into artificial liposomes. With this view, experiments were conducted to isolate and characterize Ca^{2+}-binding proteins from the brush border membrane (Chapter 6).

Chapter Five

PROBING FOR Ca^{2+}-BINDING SITES IN INTESTINAL BRUSH BORDER MEMBRANES WITH A FLUORESCENT LANTHANIDE

I. INTRODUCTION

Data presented in earlier chapters indicated that BBMV isolated from the small intestine of the rabbit resembled similar membranes from other species in their ability to bind and transport Ca^{2+}. These processes were found to respond to the lipid environment of the membrane and were sensitive to changes in fatty acid composition resulting from exposure of the luminal membranes to oleic and linoleic acids. In keeping with the current theory for the mechanism of Ca^{2+} transport in the brush border membranes some of the sites responsible for binding are thought to be proteins which have a high affinity for Ca^{2+} (K_d = 10 µM) (Miller and Bonner, 1981; Miller et al., 1982; Kowarski and Schachter, 1980; Kreutter et al., 1984). Also, it has been suggested that some of the membrane binding sites for Ca^{2+} are not proteins, but may, in fact, be phospholipids (Miller et al., 1982).

Reports to date dealing with the uptake of Ca^{2+} by BBMV (Bikle et al., 1984; Miller and Bonner, 1981; Miller et al., 1982) have described binding sites located in the interior of the membrane which did not readily transfer their ligands to added chelators unless a Ca^{2+} ionophore was present. External binding sites have not been studied because of the

limitations inherent in the membrane filtration method used for the assay of Ca^{2+} uptake. A previous study indicated that Tb^{3+} does not readily penetrate into brush border membranes and when cells are exposed to this lanthanide, binding appears to occur mainly to sites located on the exterior surface (Langer and Frank, 1972). In the study described presently, the similarity of Tb^{3+} to Ca^{2+} along with its fluorescent properties were exploited to probe and characterize Ca^{2+}-binding sites of BBMV and distinguish between binding proteins located in the interior or exterior of the membrane. This study also reveals some of the effects of oleic acid and methyl oleate on the metal binding sites of proteins found in rabbit BBMV.

II. MATERIALS

Dipicolinic acid and sodium cacodylate were purchased from Sigma Chemical Co. (St. Louis, MO.). Terbium chloride hexahydrate was obtained from Aldrich Chemical (Madison, Wis.). Urea, ultra pure grade, was purchased from Schwartz/Mann (Orangeburg, New York).

III. METHODS

A. Fluorescence measurements

Measurements of fluorescence intensity were made as stated in Chapter 4. The sample temperature was maintained constant at 25°C by a jacketed cell holder

attached to a circulating water bath. Titrations of BBMV with $TbCl_3$ were made by the appropriate additions of a stock lanthanide solution to aliquots of BBMV in borosilicate glass test tubes followed by the transfer of the equilibrated mixture to 0.5 cm quartz glass cuvettes. The wavelength of excitation was 280 nm and the emission intensity was monitored at 545 nm with a 430 nm cutoff filter in the emission beam. The excitation and emission bandwidths were 10 nm and 5 nm, respectively. Absorbance measurements were taken on a Varian Model Cary-219 Absorption Spectrophotometer.

B. Determination of Tb^{3+} concentration

Terbium concentrations were determined by using dipicolinic acid (DPA) as described in the method of Barela and Sherry (1976). However, it was found that the following modifications were required to ensure reproducibility of the assay. The final concentration of DPA was lowered from 225 to 50 µM to decrease the optical density from 0.8 to 0.16. This minimized inner filter effects at the exciting wavelength while still maintaining at least a 3:1 ratio of DPA:Tb^{3+}. In addition, it was found that certain buffers interfered with the assay. Sodium cacodylate (pH 7.5) was selected on the basis of its compatibility. The standard curves for Tb^{3+} concentration were linear in the concentration range of 20-1000 nM.

C. Separation of vesicle bound Tb^{3+} from free

Preliminary results indicated that the separation of free from bound lanthanide could not be easily accomplished. It was consequently found that the analysis of free lanthanide in the samples was complicated by its affinity for various filter materials such as Sephadex resin and nitrocellulose membrane filters.

In an effort to separate Tb^{3+} bound to BBMV from free Tb^{3+}, small 1 ml spin columns as described by Penefsky (1977) employing Sephadex G-25 (Pharmacia) as the separation medium were used with some modifications. Briefly, 1 ml tuberculin syringes were plugged with glass wool and filled with Sephadex previously equilibrated in 10 mM sodium cacodylate buffer, pH 7.5. The columns were put into 15 ml corex centrifuge tubes and centrifuged at 1600 x g for 4 minutes. To each column 200 µl of buffer was added and the columns were centrifuged again. This was repeated twice, with the final volume of the dry gel being 0.9 ml. To each column was added 200 µl of solution containing 32 µg BBMV protein which had previously been equilibrated with the appropriate lanthanide concentration in a 1.5 ml Eppendorf microcentrifuge tube. To the column effluents was added 5.5 M urea to denature the binding proteins and facilitate the chelation of Tb^{3+} by DPA (Ross et al., 1985). The amount of Tb^{3+} present in the column effluent was taken to be the amount of lanthanide specifically bound to the BBMV. It was

determined that none of the free lanthanide passed through the columns under these conditions and that 96 ± 2% of the protein was recovered in the column effluent.

IV. RESULTS

Figure 5-1 shows the emission spectrum of Tb^{3+} free in cacodylate buffer, as well as the light scattering caused by the BBMV (0.16 mg/ml) and the approximate 20 fold enhancement in fluorescence of Tb^{3+} when bound to the membrane. It was clearly evident that "resonance energy transfer"[1] from the aromatic amino acids to Tb^{3+} occurred in this system upon excitation at 280 nm, which resulted in a large increase in Tb^{3+} fluorescence.

The time course for Ca^{2+} uptake in rabbit BBMV was shown in Chapter 2. The rate of uptake of Ca^{2+} was linear for up to 5 minutes with equilibrium being established after 120 minutes at 25°C. The time course for Tb^{3+} binding by rabbit BBMV at a Tb^{3+} concentration equal to 5 µM is shown in Figure 5-2. The uptake, as monitored by an increase in the intensity of Tb^{3+} fluorescence at 545 nm, in relation to that of a sample containing no BBMV, was initially very rapid (zero time corresponded to 10 seconds) and accounted for almost one half of the final fluorescence intensity in the control. Equilibrium was reached after 2 hours.

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[1]A complete definition of this term and explanation of this phenomenon can be found in the General Introduction (section V.B.).

Figure 5-1

Various emission spectra.

(a) Emission spectrum of Tb^{3+} in 10 mM Tris-Hepes buffer, pH 7.5 containing 100 mM mannitol; (b) Emission due to light scattering by BBMV (0.16 mg protein/ml) in the same buffer; (c) Emission spectrum of Tb^{3+} in the presence of BBMV (0.16 mg protein/ml) in the same buffer.

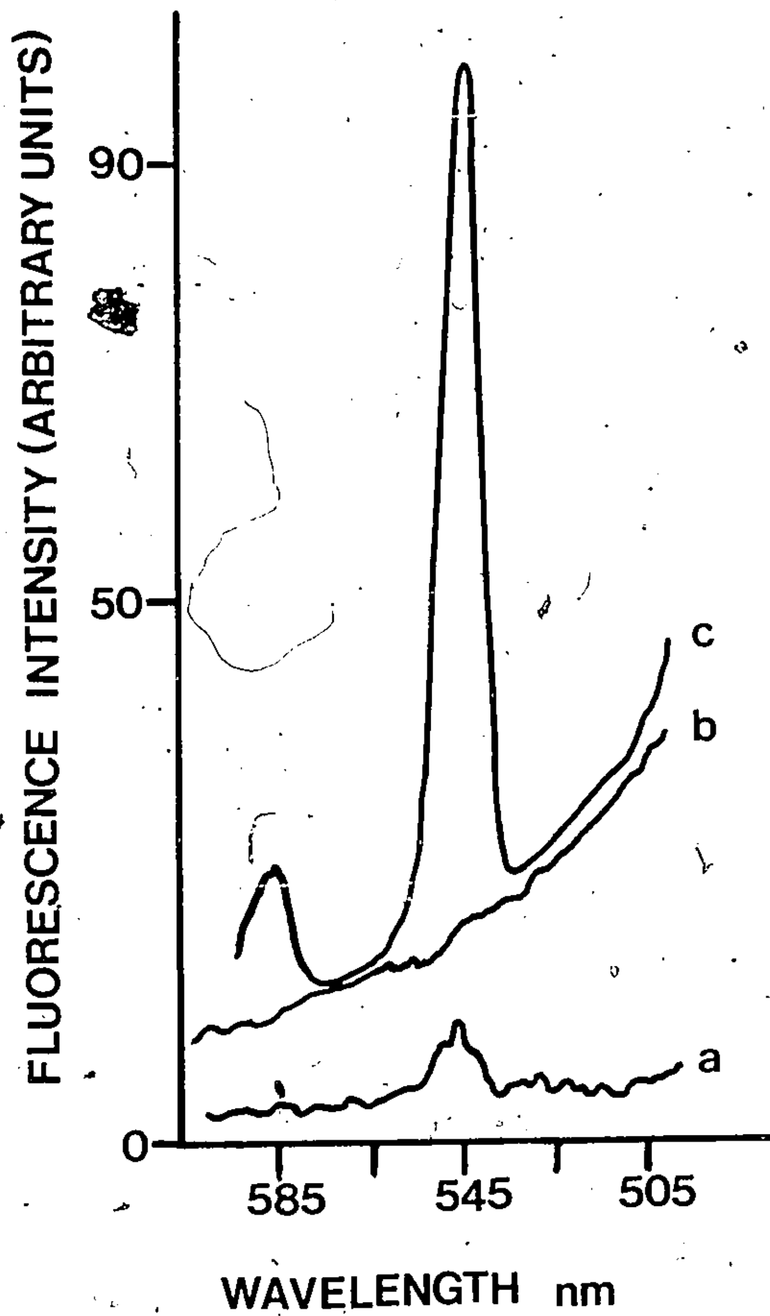
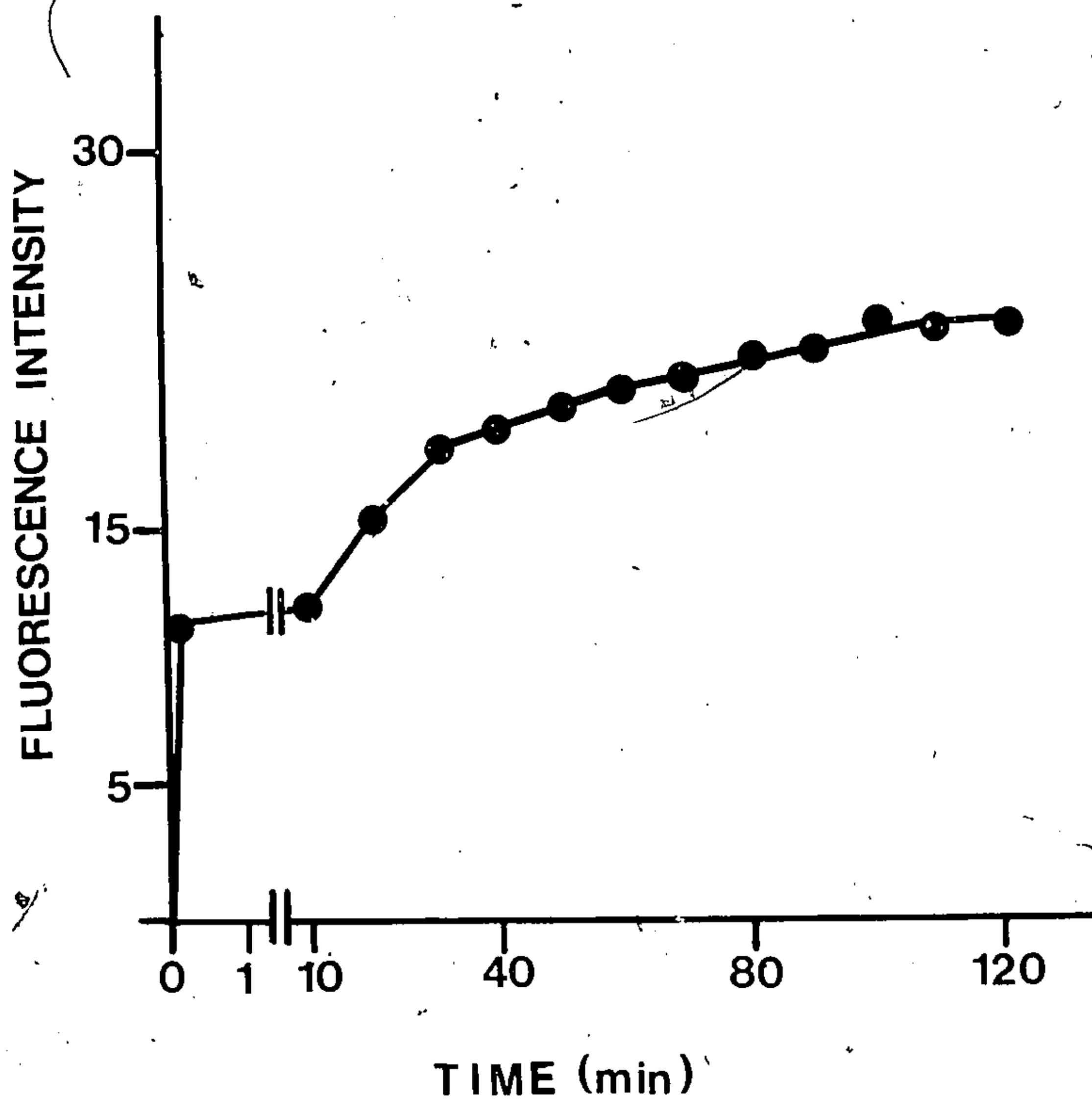

FLUORESCENCE INTENSITY (ARBITRARY UNITS)
90
50
0
c
b
a
585
545
505
WAVELENGTH nm

Figure 5-2

Time course of Tb^{3+} binding to BBMV.

Five μM Tb^{3+} was incubated with BBMV (0.16 mg protein/ml) in 10 mM Tris-Hepes buffer, pH 7.5 containing 100 mM mannitol at 25°C. Fluorescence intensity, expressed in arbitrary units was measured as a function of time.


FLUORESCENCE INTENSITY
30
15
5
0
1
10
40
80
120
TIME (min)

However, for the oleic acid-treated vesicles corresponding to an incorporation of 342 nmol of acid per mg protein the initial value was almost equal to the final value (data not shown). After the rapid initial binding there was a slower increase in the fluorescence intensity of Tb^{3+} with no further increase occurring after 2 hours. Because of the initial rapid rate and the apparent complexity of the binding processes, binding kinetic studies were not pursued further and only equilibria data were considered.

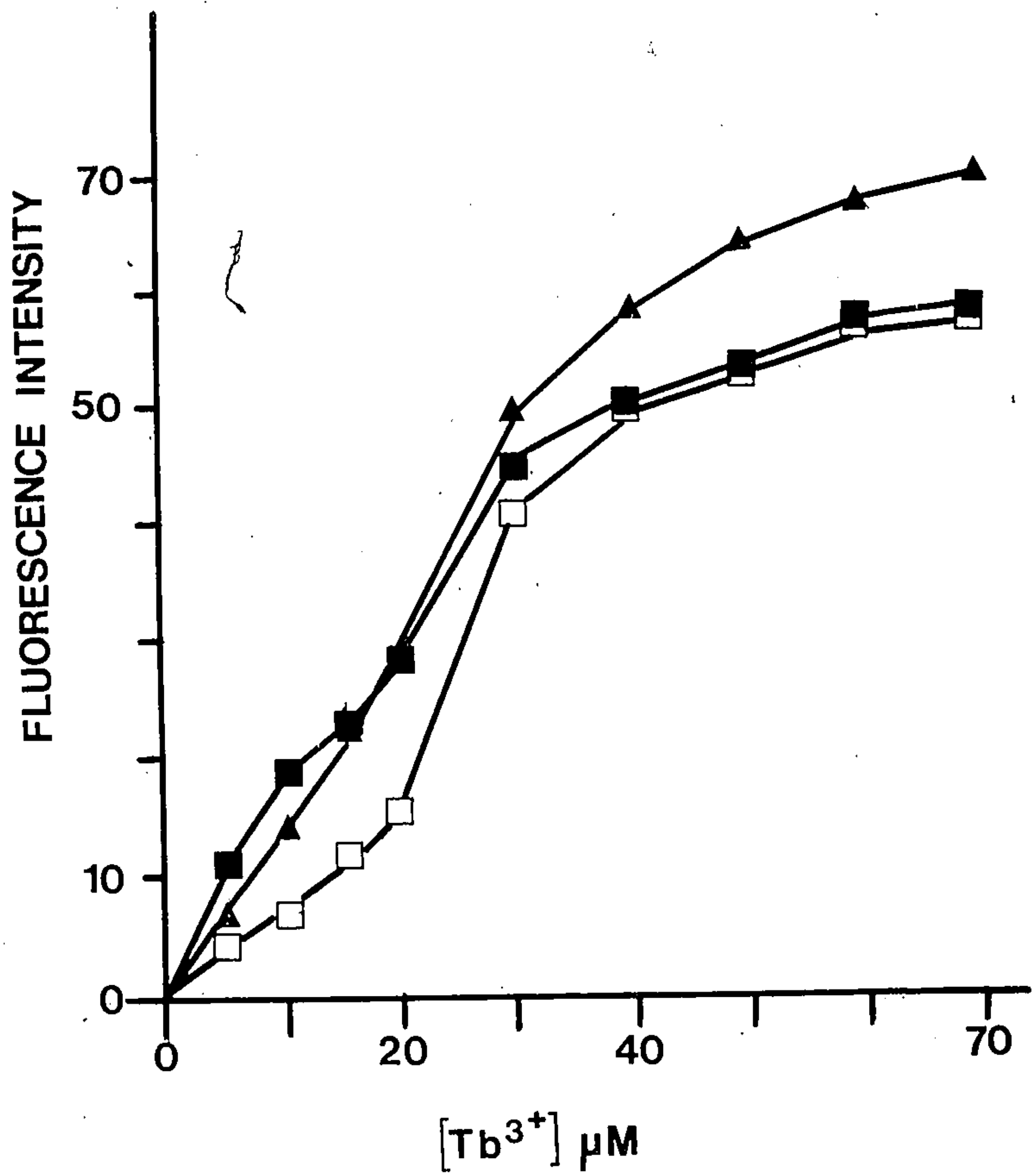
The titration of BBMV with Tb^{3+} is shown in Figure 5-3. Untreated membrane vesicles showed a steady increase in the fluorescence with increased lanthanide concentration, until 60-70 μM Tb^{3+}, at which concentration an apparent plateau was seen. It was not possible to determine the fluorescence response at concentrations of Tb^{3+} greater than 70 μM because the BBMV formed aggregates and precipitated. This phenomenon of protein precipitation upon the addition of higher concentrations of Tb^{3+} has been observed by others (Wallace et al., 1982). The titration curve was complex in form with an inflection clearly visible between 15 and 30 μM added lanthanide.

It was obvious from these results that more than a single class of binding sites were involved. Treatment of the BBMV with oleic acid resulted in significant differences in the Tb^{3+} titration curves. As greater amounts of oleic acid were incorporated into the BBMV the fluorescence at the

Figure 5-3

Effect of Tb^{3+} concentration and oleic acid treatment on binding of lanthanide to BBMV.

The conditions were as stated for Figure 5-2 except that incubations were for 2 hours and the Tb^{3+} concentration was varied. The BBMV were either untreated (■), or treated with oleic acid to give incorporations of 89 nmol/mg protein (▲) or 342 nmol/mg protein (□).


FLUORESCENCE INTENSITY
70
50
10
0
0
20
40
70
[Tb3+] μM

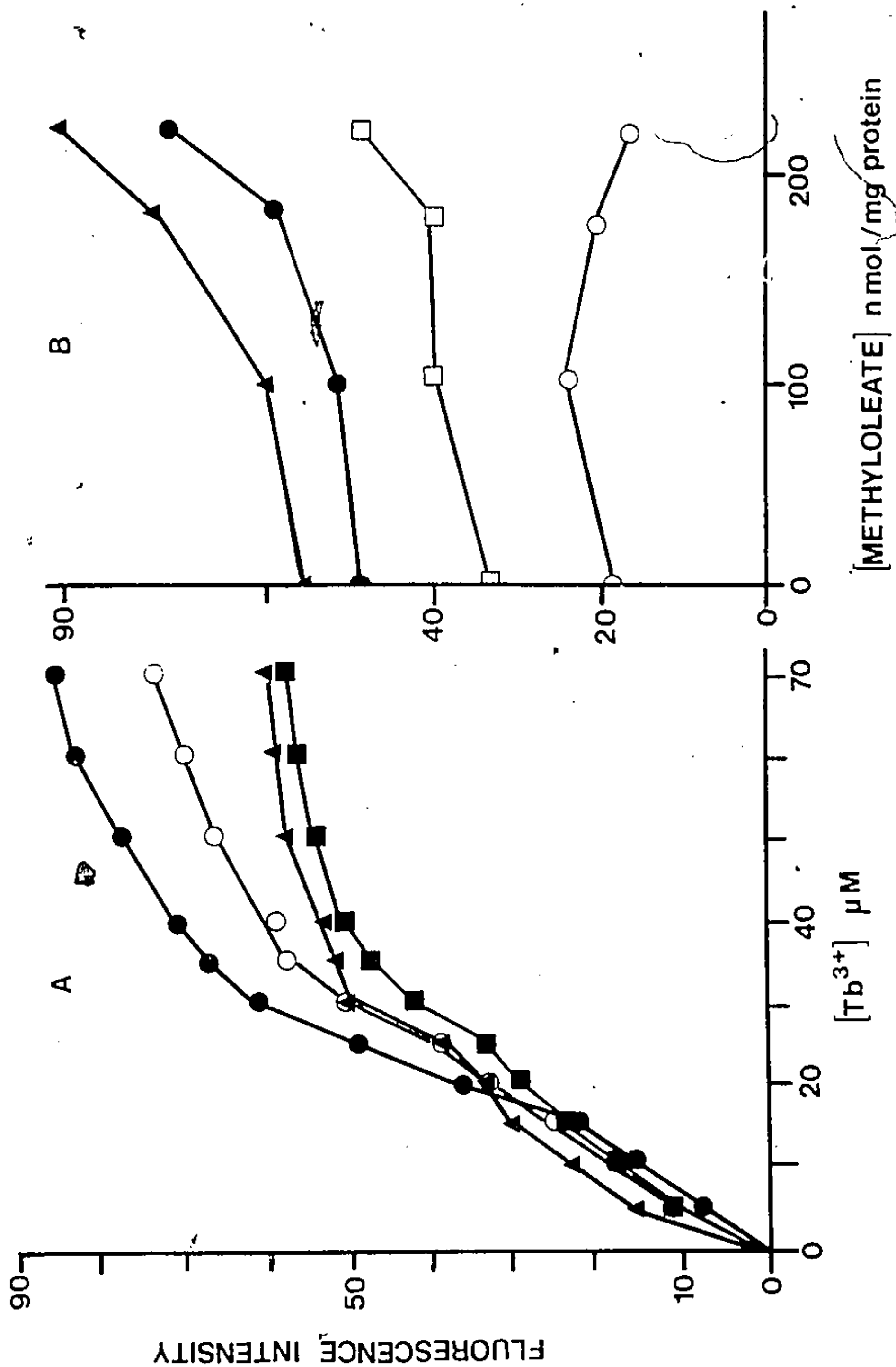
lower concentrations of Tb^{3+} was less than that of the control. There was a cross-over point in the titration curve ($[Tb^{3+}]$ = 15 µM) for the sample with a low concentration of incorporated oleic acid (89 nmol/mg protein). For these same vesicles the Tb^{3+} fluorescence reached a plateau value which was 18% greater than that for the control. However, for the highest concentration of oleic acid (342 nmol/mg protein) the fluorescence intensity never exceeded that of the control for all concentrations of Tb^{3+}, reaching a value equivalent to that of the control at high Tb^{3+} concentrations.

The Tb^{3+} titration curves of BBMV into which methyl oleate (Figure 5-4A) was incorporated were distinctly different from those in which oleic acid was incorporated. The methyl ester was employed because it provided a convenient way of assessing the effect of the free carboxyl group of incorporated oleic acid on metal binding. The methyl ester-treated vesicles corresponding to an incorporation of 101 nmol/mg protein showed higher Tb^{3+} fluorescence relative to the control throughout the entire Tb^{3+} concentration range. Furthermore, for both the control and this sample, the shape of the curves was similar with an inflection point between Tb^{3+} concentrations of 20 and 30 µM. At higher concentrations of incorporated methyl ester, (182 nmol/mg protein, 220 nmol/mg protein), the Tb^{3+} fluorescence was slightly lower than control at low

Figure 5-4

Effect of Tb^{3+} concentration and methyl oleate treatment on binding of lanthanide to BBMV.

The conditions were as stated for Figure 5-3. (A) Fluorescence intensity plotted as a function of Tb^{3+} concentration. Untreated membranes (■); BBMV containing methyl oleate 101 nmol/mg protein (▲); 182 nmol/mg protein (○); and 220 nmol/mg protein (●); (B) Fluorescence intensity plotted as a function of methyl oleate concentration in the membrane. 10 µm Tb^{3+} (○); 25 µM Tb^{3+} (□); 40 µM Tb^{3+} (●) and 70 µM Tb^{3+} (▲).


A
B
FLUORESCENCE INTENSITY
90
50
10
0
20
40
70
[Tb3+] μM
40
20
0
100
200
[METHYLOLEATE] n mol/mg protein

lanthanide concentrations. But as the $Tb3^{+}$ concentration increased the Tb^{3+} fluorescence surpassed that of the control and the plateau value increased in proportion to the amount of incorporated methyl ester.

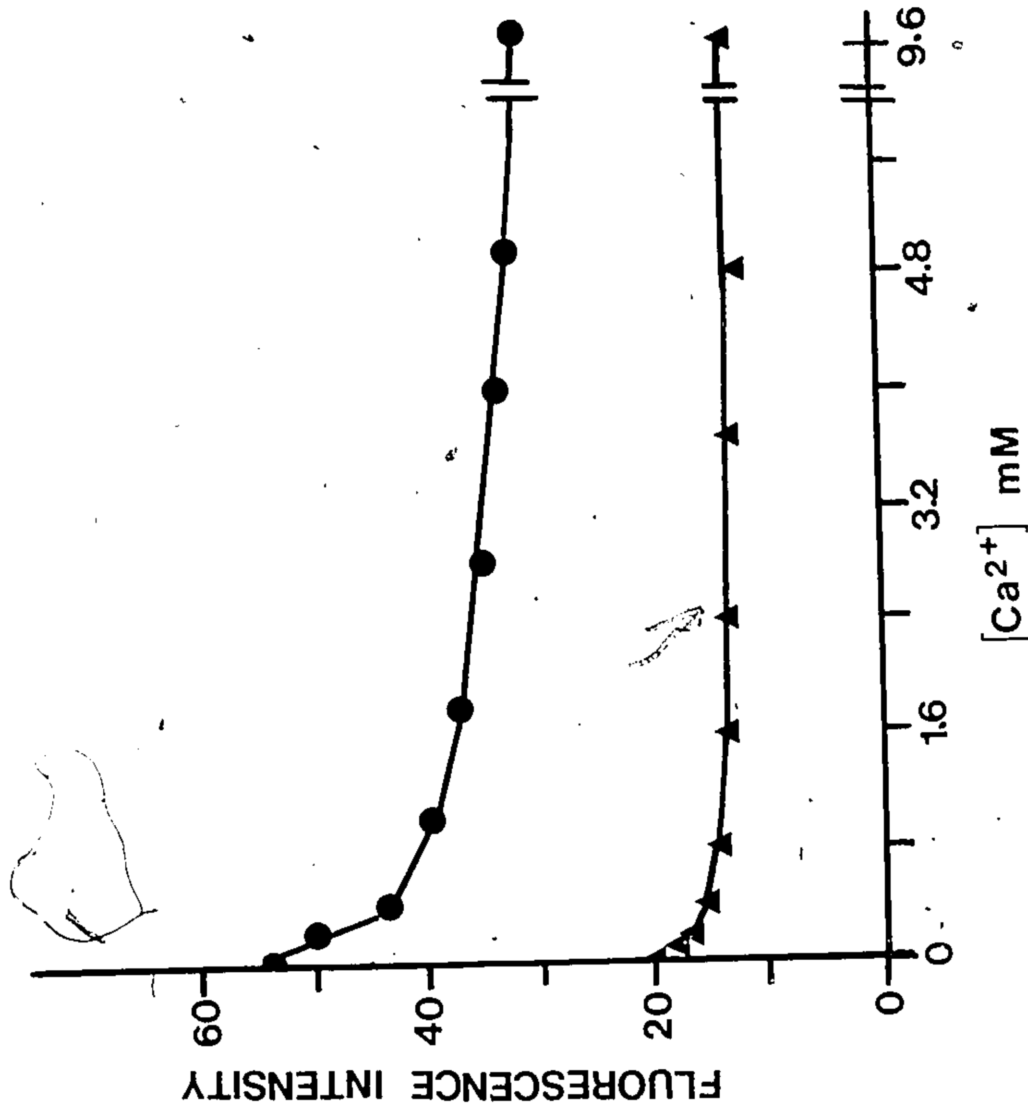
Figure 5-4B clearly shows that the incorporation of methyl ester has different effects on the Tb^{3+} fluorescence of the BBMV at low Tb^{3+} concentration than at high Tb^{3+} concentration. At low Tb^{3+} concentrations (10 µM) incorporation of methyl ester caused an initial increase in fluorescence followed by a decrease as higher concentrations of methyl ester were incorporated. At higher concentrations of added lanthanide, (>25 µM), the total fluorescence signal increased with the amount of methyl oleate incorporated into the bilayer. In vesicles containing 220 nmoles of methyl ester/mg protein there was an increase of 48% in the Tb^{3+} fluorescence at an added $Tb3^{+}$ concentration of 70 µM when compared with the control.

The results illustrated in Figure 5-5 clearly show that not all of the bound Tb^{3+} can be displaced by Ca^{2+}. The maximum displacement occurred at a Ca^{2+} concentration of almost 1 mM for 10 µM Tb^{3+} and 5 mM at 50 µM Tb^{3+}. The addition of Mg^{2+} instead of Ca^{2+} to Tb^{3+}-equilibrated BBMV under similar conditions resulted in only a small displacement of lanthanide (maximum 5-6%, results not shown).

Figure 5-5

Displacement of Tb^{3+} from BBMV by Ca^{2+}.

BBMV were incubated with 10 μM Tb^{3+} (▲) and 50 μM (●) as stated for Figure 5-3 and were then exposed another 120 min to increasing concentrations of $CaCl_2$.


FLUORESCENCE INTENSITY
60
40
20
0
0
1.6
3.2
4.8
9.6
[Ca2+] mM

Terbium-equilibrated BBMV (50 μM Tb^{3+}) control samples were treated with 100 mM EGTA and the fluorescence was monitored as a function of time (Figure 5-6). There was a rapid decrease in the intensity of the Tb^{3+} fluorescence for the first two minutes after which time the fluorescence continued to decrease slowly. This system reached equilibrium after 20 minutes when there was no further decrease in intensity. There was a significant difference in the effect of added EGTA on the Tb^{3+} fluorescence between the control and the methyl oleate-treated samples (Table 5-1). The fluorescence value for the methyl ester-treated vesicles (182 nmol/mg protein) was slightly more than three times that of the control after EGTA was added. In the absence of chelator the ratio of the Tb^{3+} fluorescence for these samples was only 1.25. In the EGTA-treated vesicles the fractional change in fluorescence was greatest for the control (0.74). As increasing amounts of methyl ester were incorporated into the vesicles the fractional change in fluorescence caused by the addition of EGTA decreased. For example, in samples enriched with 182 nmoles of methyl oleate/mg protein the change was 0.30.

Binding of Tb^{3+} as determined by the DPA assay is shown in Figure 5-7. Enrichment with methyl oleate to 101 and 182 nmol/mg protein each produced almost a two fold stimulation of Tb^{3+} uptake for lanthanide concentrations of up to 35 μM. The Tb^{3+} titration curve for methyl oleate-treated membranes

Figure 5-6

The effect of EGTA on Tb^{3+} binding by BBMV.

BBMV containing no methyl oleate (●); or containing methyl oleate, 14 nmol/mg protein (□); 101 nmol/mg protein (○) and 182 nmol/mg protein (▲) were incubated 50 µM Tb^{3+} for 120 min after which time they were treated with 0.1 M EGTA for various times.

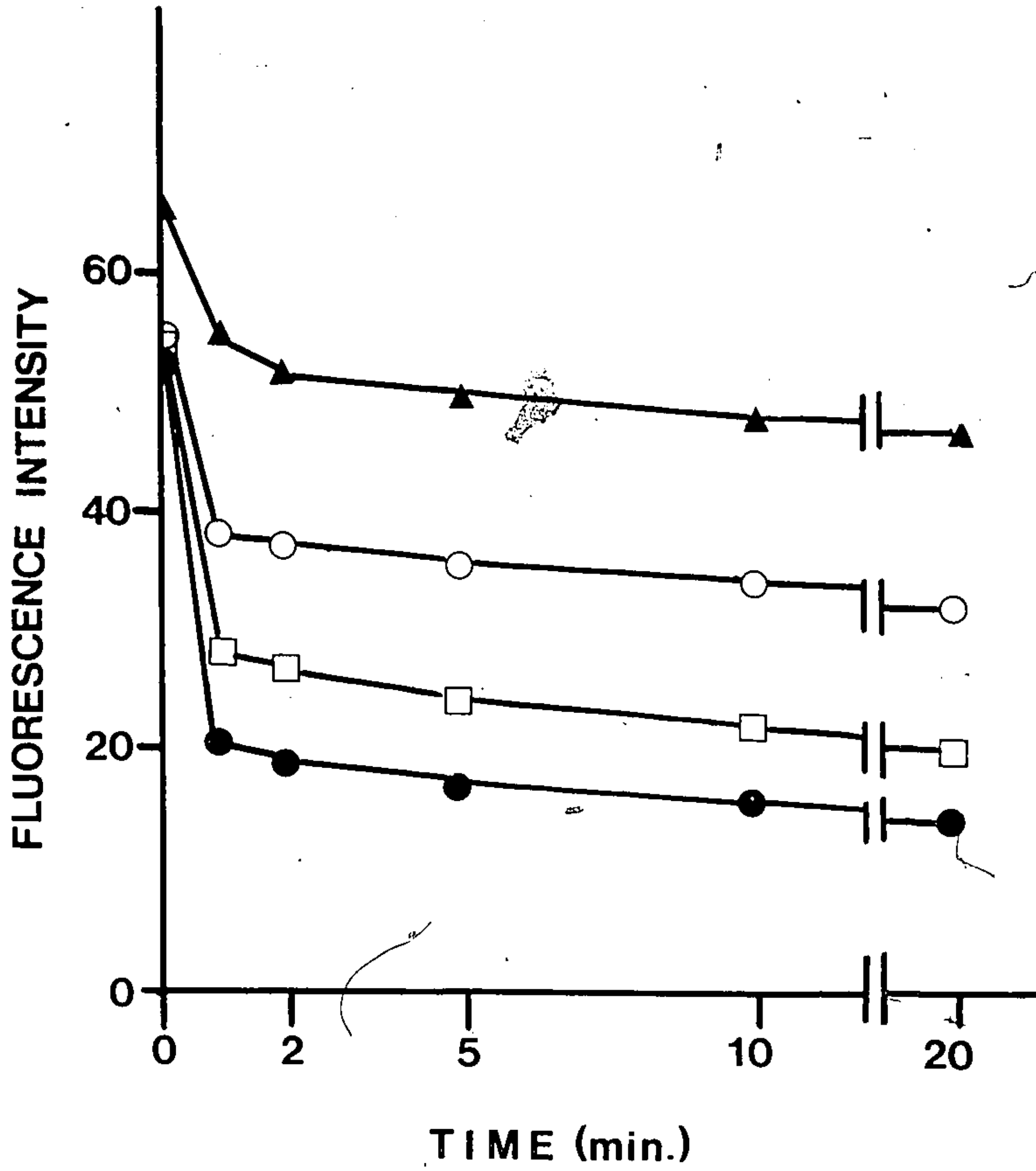

FLUORESCENCE INTENSITY
60
40
20
0
0
2
5
10
20
TIME (min.)

TABLE 5-1

The Effect of EGTA on the Tb^{3+} Fluorescence of Methyl oleate-Treated BBMV

Concentration of Incorporated Methyl Ester (nmol/mg protein)	F_0	F_{EGTA}	$\Delta F/F_0$*
0 (control)	54.0	14.0	0.74
14	55.5	20.0	0.64
101	56.5	32.0	0.43
182	67.0	47.0	0.30

$$*\Delta F/F_0 = \frac{F_I(\text{no EGTA}) - F_I(\text{EGTA})}{F_I(\text{no EGTA})}$$

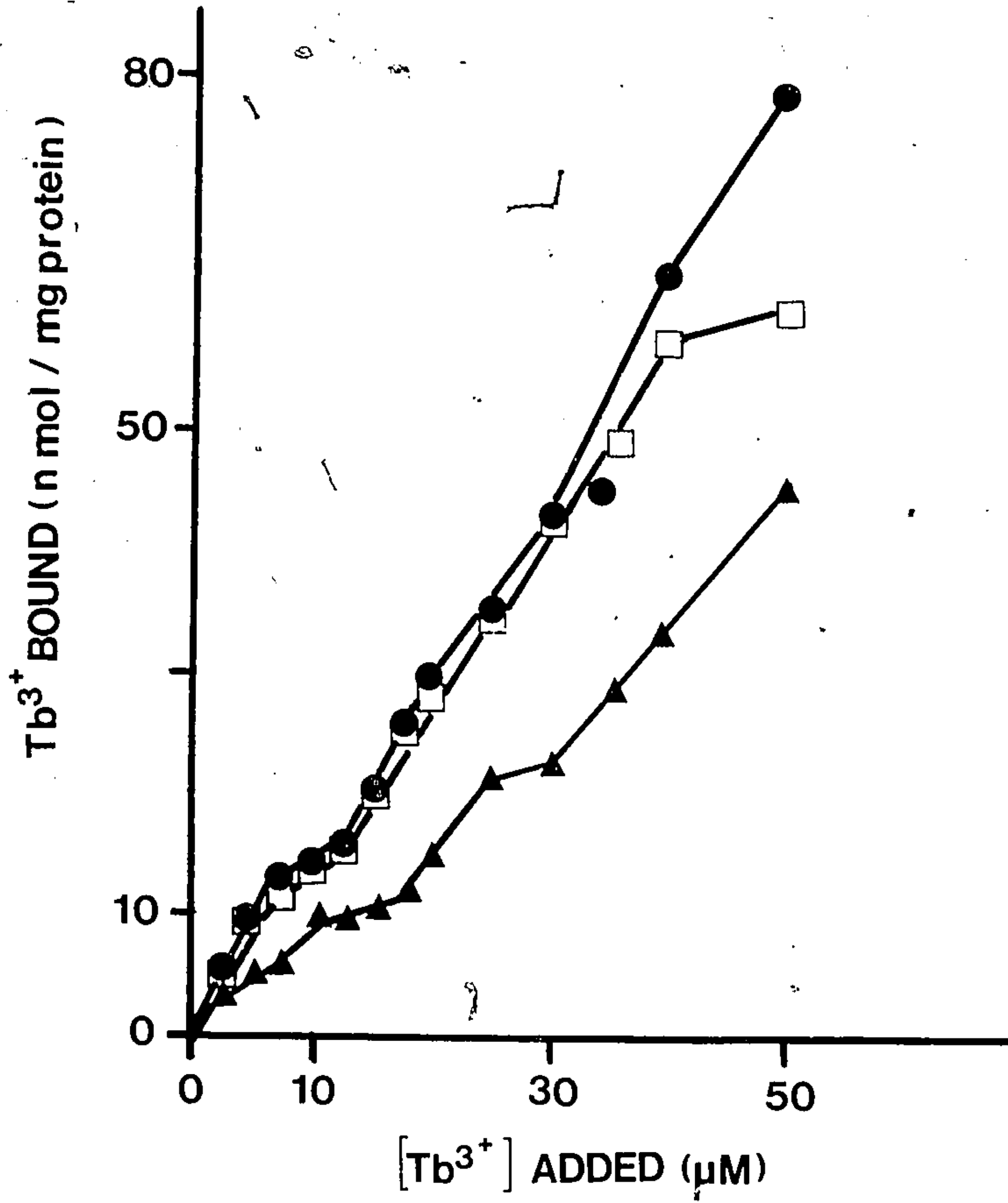
$[Tb^{3+}]$ = 50 μM

Time of incubation of samples with Tb^{3+} was 120 minutes at 25°C followed by a 20 minute incubation with EGTA after which no further change in fluorescence intensity occurred.

Figure 5-7

Titration of BBMV with Tb^{3+} as measured by DPA complexing.

Incubations of BBMV with lanthanide were as stated for Figure 5-3. Untreated membranes (▲); BBMV containing methyl oleate, 101 nmol/mg protein (□); 182 nmol/mg protein (●).


80
50
10
0
Tb^{3+} BOUND (n mol / mg protein)
0
10
30
50
[Tb^{3+}] ADDED (μM)

determined by the DPA assay differs from that obtained following the fluorescence at 545 nm. In the latter case, the effect of low concentrations of incorporated methyl oleate (101 nmol/mg protein) on the Tb^{3+} fluorescence was small at all Tb^{3+} concentrations while at higher concentrations (182 nmol/mg protein) of incorporated methyl oleate, a marked enhancement in the fluorescent signal was noticed only at concentrations of Tb^{3+} above 15-20 μM.

V. DISCUSSION

An unanswered question in the investigation of the nature of the Ca^{2+}-binding sites in the brush border membrane of the columnar epithelial cell is whether such sites are exclusively protein and if so what is the nature of the protein binding and transporting species found within this natural membrane bilayer? Also, what role do non-protein species play in the movement of the Ca^{2+} across the brush border membrane? The present results, along with those of others (Ohyashiki et al., 1985; Bikle and Munson, 1985), demonstrated the existence of protein sites capable of binding Tb^{3+} in such a manner as to allow resonance energy transfer from the aromatic amino acids to the Tb^{3+}. The fluorescence enhancement in the Tb^{3+} emission spectrum seen upon the addition of Tb^{3+} to BBMV indicated that Tb^{3+} was bound within 10-20 Å of these aromatic amino acid residues (Richardson, 1982). These results agreed, in part,

with the previous findings in porcine BBMV (Ohyashiki et al., 1985) which suggested that the sites in the membrane to which Tb^{3+} was bound were located on Ca^{2+}-binding proteins.

The Tb^{3+} fluorescence titration curves (Figure 5-3 and 5-4) clearly show that there are at least two classes of Tb^{3+} binding sites in control, oleic acid- and methyl oleate-treated vesicles. These may be discussed in terms of low affinity and high affinity binding sites. At low Tb^{3+} concentrations the high affinity sites will become occupied first. In the case of oleic acid-treated vesicles, a lower Tb^{3+} fluorescence than for the control was observed at Tb^{3+} concentrations less than or equal to 30 µM. Such a case could result from a complex formed between Tb^{3+} and the oleic acid carboxylate group, if the affinity of Ca^{2+} for oleic acid was stronger than for any high affinity protein site. Unless the oleic acid-Tb^{3+} complex was close to an aromatic amino acid residue in a protein, total fluorescence in the acid-treated samples would be significantly reduced compared with the control. The results with methyl oleate-incorporated vesicles support this rationalization. In these latter systems, the Tb^{3+} fluorescence was always higher than samples with nearly equivalent amounts of incorporated oleic acid. When the amount of bound Tb^{3+} was assayed by the DPA method, there was a substantial increase in bound Tb^{3+} when oleic acid was present. Virtually all the Tb^{3+} was bound. While for methyl oleate-treated

vesicles approximately 20% of the Tb^{3+} added was bound. The most reasonable conclusion based on these results is that Tb^{3+} like Ca^{2+} (Chapter 3) forms an ionic complex with the carboxylate group of incorporated oleic acid. The possibility cannnot be ruled out, however, that in methyl oleate-treated vesicles the Tb^{3+} also bound to membrane constituents which could not undergo resonance energy transfer to the lanthanide, possibly due to the lack of a nearby donor chromophore. Indeed, the DPA assay showed that Tb^{3+} likely binds to such sites as well as to proteins, which possess aromatic amino acids close to the Tb^{3+} binding segment, because in the former instance the amount of Tb^{3+} bound increased in proportion to the amount of incorporated oleic acid.

As the Tb^{3+} concentration increased ($[Tb^{3+}] \geq 30\mu M$), the high affinity sites became saturated and the low affinity sites dominated the fluorescence behaviour. For low concentrations of oleic acid the Tb^{3+} fluorescence exceeded that of the control. This implied that at low incorporation levels of oleic acid more Tb^{3+} and/or Ca^{2+} binding sites became exposed. At the highest concentration of incorporated oleic acid the number of fluorescent sites was slightly reduced when compared with the control sample. It can be considered likely that the Tb^{3+}-oleic acid complex (high affinity binding) may reduce the binding to the lower affinity binding sites. This would parallel the earlier

finding that high concentrations of incorporated oleic acid reduced the equilibrium concentration of Ca^{2+} uptake, (Chapter 3). It was shown in this same chapter that at low concentrations of incorporated oleic acid there was an enhanced rate of Ca^{2+} uptake and no effect on the equilibrium concentration. One explanation, put forth in Chapter 3 was that oleic acid possessed ionophoric properties similar to those reported for another cis-unsaturated fatty acid, ricinoleic, by Maenz and Forsyth (1982). Alternatively, this behavior could be explained in terms of the existence of surface sites and/or internal Ca^{2+}-binding sites, (internal sites are considered to be all those sites not exposed to the external environment). Low concentrations of oleic acid might expose more metal binding sites which are oriented towards the outside of the vesicles and make them more accessible to the metal ion. The increase in the number of these outwardly-oriented binding sites would increase the rate at which Ca^{2+} could be transferred to the internal sites and this would result in the enhanced rate of Ca^{2+} uptake.

Whether the fluorescent Tb^{3+} binding sites in the oleic acid-treated vesicles were external or internal cannot be clearly established since these vesicles were not washed with chelator to remove any externally bound lanthanide. However, the data presented in Chapter 2 (Figure 2-9) showed that Tb^{3+} was not able to displace Ca^{2+} when the vesicles

had previously been equilibrated with the divalent cation. This indicated that Tb^{3+} was not able to penetrate into the interior of the vesicle or, at least, not able to displace Ca^{2+} from internal binding sites. Nonetheless, based on the oleic acid studies alone we cannot rule out the possibility that internally oriented Ca^{2+}-binding sites became more accessible at low oleic acid concentrations.

The results with the methyl oleate-treated vesicles support the proposal that additional protein sites may become accessible in the presence of oleic acid or its methyl ester derivative. As greater amounts of methyl ester were incorporated into the vesicles the intensity of Tb^{3+} fluorescence ($[Tb^{3+}] \geq 15$ µM, Figure 5-4B) progressively increased, indicating that the methyl ester was exposing protein binding sites and facilitating the binding of lanthanide. This was consistent with what was shown previously, that there was an increase in the equilibrium concentration of bound Ca^{2+} at all methyl oleate concentrations with no inhibition in binding being observed (Table 3-3, Chapter 3). Hence it would be reasonable to correlate the Tb^{3+} fluorescence data and Ca^{2+} uptake results, and confirm the proposal that methyl oleate when incorporated into BBMV increased the accessibility of $Ca2^{+}$ to binding proteins by rendering the membranes more fluid.

The observations of the changes in the Tb^{3+} fluorescence when Tb^{3+} vesicle complexes are treated with

EGTA or excess Ca^{2+}, provide further support for this rationalization and some evidence for the location of these binding proteins in the membrane. In the case of EGTA-treated vesicles the fractional decrease in Tb^{3+} fluorescence was greatest in the control sample (0.74). As increasing amounts of methyl ester were incorporated into the vesicles the fractional fluorescence decrease upon the addition of EGTA reached a value of 0.3 for 182 nmoles incorporated methyl ester per mg protein. This implied that the methyl ester exposed cryptic sites within the membrane and metal ions which are bound to these sites cannot be extracted with EGTA. Lysis of control untreated BBMV in cold distilled water followed by equilibration with fluorescent lanthanide, resulted in a further 10-15% enhancement in the Tb^{3+} fluorescence. Sonication of the untreated BBMV preparation in the presence of Tb^{3+} under the same conditions caused a 30% fluorescence enhancement over the unsonicated sample. Vesiculation, using a Potter Elvehjeim homogenizer, of BBMV in the presence of Tb^{3+} also enhanced the Tb^{3+} fluorescence by 20% when compared to a control sample, which had not been homogenized. Hence, in the untreated vesicles the majority of the binding sites for metals were located close to the surface of the membrane. It follows, therefore, that a significant number of metal-binding sites in untreated vesicles were buried but became

accessible to Tb^{3+} binding upon incorporation of methyl oleate.

Earlier results (Chapter 3) demonstrated that Tb^{3+} blocked the Ca^{2+} binding sites in the BBMV inhibiting the binding of Ca^{2+}. It may be that Tb^{3+} was binding to a passive carrier protein within this membrane, one that was responsible for the binding and subsequent transfer of Ca^{2+} to the cytoplasm of the epithelial cell (Wilson and Lawson, 1980).

The reliability of the calculation of the binding constants of such sites for Tb^{3+} was not fully satisfactory because of the nature of the system under study. As previously mentioned, it was not possible to exceed a Tb^{3+} concentration of 80 µM without aggregation of the BBMV. This necessitated working at Tb^{3+} concentrations of 70 µM or less. Under these conditions the total amount of Tb^{3+} bound represented a significant fraction of the total Tb^{3+} present. This made the determination of the binding constants from the data difficult since appropriate assumptions could not be made, as those required in the case of the double reciprocal plot for the treatment of binding data (Leen and Rodbard, 1984). In order for such a treatment of the binding data to be made it must be assumed that the amount of free Tb^{3+} be nearly equivalent to the total Tb^{3+}. However, in this study the values of bound Tb^{3+} usually represented less than 15% of the total Tb^{3+} added.

Interestingly, two affinity constants were reported by Ohyashiki et al. (1985) for Tb^{3+} binding to porcine BBMV. The high affinity constant was shown to be in the µM range whereas the lower constant was millimolar. In our hands, the complexity of the binding of Tb^{3+} to rabbit BBMV made any estimation of the binding parameters pointless. Still, it was evident that the affinity of Tb^{3+} for rabbit BBMV was much greater than the affinity of Ca^{2+} binding to the same membrane as evidenced by the concentration of Ca^{2+} required to displace Tb^{3+} from the membranes (1 mM and 10 µM for Ca^{2+} and Tb^{3+}, respectively; Figure 5-5). Leavis et al. (1980) have found that Tb^{3+} has at least an 100 fold greater affinity for the high affinity Ca^{2+}-binding sites in troponin-C than Ca^{2+}. The addition of magnesium to Tb^{3+}-equilibrated BBMV resulted in only a small amount of displacement of Tb^{3+} from the fluorescent sites (<20% of that seen for Ca^{2+}, data not shown) indicative of the Ca^{2+}-specific nature of the Tb^{3+}-displacable sites.

The results were consistent with the proposal that the incorporation of fatty acid modified the conformation, structure and fluidity of the brush border membrane resulting in an increased exposure of cryptic protein metal-binding sites. The observed rate enhancement of Ca^{2+} uptake by vesicles treated with the low concentration of oleic acid and all of the methyl oleate-treated vesicles may partially be due to this exposure. The inhibition of Ca^{2+} uptake seen

at the high concentration of oleic acid was probably due to the effects of the binding of Ca^{2+} to incorporated oleic acid which likely was oriented towards the exterior of the vesicles.

This study indicates that Tb^{3+} can be used as a probe for certain Ca^{2+}-binding sites on proteins if interpreted with extreme caution, but cannot be used indiscriminately as a replacement for Ca^{2+}. It is clear that both Tb^{3+} and Ca^{2+} bind to some of the same sites as revealed by the displacability of the lanthanide by Ca^{2+} (Figure 5-5). However, there exist some sites which appear to be Tb^{3+} specific and which cannot be displaced by Ca^{2+}. The reason for this is not certain but may be at least partly due to the difference in the affinities of the binding sites for the two cations. Nonetheless, these results do provide sufficient data to suggest that a significant portion of the binding sites for Ca^{2+}, namely those sites common to both cations, are protein in nature.

Chapter Six

PARTIAL CHARACTERIZATION OF Ca^{2+}-BINDING PROTEINS FROM PORCINE INTESTINAL BRUSH BORDER MEMBRANES

I. INTRODUCTION

Much work has been directed at elucidating the protein component(s) responsible for the translocation of Ca^{2+} across the brush border membrane (Wasserman and Fullman, 1983; Miller et al., 1982; Kowarski and Schachter, 1980). As research in this area progressed, several proteins were suggested as part of the Ca^{2+} transport and binding mechanism namely, CaBP (Taylor 1981); CaATPase-alkaline phosphatase (Schiffle and Binswanger 1980); and calmodulin, (Bikle and Munson, 1986). However, this subject remains controversial and certainly somewhat of an enigma. Notwithstanding this, one integral membrane protein, IMCal, was characterized and proposed to be part of a Ca^{2+}-binding complex of the brush border membrane that has a high affinity for Ca^{2+} (Schachter and Kowarski, 1980). Very little is known about the structure of IMCal and even less concerning its potential role in Ca^{2+} movement across the brush border membrane. To date this membrane-bound Ca^{2+}-binding entity has been identified only in rat intestine.

A group of proteins of similar molecular weights have been identified in Ca^{2+} chelator extracts of both lymphocyte plasma membranes and intestinal brush border membranes (Gerke and Weber, 1984). These proteins are preferentially-

associated with the non-ionic detergent-soluble fraction of the membrane and were resolved as three Ca^{2+}-binding polypeptides with molecular weights of 68,000, 33,000, and 28,000. No function has been assigned as yet to these proteins.

It is remarkable that Ca^{2+} transport proteins have not been isolated and characterized as such from the brush border membrane. To date, only Ca^{2+}-binding proteins have been identified and their relationship with transport remains undetermined. Only reconstitution studies can eventually resolve this problem. In the present chapter however, this aspect will not be considered. Instead, attempts to characterize the binding proteins in porcine BBMV will be made in the hope that this will yield sufficiently pure and well-characterized material which can eventually be used in reconstitution studies.

II. MATERIALS

The detergents, Triton X-100, RTX-100 (reduced Triton X-100), Triton X-114, 3-[3-(cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), Lubrol-PX and octyl-glucoside, and bis-Tris, bovine brain calmodulin, $NaCNBH_3$, ^{14}C-formaldehyde, elastase, chymotrypsin, dinitrophenol-lysine, ninhydrin, guanidine HCl, cyclic AMP, cobra snake venom (Ophiophagus hannah) and activator-free phosphodiesterase (lot no. 83F-96251) were all obtained from

Sigma Chemical Ltd (St. Louis, MO). The detergents, deoxycholate and sodium glycocholate, were purchased from K & K Laboratories (Plainsview, NY). The cellulose thin-layer chromatography plates were from Eastman, Kodak (Rochester, NY). The gels and resins, Sephadex G-150 and G-25, phenyl-Sepharose and DEAE-Sephacel, and Polybuffer-74 were purchased from Pharmacia Ltd (Dorval, Que). The following chemicals were obtained from Biorad Ltd (Mississauga, Ont.): Coomassie blue R-250, bromophenol blue, SDS standard proteins (LMW 10,000-100,000 and HMW 50,000-200,000) and AG1-X2 resin. The electrophoresis grade acrylamide and bis-acrylamide were procured from Terochem Laboratories (Mississauga, Ont.). The ampholyte buffers, pH range 3-10 and 2.5-5, and ammonium bicarbonate buffer were bought from Fischer Scientific Company (Don Mills, Ont.). Cyclic-^{3}H-AMP and ^{125}I-calmodulin were supplied by Amersham Radiochemicals Ltd (Montreal, Que.) New England Nuclear (Boston, MA) was the supplier of ^{3}H-formaldehyde.

III. METHODS

A. Preparation of porcine BBMV

The method of Christiansen and Carlsen (1981), already adapted for porcine intestine, was used to prepare BBMV from the first 2/5 of the small intestine (duodenum and jejunum) of 6 month old pigs obtained from a local abattoir. These animals were raised at the Agriculture

Canada Experimental Farm, Ottawa Ontario and were disease and parasite free. The BBMV were assayed for the presence of the marker enzymes, alkaline phosphatase and sucrase, and were found to display a similar degree of purification relative to the homogenate (12 and 15 fold, respectively) as reported by Christiansen and Carlsen. Routinely, cells from six animals were collected and used for each preparation of purified BBMV.

B. Extraction of BBMV with detergent

Initially, brush border membrane proteins were extracted from freshly prepared BBMV using Triton X-100. However, this non-ionic detergent possessed significant absorbance at 280 nm, so it was substituted with hydrogenated Triton X-100 (RTX-100) which had 0.01 the absorbance at 280 nm. Eventually, another detergent, Lubrol-PX, was adopted for the solubilization of brush border membrane proteins, chosen for its superior non-denaturing qualities and negligible ultraviolet absorbance. BBMV, 6 mg/ml (final protein concentration) were stirred slowly overnight in 1.0% Lubrol-PX, 100 mM KCl, 50 mM mannitol, 30 μM PMSF and 40 mM Tris/HCl, pH 7.4. After stirring for 16h the extract was centrifuged at 10,000 x g for 30 minutes to pellet insoluble material and the supernatant was withdrawn.

C. <u>Delipidation of brush border membrane protein extract with 1-butanol</u>

To the detergent-solubilized solution was added 30% (v/v) ice-cold 1-butanol and the resulting suspension was stirred vigorously at 4°C for 30 min. This mixture was centrifuged at 105,000 x g for 60 min in a Beckman L8-M Ultracentrifuge (Beckman Instruments Inc., Mississauga, Ont.) using a Beckman 55.2 Ti titanium fixed-angle rotor. Upon centrifugation the extract separated into two phases, a lower aqueous phase and an upper organic phase. A small pellet always formed at the bottom of the centrifuge tube and a white interfacial layer, probably consisting of denatured protein, developed between the aqueous and organic phases. Protein analysis of the two phases revealed a lack of detectable protein in the butanol (upper phase) and that greater than 80% of the total protein added was found in the lower aqueous phase. This delipidated extract was concentrated by a volume factor of 10 with a 200 ml ultrafiltration cell (Amicon Ltd, Oakville, Ont.) equipped with a 10,000 MW cut-off filter (YM type) and the resulting, somewhat cloudy, concentrated extract was recentrifuged at 105,000 x g for 60 min. After centrifugation, this extract was very clear and exhibited a pale yellow color with a slight odor of butanol.

D. Assay of Ca^{2+}-binding protein activity

To determine the relative Ca^{2+}-binding activity of the various protein samples a simple assay was developed based on the spin column method of Penefsky (1977). Small circular disks of porous polyethylene mesh were cut from a large sheet (30 µm pore size, Fischer Scientific) and placed in the bottom of 1 cc plastic disposable tuberculin syringes. Previously swollen Sephadex G-25 (fine) was pipetted as a slurry into the syringes until the drained volume of gel was 1.0 cc. The columns were then spun at room temperature in a table top centrifuge (International Equipment Co., Boston, MA) at 1500 rpm for 3 min collecting the effluent in 1.5 ml Eppendorf microcentrifuge tubes. Next, 200 µl of buffer containing 150 mM NaCl, 0.1% Lubrol-PX, 20 mM Tris, pH 7.5 was applied to the top of the gel in the columns and the centrifugation step repeated. This last step was repeated a total of three times. To the equilibrated spin columns was added a mixture of protein and $^{45}Ca^{2+}$ ($5 \cdot 10^5$ cpm) which had been incubated 15 minutes previously. After application, the contents were spun again for 3 min at 1500 rpm. The effluent (192 ± 6 µl) was pipetted directly into 20 ml plastic scintillation vials and the radioactivity associated with the protein detected. It was determined that all or nearly so (96 ± 4 %) of the protein in the incubation mixture was recovered in the

effluent while the free $^{45}Ca^{2+}$ was retained in the upper portion of the gel.

E. Sephadex G-150 chromatography

Sephadex media, G-150, was allowed to swell for several h in Buffer A (see below), then packed into appropriate columns and equilibrated with two column volumes of buffer. Initially, size exclusion chromatography was performed with a relatively small analytical column (1.6 cm dia. x 54 cm length), volume equal to 115 ml, which was run at a flow rate of 14 ml/h with sample volume application equal to 2.5 ml. Later, a larger preparative column was used (5.0 cm dia x 60 cm length), volume equal to 1178 ml, and run at a flow rate of 30 ml/h with the applied sample volume being equal to 30 ml. The elution buffer (Buffer A) used for both columns was 0.1% Lubrol-PX, 30 μM PMSF, 20 mM Tris/HCl, pH 7.4. These columns, as well as all others described later, were run in a cold room at 4°C.

F. Ion-exchange chromatography

DEAE-Sephacel, supplied pre-swollen in methanol, was equilibrated at the appropriate pH, 7.5, by allowing the gel to settle and decanting the methanol followed by the addition of two settled-gel volumes of Buffer A. The mixture was swirled gently to make a slurry which was then allowed to settle. The buffer was decanted and the whole procedure repeated several times until the pH of the mixture was 7.5. The titrated resin was packed into a 1.0 cm dia. x

30 cm long column (column volume approx. 24 ml) and equilibrated by passing two column volumes of Buffer A through the column, while verifying that the pH of the effluent was identical with that of the starting buffer. Samples of membrane protein, obtained after sizing on Sephadex G-150, were applied directly, without concentration between separation steps, and upon complete loading of the sample, Buffer A was pumped through the column (flow rate = 16 ml/h) until no absorbance at 280 nm was detected in the effluent. A linear gradient of NaCl (0 - 0.5 M, or 0 - 0.4 M) in a volume of 200 ml was run through the column collecting 1.3 ml fractions.

G. <u>FPLC of brush border membrane protein</u>

Selected peaks of brush border membrane protein eluted from DEAE-Sephacel were dialyzed into Buffer B (0.025 M bis-Tris/HCl pH 7.1) and applied to a Mono-P HR 5/20 chromatofocussing column connected to a Pharmacia FPLC System (Pharmacia, Dorval, Que.) equipped with a high performance pump (P-3500), an UV monitor (UV-M), and a single channel recorder (REC-481). Sample volume was routinely 2.0 ml and was applied to the column by means of a capillary sample loop attached to a Pharmacia PV-7 valve. The chromatofocussing column was developed with Polybuffer-74 adjusted to pH 5.0 with HCl, and ninety, 0.3 ml fractions were collected in a Pharmacia Frac-300 fraction collector. The pH gradient thus formed was determined by reading every

third or fourth fraction with a pH meter. Immediately upon determining the pH gradient 50 µl of 100 mM Tris/HCl buffer, pH 7.5, was added to each fraction to bring the pH back to 7.5 in order to avoid potential loss of activity due to the insolubility and instability of various proteins at their isoelectric points.

H. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Newbold et al. (1982). The size of the resolving gel was 1.5 mm thick by 19 cm wide and the dye front was run routinely for a distance of 13 cm. A 4% acrylamide stacking gel was poured on top of the resolving gel (dimensions: 19 cm wide x 4 cm deep). Proteins were stained with Coomassie Blue R-250 in 7.5% acetic acid/ 25% isopropanol/ water (v/v/v) and destaining involved two steps: (1) removal of background stain in a rapid destain solution (7.5% acetic acid/25% isopropanol/ water, v/v/v) and then (2) further clearing of background staining with regular destain consisting of 7.5% acetic acid only. Samples and standards were prepared by the addition of sample buffer (10% glycerol, v/v; 1% SDS, 4 mM PMSF; 50 mM Tris/HCl; 10% β-mercaptoethanol, v/v; 2 mM EDTA; 0.02% bromophenol blue dye) to the samples followed by boiling of the samples in reducing conditions for 5 min at 100°C. The proteins were run at constant current (40 mA/gel) with power being

supplied by a D.C. power supply (Gelman Instrument Co., Model 38201, Ann Arbor, Michigan), running time at room temperature usually being 5.5 h, but varied according to the acrylamide concentration in the gels.

I. Isoelectric focussing

Brush border membrane proteins were subjected to isoelectric focussing by modifying the method of Schmidt-Ulrich et al. (1975). This was deemed necessary because various brush border membrane protein samples would not enter the gels when prepared as outlined by Schmidt-Ulrich et al. The acrylamide concentration used was 4% and bis-acrylamide, 0.1%. The ampholyte buffers were in the pH ranges 3 - 10 and 2.5 - 5.5. It was established that 12 and 16 h were needed for the proteins to reach their isoelectric points in the gels (rod gel dimensions were 0.5 cm dia. x 8.5 cm long) when ampholytes 2.5 - 5.5 and 3 - 10 were used, respectively. To determine the continuity of the pH gradient in the rod gels, one control was routinely run which had no sample applied and was sliced into 0.5 cm slices. Each slice was placed into a test tube to which was added 2 ml of a 150 mM KCl solution, then the samples were mixed and allowed to equilibrate for 1 h before the pH was read. The proteins after isoelectric focussing were fixed in 10% trichloroacetic acid (TCA) for 6-8 h in 16 x 100 mm test tubes. Following fixation, the rod gels were washed with 25% isopropanol, 10% acetic acid, 65% water (v/v/v) for

8 h, then with water only for 2-3 h. The rods were stained in 25% ethanol, 10% acetic acid, 65% water (v/v/v) containing 0.05% Coomassie Blue and 0.05% $CuSO_4$ for 4 h and then destained with 10% ethanol, 10% acetic acid, 80% water (v/v/v) until background stain was eliminated.

J. Phosphodiesterase assay

Phosphodiesterase assays were performed according to the method of Thompson et al. (1974) with one major modification being that the pH of the ion exchange resin was adjusted to 3.0 to optimize the conditions for recovery of labelled nucleotides as outlined by Boudreau and Drummond (1975). The enzyme activity was assayed in a final volume of 400 µl containing 40 mM Tris/HCl (pH 8.0), 3.75 mM β-mercaptoethanol, 5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, cyclic ^{3}[H]-AMP (38 Ci/mmol, $2 \cdot 10^5$ cpm/tube) and 50 µM unlabelled cyclic AMP. Enzyme basal activity was measured in the absence of the activator, calmodulin, and in the presence of EGTA. Blank values, always less than 7% of the total ^{3}[H]-cyclic AMP added, were determined by substituting the active enzyme with a boiled enzyme preparation. Briefly, tubes were incubated at 30°C for 10 min and the reaction was stopped by boiling the samples for 2.5 min. The sample tubes were then cooled for 10 min on ice and 100 µl of cobra snake venom was added to each tube. The tubes were incubated for another 10 min at 30°C and the reaction was terminated by adding 1.0 ml of the ion-exchange resin,

AG1-X2, which had previously been prepared as a 1:3 slurry titrated to pH 3.0 with glacial acetic acid. The resulting suspensions were allowed to equilibrate for 15 min at 4°C and then were centrifuged at 500 x g for 10 min. From each tube, 0.5 ml aliquots of the supernatant were removed and added to 10 ml of 1:1 toluene:Liquiscint (Diamed Laboratories, Missassauga, Ont.) and counted.

K. Reductive methylation of brush border membrane proteins

Various proteins, extracted from the brush border membrane, and bovine brain calmodulin were labelled as described by Jentoft and Dearborn (1983). The proteins were prepared for labelling by dialyzing against 6M guanidine HCl, 0.1 M HEPES buffer, pH 7.0. The dialyzed samples, containing 200 µg protein in 200 µl, were incubated for 2 h at room temperature in the presence of 20 µl of 20 mg/ml $NaCNBH_3$ and 10 µl ^{3}[H]-HCHO for the brush border membrane proteins, with the exception that for calmodulin ^{14}C-formaldehyde was used. The labelling reaction was terminated by the addition of 20 µl 100 mM Tris buffer, pH 7.0. Each reaction solution was next subjected to size exclusion chromatography with Sephadex G-25, swollen in 0.5% ammonium bicarbonate, pH 7.0 and packed in small plastic disposable columns (Biorad, Missassauga Ont.). The labelled protein peak (void volume) was separated from the free label peak and collected. A small aliquot, 5 µl, of each collected fraction was counted and the appropriate fractions

were pooled. The pooled, labelled proteins were lyophilized overnight attached to a Virtis Freezemobile-12 (Gardener, NY) and stored at -20°C until use.

L. Peptide fingerprint mapping of brush border membrane proteins and calmodulin

The labelled proteins were subjected to protease digestion by incubating 20 µl of ^{14}C-labelled calmodulin (5000 cpm) with 10 µl of a ^{3}H-porcine intestinal Ca^{2+}-binding protein, isolated as described in this chapter, (≈20,000 cpm purified Sephadex G-150 Peak 1 activity). To these was added 20 µg (20 µl) of unlabelled bovine brain calmodulin, the total mixture being buffered in 20 mM ammonium bicarbonate, pH 8.8. The proteases, elastase and chymotrypsin were dissolved in $1 \cdot 10^{-3}$ M HCl at a protein concentration of 10 mg/ml and were diluted 10 fold with ammonium bicarbonate buffer, pH 7.0. To each mixture of labelled calcium-binding proteins was added 10 µl of one of the protease solutions and the whole mixture was incubated for 3 h at 37°C.

The digested protein samples were spotted 1 cm from the bottom and 1 cm from the right side of cellulose plates (20 cm x 20 cm). The plates were also spotted with 2 µl of a dinitrophenol-ε-labelled lysine standard solution to serve as a marker during peptide migration. The cellulose plates were gently blotted with a piece of filter paper until wet with an electrophoretic buffer consisting of acetic acid, formic acid and water (64:16:720, v/v/v

respectively). Voltage (800 V) was applied to the cellulose plates and the marker was allowed to migrate 40% of the length of the plates. The plates were then air dried for several hours and developed in the second dimension by chromatography with a solvent system consisting of n-butanol, acetic acid, water and pyridine (75:15:50:60, v/v/v/v respectively), allowing the solvent to run to 80% of the length of the plate. After drying thoroughly, the plates were sprayed with a 1% ninhydrin in acetone solution to visualize the spots, which formed 5-10 minutes after spraying.

The plates were exposed to X-ray film, Chronex type (Picker International, Ottawa Ont.) and autoradiographed for 3-4 days for detection of ^{14}C-labelled peptide spots. The spots on the film were used as a guide to scrape the corresponding spots on the cellulose plates and the cellulose scrapings were counted for both ^{14}C and ^{3}H using a double-label counting program.

M. <u>Triton X-114 phase-partitioning assay</u>

The hydrophobic nature of calmodulin-binding protein(s) from the brush border membrane was determined using the phase separation assay of Bordier (1981), which consists of partitioning proteins between a detergent phase and a non-detergent phase. Basically, Triton X-114 was washed with 10 mM HEPES, 150 mM NaCl, pH 7.5 buffer and the washed Triton (lower phase) was collected and used as the

stock detergent solution. The detergent concentration in the stock solution was determined by its absorbance at 277 nm ($A_{1\%}$ = 22.2) and was routinely found to be 10-11% after washing with buffer as described. Routinely, 200 µg of membrane protein was incubated with various concentrations of ^{125}I-calmodulin (stk ^{125}I-calmodulin, 55 µCi/µg) in 1% Triton X-114, 150 mM NaCl and 10 mM HEPES, pH 7.5 (total volume 200 µl) for 60 minutes on ice. The incubation mixture was gently layered onto a sucrose cushion (6% sucrose, 0.006% Triton X-114, 150 mM NaCl, and 10 mM HEPES buffer, pH 7.5) in the bottom of a 1.5 ml Eppendorf microcentrifuge tube and placed in a water bath at 30°C for 3 min. The cloudy solution was centrifuged at 300 x g for 3 min in a microcentrifuge (Brinkman-Eppendorf Model 5415), after which a pellet of concentrated detergent could be seen in the bottom of the tube. The top portion of the mixture (200 µl) was drawn off and Triton X-114 was added to this portion to make it 1% again and the incubation and centrifugation steps were repeated. Subsequently, the top two layers of solution (500 µl, including the sucrose cushion) were drawn off the detergent pellet, then 1 ml of distilled water was added to each tube and the detergent pellet was resuspended. The solution was then transferred to plastic tubes (12 mm dia. x 75 mm long) and a second ml of distilled water was added to each centrifuge tube followed by a repetition of the rinsing and transfer steps.

The samples were counted in a Beckman L-5500 gamma counter (Beckman Instruments Inc., Mississauga, Ont.).

1. Analysis of ^{125}I-calmodulin binding to BBMV

The determination of the dissociation constant (K_d) and the amount of calmodulin bound to the BBMV on a per mg protein basis (B_{max}) was completed with the assistance of a computer program, designated Ligand, designed for the analysis of such parameters in binding studies (Peter J. Munson, Endocrinology and Reproduction Research Branch, National Institutes of Child Health and Human Development, National Institutes of Health, Bethesda, Md. 20205). Instead of treating the "infinite" dose binding as an exact measure of non-specific binding, the computer program treats this "infinite dose" data point (the last point in the binding assay corresponding to infinite dilution of the labelled calmodulin with cold protein) just like the other ligand doses and estimates the non-specific binding as a data-determined parameter.

N. ^{125}I-calmodulin gel overlay technique

For the detection of calmodulin-binding proteins in the brush border membrane the gel overlay method of Slaughter and Means (1987) was followed. Various extracts from the BBMV were run on SDS-PAGE as outlined above (section H, Methods) except that the gel dimensions were reduced to lower the amount of labelled substrate needed in the subsequent incubation procedure. To facilitate this, the gels were run with the use of the Biorad Protean II minigel apparatus (Biorad Laboratories, Mississauga, Ont.), the dimensions of the gel being 0.75 mm thick, 8.5 cm wide by 6 cm long (resolving gel) with a 2 cm stacking gel formed on the top. After electrophoresis, the SDS was removed as outlined by Slaughter and Means, the proteins fixed to the gel and incubated with ^{125}I-calmodulin, 5 ml/gel (1 µg calmodulin/ml, $1 \cdot 10^6$ cpm/µg) for 15 . After thorough washing of the gel (four separate washes) with calmodulin-free incubation buffer until only background radioactivity was detected, the gels were stained for protein, dried for 2 h under vacuum and autoradiographed with Chronex X-ray film for 3 days at -70°C.

O. Chromatography assay for calmodulin

Various proteins (200 µg each) were ^{3}H-labelled as described in Methods section K. Following labelling, approximately 20,000 cpm of ^{14}C-calmodulin was added to $5-7 \cdot 10^5$ cpm of the test protein in a total volume of 1.5 ml.

These protein samples were applied to Biorad plastic mini-columns containing DEAE-Sephacel in 1% N-ethyl morpholine acetate buffer, pH 7.0. The samples were eluted with 0.7 M NaCl and an aliquot of each sample effluent was taken and counted. The resulting effluents were next made 10 mM Ca^{2+} by the addition of a concentrated solution of this cation in elution buffer and were applied to phenyl-Sepharose, which also had been packed into mini-columns. The proteins were eluted with NEMAc buffer containing 10 mM EGTA and again an aliquot of each sample effluent was counted. Following this last column chromatography step the proteins were desalted, concentrated and subjected to SDS-PAGE (12.5%). The stained bands were excised from the gel and the proteins were extracted with TCA and an aliquot counted.

P. Protein Determination

Protein concentrations were assayed by the Modified Lowry Method which involves the addition of SDS as described by Peterson (1983) to eliminate interference from detergents etc.

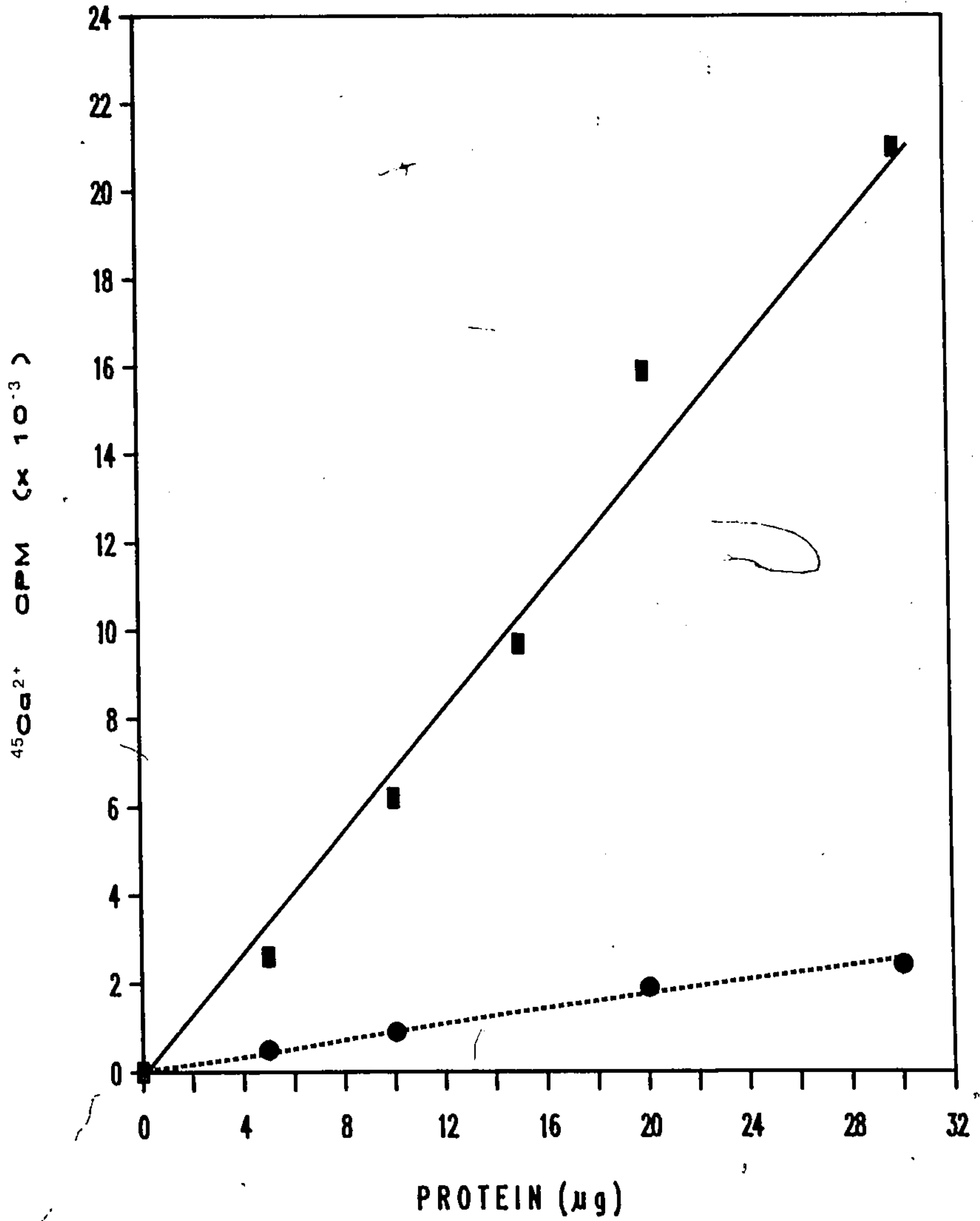
IV. RESULTS

Figure 6-1 shows a typical standard curve constructed as validation of the method used for the detection of Ca^{2+}-binding activity of brush border membrane proteins. In this particular experiment bovine brain calmodulin was used as a standard to determine the relationship between the amount of

Figure 6-1

Calcium-binding assay standard curve using Sephadex G-25 spin column method.

Spin columns were prepared as described in Methods section D. Various amounts of protein, bovine brain calmodulin (■) or brush border membrane extract (●) were mixed with $^{45}Ca^{2+}$ ($5 \cdot 10^5$ cpm) and incubated at room temperature for 15 min in Ca^{2+}-binding assay buffer (150 mM NaCl, 0.1% Lubrol-PX and 20 mM Tris/HCl, pH 7.5) in a volume of 200 µl. The incubation mixture was subsequently applied to pre-equilibrated spin columns (1 cc, gel volume) and centrifuged at 1500 x g for 3 min. The effluent was collected in plastic 1.5 ml Eppendorf microcentrifuge tubes and the amount of $^{45}Ca^{2+}$ in the effluent was determined by scintillation counting.



24
22
20
18
16
14
12
10
8
6
4
2
0
⁴⁵Ca²⁺ CPM (x 10⁻³)
0
4
8
12
16
20
24
28
32
PROTEIN (µg)

binding protein and the $^{45}Ca^{2+}$ cpm in the spin column effluent. This data reveals that the method was sufficient to detect Ca^{2+}-binding activity of a known protein, calmodulin, and also was satisfactory for the detection of Ca^{2+}-binding activity in the brush border membrane extract. However, it should be stressed that this assay procedure provided only relative binding information under these conditions and could not supply data for the calculation of binding constants. This was the case because the degree of Ca^{2+} saturation of the proteins in the assay was not determined, i.e., the apo form of the protein(s) was not generated and likely varied in amount from preparation to preparation.

The optimal conditions concerning membrane protein and detergent concentrations for the solubilization of Ca^{2+}-binding activity from porcine brush border membranes were determined and the results are plotted in Figure 6-2. As the concentration of the detergent, Lubrol-PX, was increased a proportionately greater amount of protein was extracted from the membrane. This is true for all concentrations of protein used. However, the percent increase in the amount of protein extracted at the higher detergent concentrations (0.5% - 1.0%) is much smaller than the increase seen at lower concentrations (0.01 - 0.1%). A similar trend was seen for the extraction of Ca^{2+}-binding activity as a function of detergent concentration, except in this case at

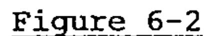
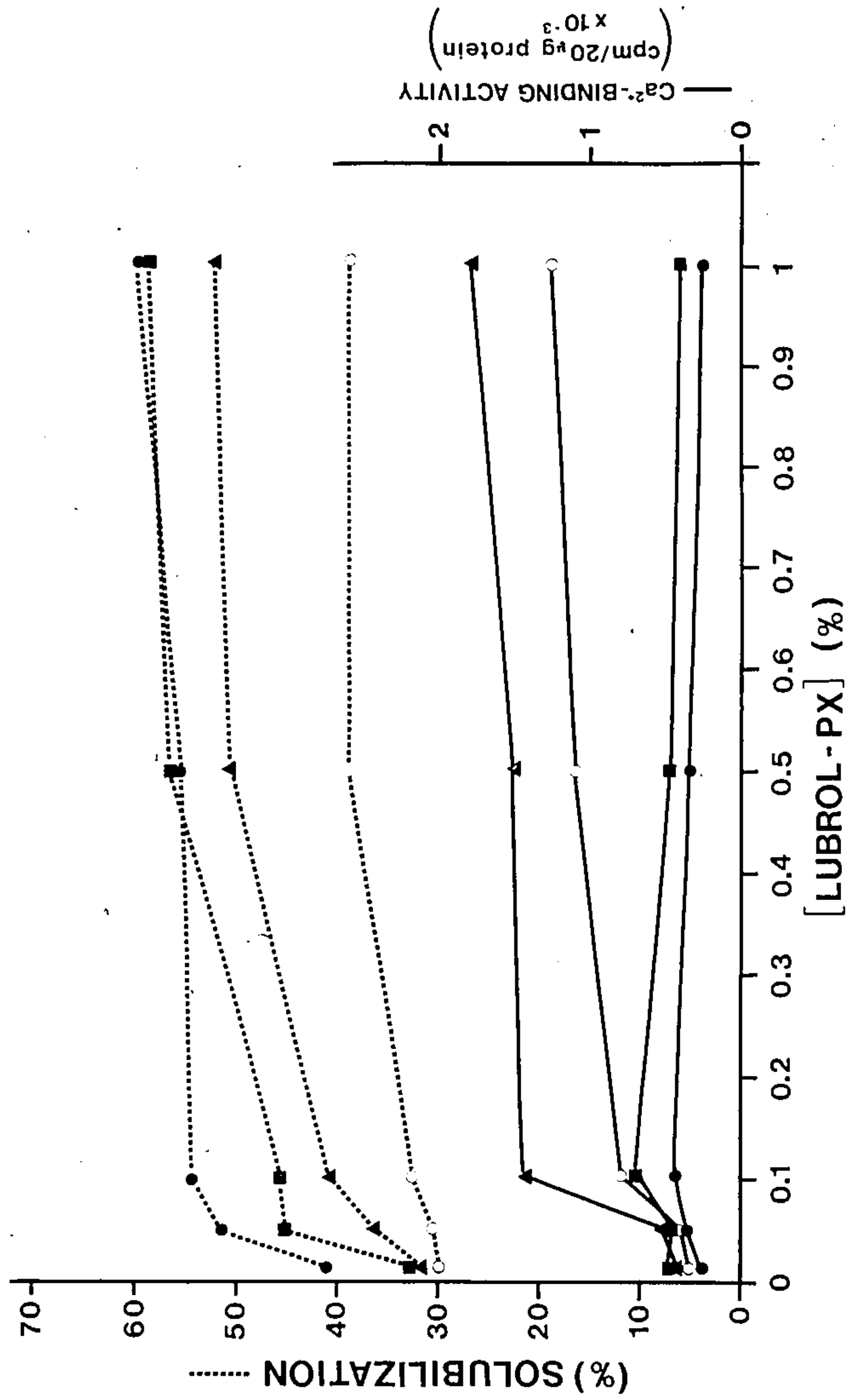


Figure 6-2

Optimization of solubilization conditions for Ca^{2+}-binding activity using the non-ionic detergent, Lubrol-PX.

Brush border membrane vesicles at various protein concentrations, 1 mg/ml (●); 3 mg/ml (■); 6 mg/ml (▲); and 9 mg/ml (○) were mixed with various concentrations of Lubrol-PX and the percentage of total protein added that was soluble after centrifugation at 105,000 x g for 60 min, was determined (••••••••) along with the specific Ca^{2+}-binding activity of the soluble extract (——). Total volume of the mixtures was 1.5 ml and the solubilization was performed at 4°C for 12 h with mild shaking in 100 mM KCl, 30 µM PMSF, and 40 mM Tris/HCl buffer, pH 7.5.



(%) SOLUBILIZATION
0
10
20
30
40
50
60
70
[LUBROL-PX] (%)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Ca2+-BINDING ACTIVITY
(cpm/20 μg protein x 10-3)
0
1
2

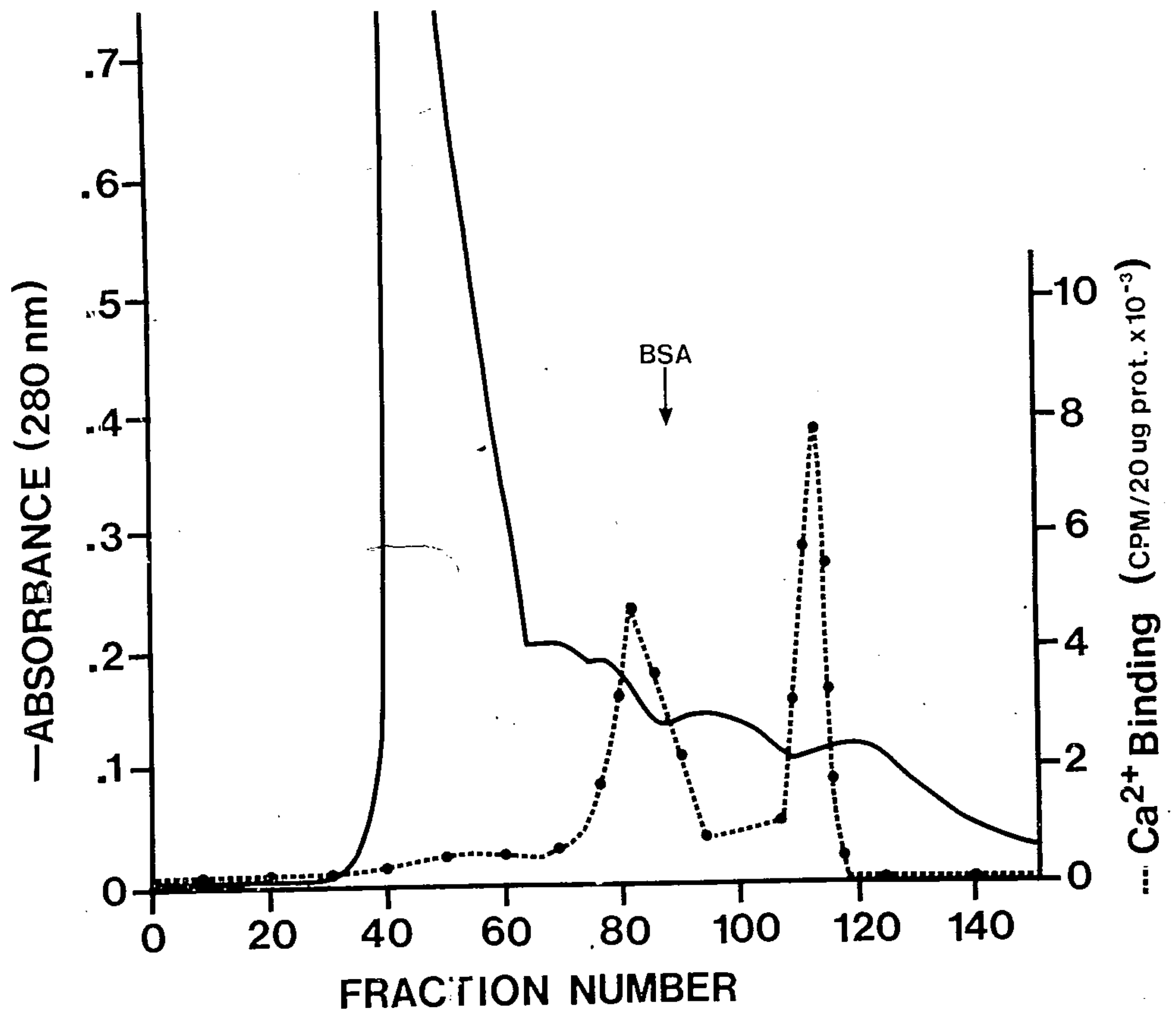
some of the higher detergent concentrations (0.5% and 1%, protein concentrations 1 and 3 mg/ml), the specific activity of the solubilized extract was lower than for the reduced detergent concentrations. Also, the percentage of membrane protein solubilized decreased with increasing protein concentration while, at the same time, the specific Ca^{2+}-binding activity increased for membrane protein concentrations 1 mg/ml to 6 mg/ml, but this trend was reversed with respect to Ca^{2+}-binding activity at the highest membrane protein concentration used, 9 mg/ml. At this protein concentration, the specific Ca^{2+}-binding activity of the extract decreased as compared to the same binding activity at 6 mg/ml protein. These results suggested that the optimal protein and detergent concentrations for solubilization of Ca^{2+}-binding activity were 6 mg/ml and 1.0%, respectively.

Figure 6-3 shows the elution profile of porcine brush border membrane extract prepared with Lubrol-PX under optimal conditions as just stated and then run through a Sephadex G-150 column. The protein elution pattern revealed that there was large proportion of the extract which eluted off the column in the void volume (fractions 35-63) which represented large molecular weight complexes or aggregates (greater than 300,000 MW). There were four peaks that were resolved although not sharply. The Ca^{2+}-binding activity of the resolved extract appeared primarily in the

Figure 6-3

Sephadex G-150 elution profile of porcine BBMV extract.

To an equilibrated Sephadex G-150 column (1.6 cm dia. x 54 cm length) was applied 2.5 ml of BBMV protein extract (10 - 15 mg/ml protein). The elution buffer, 0.1% Lubrol-PX, 30 μM PMSF and 20 mM Tris/HCl buffer, pH 7.5, was pumped through the column at 4°C by means of a peristaltic pump with a flow rate of 22 ml/h. Fractions were collected by means of a LKB fraction collector, each fraction being 0.75 ml. The absorbance (280 nm) of every second tube was read to determine the amount of protein present. Calcium-binding activity of the various fractions was determined by the spin column method (cf. Methods section D).



—ABSORBANCE (280 nm)
.7
.6
.5
.4
.3
.2
.1
0
BSA
FRACTION NUMBER
0
20
40
60
80
100
120
140
--- Ca^{2+} Binding (CPM/20 ug prot. x 10^{-3})
10
8
6
4
2
0

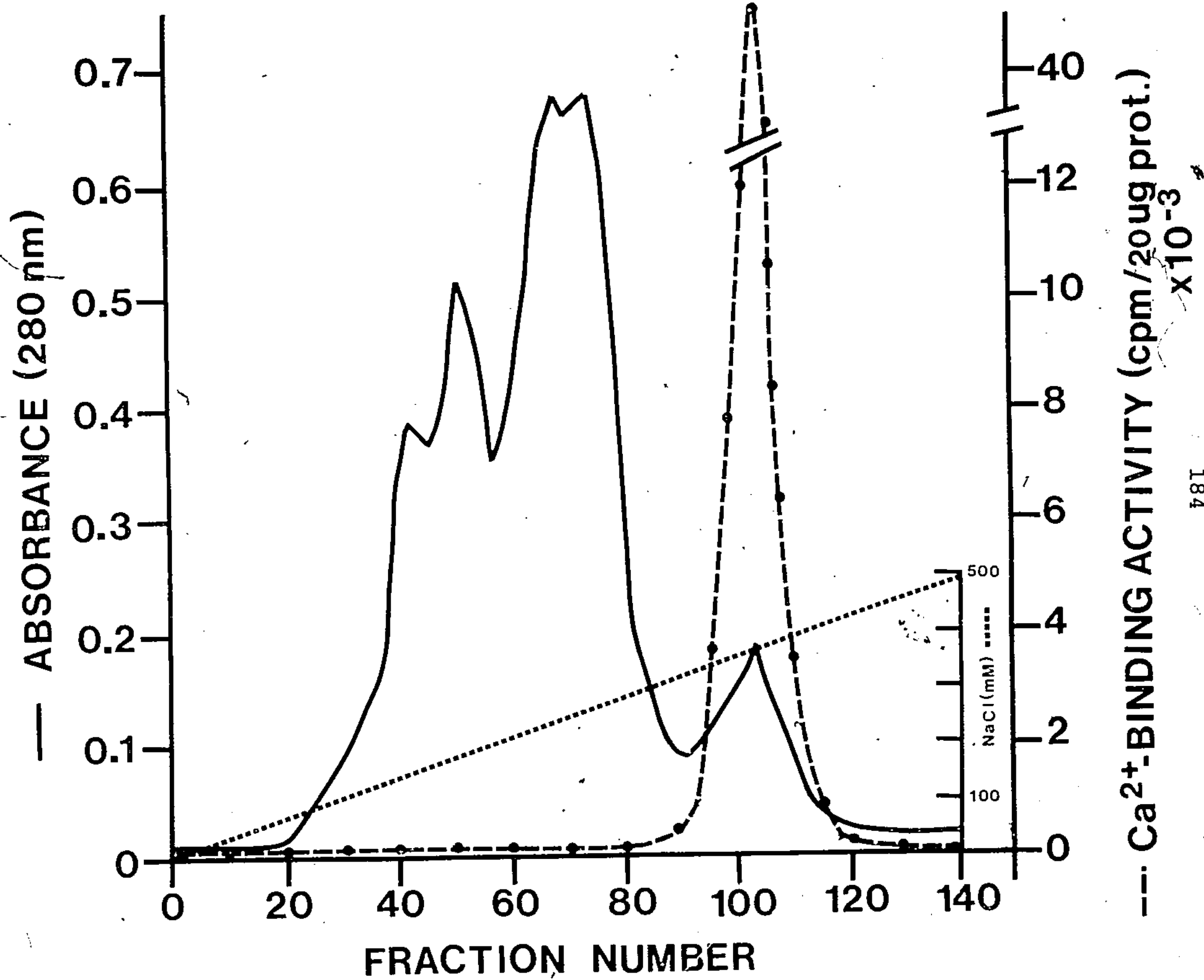
form of two peaks, arbitrarily designated peaks 1 and 2. Peak 1 activity corresponded to a molecular weight of 90,000 daltons while peak 2 showed a molecular weight of 30,000. It was always noted that peak 2 possessed the higher specific Ca^{2+}-binding activity, but in total units of activity peak 1 was greater.

Figure 6-4 reveals the elution pattern of peak 1 (G-150) activity applied to DEAE-Sephacel at pH 7.5 and eluted with a linear gradient of NaCl from 0 to 0.5 M. Several peaks were partially separated but the protein peak corresponding to the Ca^{2+}-binding activity was the best resolved. When sample from this peak was subjected to SDS-Page a single band (96% homogeneous, SDS-PAGE) was identified corresponding to a MW of 20,500 (Figure 6-5A). It was established the two minor protein contaminants could be removed by subjecting the pig Ca^{2+}-binding protein to hydrophobic interaction chromatography with phenyl-Sepharose (cf. Figure 6-7). When this protein was focussed in polyacrylamide (pH range 3.5-10) it was found that this Ca^{2+}-binding protein possessed an isoelectric point of 3.8, indicating that it was a very acidic protein (Figure 6-5B). When compared with another protein, bovine brain calmodulin, in the same system there was not much difference; however, bovine brain calmodulin always focussed 0.3 pH units more basic than this porcine intestinal Ca^{2+}-binding protein.

Figure 6-4

Ion-exchange chromatography of Sephadex G-150 peak 1 Ca^{2+}-binding activity.

DEAE-Sephacel was prepared as described in Methods section F and was packed and equilibrated in a glass column (1.0 cm dia. x 30 cm length). Column effluent from the Sephadex G-150 column corresponding to peak 1 Ca^{2+}-binding activity was applied without concentration to the ion exchange resin. The column was then washed with Buffer B (Methods section F) until no absorbance was detected at 280 nm. Subsequently, a linear gradient (200 ml total volume) of NaCl in Buffer A (0 - 0.5 M) was run at a pumped flow rate of 16 ml/h with the collected fraction volume being 1.3 ml each. Again, the relative amounts of protein were determined by reading every second fraction (absorbance at 280 nm) and Ca^{2+}-binding activity measured by the spin column method.



— ABSORBANCE (280 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
--- Ca2+-BINDING ACTIVITY (cpm/20ug prot.)
x10-3
40
12
10
8
6
4
2
0
NaCl(mM)
500
100
FRACTION NUMBER
0
20
40
60
80
100
120
140

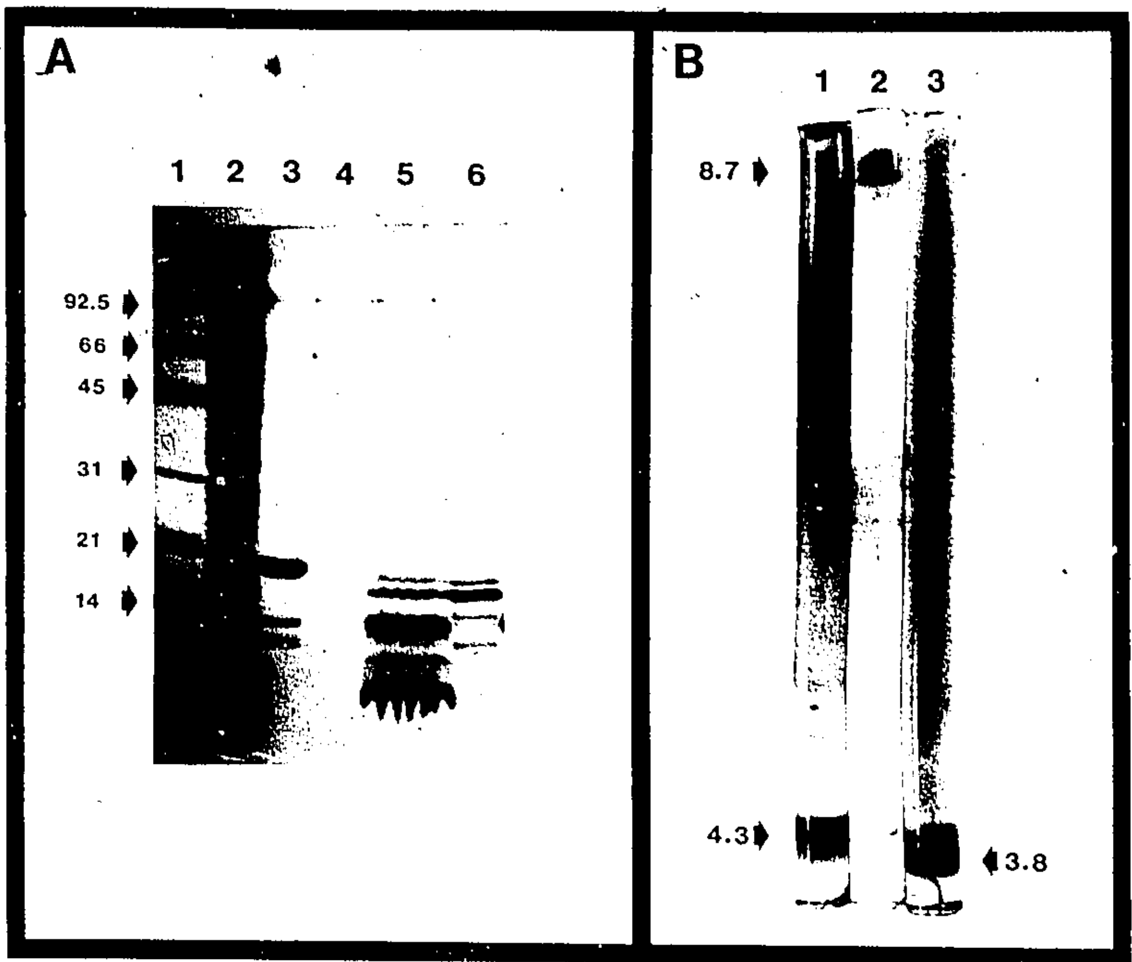
Figure 6-5

A. Electrophoretic analysis of Sephadex G-150 peak 1 Ca^{2+}-binding protein at various steps in purification.

Various protein fractions were prepared for electrophoresis and run as described in Methods section H. The acrylamide concentration was 12.5% with the acrylamide/bisacrylamide being 75:1. Lane 1, low molecular weight standards; lane 2, total BBMV extract; lane 3, purified pig Ca^{2+}-binding protein eluted from DEAE-Sephacel; lane 4, Sephadex G-150, peak 1 Ca^{2+}-binding activity; lane 5, Sephadex G-150, peak 2 Ca^{2+}-binding activity; lane 6, semi-purified peak 2 Ca^{2+}-binding activity after elution from DEAE-Sephacel. The positions of the standard protein markers are indicated by the arrows and the molecular weight of each standard is given in kilodaltons.

B. Isoelectric focussing gels of purified pig Ca^{2+}-binding protein.

The proteins were subjected to isoelectric focussing at 4°C as described in Methods section I. Three rod gels are depicted to which various proteins have been added: 1, bovine brain calmodulin; 2, rabbit muscle aldolase; 3, peak 1 (Sephadex G-150) purified. The concentration of acrylamide in the gels was 4%, bisacrylamide 0.1%. The ampholyte used was sufficient for the formation of a pH gradient from 3.5 to 9.5. The pH at which the various proteins focussed are indicated by the arrows at the side of the rod gels.


A
1
2
3
4
5
6
92.5
66
45
31
21
14
B
1
2
3
8.7
4.3
3.8

A summary, typical of the purification steps and degree of purification of this pig Ca^{2+}-binding protein is described in Table 6-1. The final purified protein represents an eight hundred fold purification with respect to the original cell homogenate, 19% of the total brush border membrane Ca^{2+}-binding activity being recovered, and less than 1% of the brush border membrane protein. A typical purification yielded 4.5 mg of this protein from over 1.5 gram of membrane protein and this yield as well as the purification protocol was found to be highly reproducible from preparation to preparation. The similarity of this pig Ca^{2+}-binding protein to bovine brain calmodulin with respect to molecular weight (20,500 for the porcine protein cf. 19,400 for bovine brain calmodulin) and isoelectric point (3.8 and 4.3, respectively) led to a more in-depth comparison of the two proteins in terms of their biological activities, behavior during a purification assay, and susceptibilities to various proteases.

The ability of the pig protein to activate the calmodulin-sensitive enzyme, phosphodiesterase was tested and the results are presented in Figure 6-6. It was clearly evident from the activation of phosphodiesterase by the pig Ca^{2+}-binding protein (half maximal activation being 10 ng for this protein cf. 8 ng for bovine brain calmodulin) and from the similarity of the activation curve of the pig protein to that of calmodulin that the biological activities

TABLE 6-1

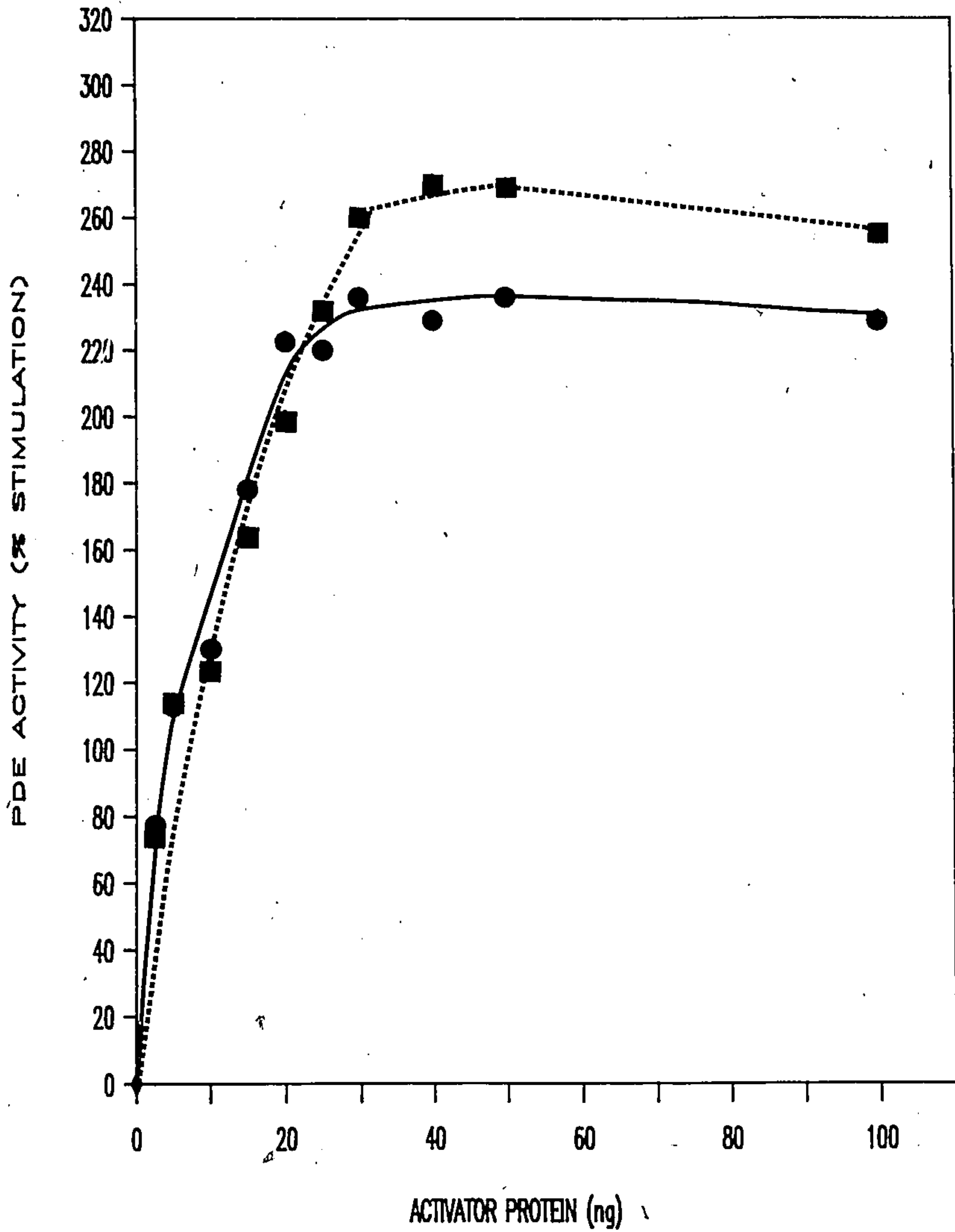
Summary of the Purification Steps for
Peak #1 (Sephadex G-150) Ca^{2+}-Binding Activity

Purification Step	Protein (mg)	% Protein Recovery	% of Total Activity	Specific Activity CPM/20µg	Purification Factor
BBMV	1610	100	100	634	12
Lubrol-PX extract	719	44.7	86.9	1,234	23
Butanol extract	444	27.6	80.5	1,850	35
Sephadex G-150	78	4.8	23.7	3,100	59
DEAE-SEPHACEL	4.5	0.3	18.6	42,100	801
	M_r	20,500	SDS - PAGE		
	P_I	3.5 - 3.8	PAGE - IEF		

Figure 6-6

The Ca^{2+}-dependent activation of PDE by calmodulin and by purified pig Ca^{2+}-binding protein (G-150, peak 1).

The enzyme, phosphodiesterase (PDE) was measured in the presence of varying amounts of bovine brain calmodulin (●) or purified pig Ca^{2+}-binding protein (■) as described in Methods section J.


PDE ACTIVITY (% STIMULATION)
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0
20
40
60
80
100
ACTIVATOR PROTEIN (ng)

of these two Ca^{2+}-binding proteins were comparable.

To further substantiate this finding an assay for calmodulin, based on the unique affinity and specificity of two resins for this protein, was used to determine the similarity of the pig Ca^{2+}-binding protein to bovine brain calmodulin. Bovine brain calmodulin was labelled with ^{14}C-formaldehyde while the porcine protein was labelled with ^{3}H-formaldehyde, providing a convenient way of distinguishing between the two proteins. The proteins were mixed and subjected to two chromatography steps, one based on the inherent hydrophobicity of the proteins using phenyl-Sepharose and the other on the protein's net charge at pH 7.5 (DEAE-Sephacel). After the column procedures, the labelled protein effluent from the two-step column procedure was run on 12.5% SDS-PAGE and the gel was sliced and counted for the presence of the ^{14}C-labelled calmodulin and the ^{3}H-labelled pig Ca^{2+}-binding protein. The results shown in Table 6-2 demonstrated co-migration of both proteins through all three steps, again indicating the similarity of the two proteins. Additionally, when these labelled proteins were run in SDS-PAGE they were found to migrate identically (Figure 6-7).

An additional proof was secured to further verify the identity of the pig intestinal protein. This was based on the sensitivity of both Ca^{2+}-binding proteins to protease activity. Two representative drawings of the autoradiograms

TABLE 6-2

Two-Step Column Assay for Calmodulin

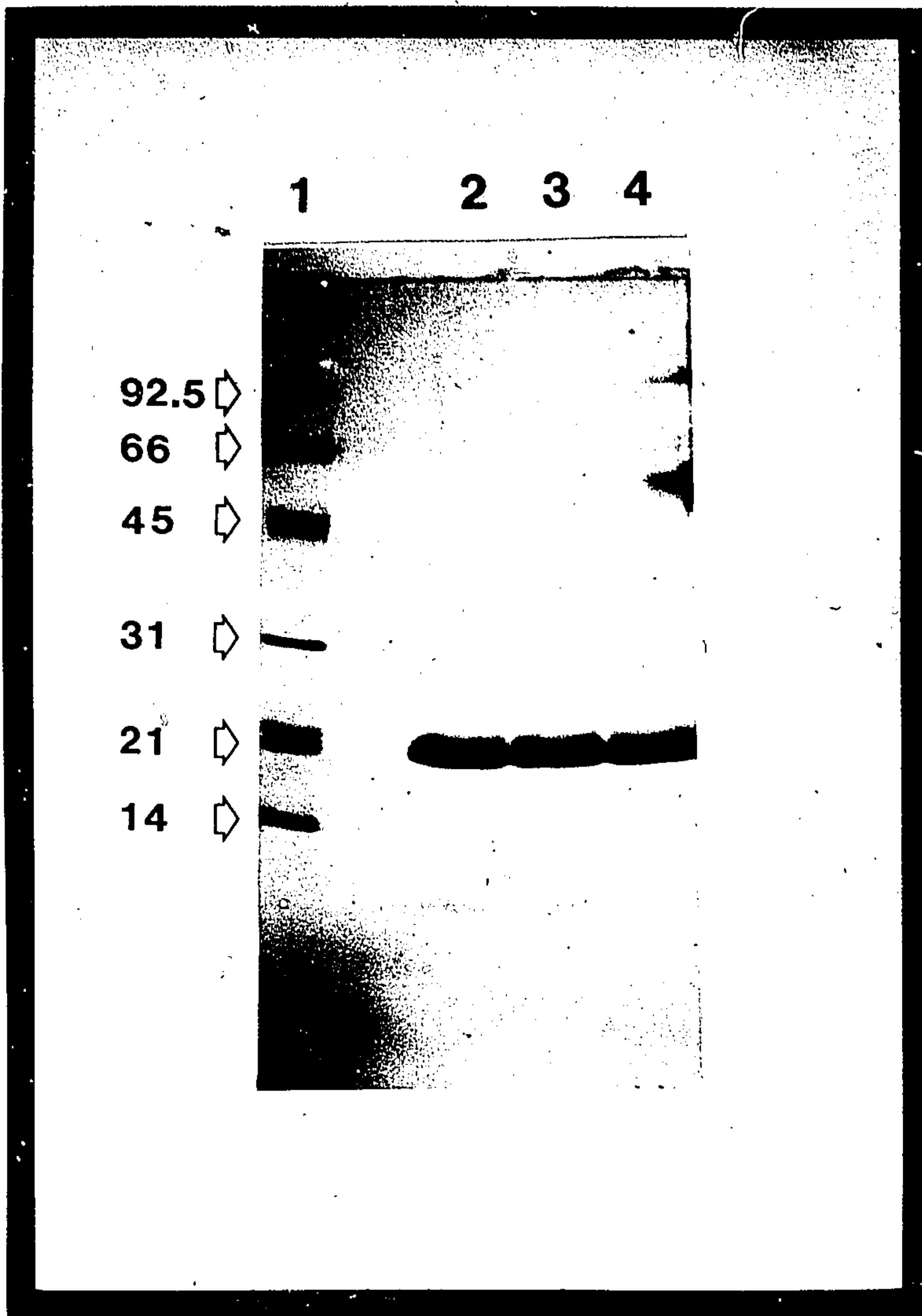
Sample	$^3H/^{14}C$ CPM			
	Before Chromatography {25/1500 µl}	After phenyl-Sepharose {50/2000 µl}	After DEAE {250/2000 µl}	After SDS-PAGE {25/200 µl}
BBMV	(12357/368) 33.6	(291/154) 1.9	(135/144) 0.9	-
BBMV extract	(12514/406) 30.8	(431/148) 2.9	(204/132) 1.5	-
Purified pig Ca^{2+}-binding protein	(7951/303) 26.2	(4782/266) 18.0	(6690/313) 21.4	(2736/135) 20.3

The experimental protocol is described in the Methods section O. The numbers in the brackets represent the volume of protein solution counted/the total volume of the solution. Those values in parenthesis denote the actual $^3H/^{14}C$ cpm of the samples whereas the numbers directly below those in parenthesis indicate the actual $^3H/^{14}C$ ratio. The values in the last column represent the ratios for cpm obtained after subjecting the purified pig Ca^{2+}-binding protein to SDS-PAGE.

Figure 6-7

SDS-PAGE of calmodulin and pig Ca^{2+}-binding protein after the calmodulin two-step column assay procedure

Following the two-step column assay procedure for calmodulin, the Ca^{2+}-binding proteins were subjected to SDS-PAGE in 12.5% acrylamide. The gel was stained as previously outlined (cf. Methods section H) and the following samples were applied to the gel: lane 1, low molecular weight standard proteins; lane 2, unlabelled pig Ca^{2+}-binding protein (30 µg); lane 3, ^{3}H-labelled pig Ca^{2+}-binding protein (30 µg); lane 4, ^{14}C-labelled bovine brain calmodulin (30 µg). The molecular weight markers are indicated by arrows and their values are given in kilodaltons.


1
2
3
4
92.5
66
45
31
21
14

of the peptide fingerprint maps of bovine brain calmodulin are shown in Figure 6-8. The areas on the cellulose plates corresponding to the autoradiographic spots were scraped and counted for the presence of both ^{3}H and ^{14}C and the results are listed in Table 6-3. This table shows the various $^{3}H/^{14}C$ ratios for seven peptides generated by treatment of ^{14}C-labelled bovine brain calmodulin and ^{3}H-labelled pig Ca^{2+}-binding protein with elastase and for eight peptides generated from chymotrypsin treatment of the two proteins. These two Ca^{2+}-binding proteins were mixed such that the ratio of ^{3}H to ^{14}C was 4:1 in the original mixture. The results point to the co-migration of fragments generated of the two proteins by both proteases. This data, along with the previous data strongly suggested the similarity of the pig protein with calmodulin.

The presence of calmodulin in the intestinal cell brush border membrane was an interesting finding. However, further experiments were necessary to establish the nature of the association of this ubiquitous, water-soluble protein with the membrane. The protein pattern of the gel shown in Figure 6-9A indicated that calmodulin could be extracted from the membrane by treatment with various detergents but not by increasing concentrations of KCl (high ionic strength, Figure 6-9B). This finding ruled out the possibility of calmodulin being adsorbed to the bilayer or to surface proteins (as an artifact of the membrane

Figure 6-8

Peptide fingerprint maps of bovine brain calmodulin and pig Ca^{2+}-binding protein.

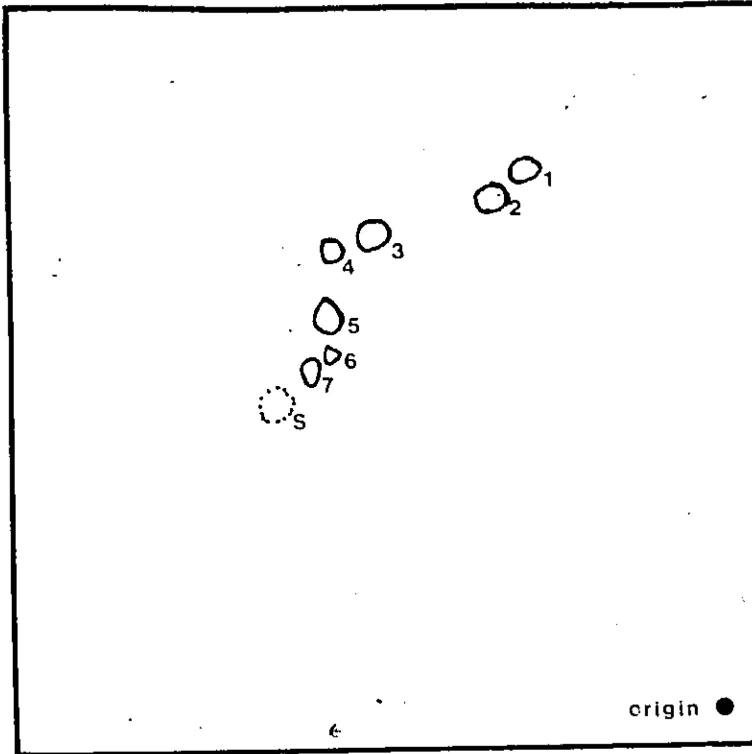
A. Tracing of an autoradiogram of an elastase digest of ^{14}C-calmodulin and ^{3}H-pig Ca^{2+}-binding protein.

The conditions were those specified in Methods section L.

B. Tracing of an autoradiogram of chymotrypsin digest of ^{14}C-calmodulin and ^{3}H-pig Ca^{2+}-binding protein.

The conditions were identical with those specified in A.

Representative schetches of the two dimensional chromatograms of the two protease digests of the two Ca^{2+}-binding proteins are shown. The spots on the plates corresponding to those on the X-ray film were scraped into scintillation vials and the $^{3}H/^{14}C$ ratio was determined and the results are presented in Table 6-2. The s refers to the location of the standard, dinitrophenol-lysine.


A. ELASTASE
SECOND
1
2
3
4
5
6
7
S
origin
FIRST

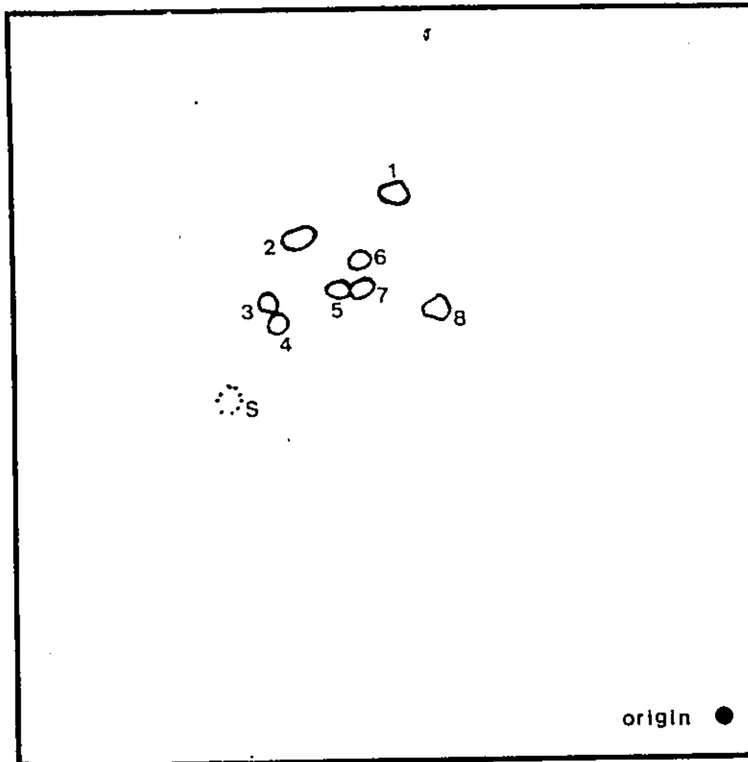

B. CHYMOTRYPSIN
SECOND
1
2
6
7
5
3
4
8
S
origin
FIRST

TABLE 6-3

Protease Digests of Bovine Brain Calmodulin and Pig Intestinal Ca^{2+}-Binding Protein (Peak 1, G-150)

Spot	$^{3}H/^{14}C$ Ratio
A. Elastase Digest	
1	6.2
2	5.6
3	6.7
4	5.6
5	4.9
6	4.7
7	5.6
B. Chymotrypsin Digest	
1	4.6
2	4.0
3	6.7
4	6.2
5	4.3
6	4.1
7	3.1
8	3.9

The labelled proteins, ^{14}C-calmodulin (bovine brain, 5000 CPM) and ^{3}H-porcine intestinal Ca^{2+}-binding protein (Sephadex G-150 peak 1 activity, 22,000 CPM) were incubated with 20 µg unlabelled calmodulin, 10 µl of 1mg/ml elastase (A) or chymotrypsin (B), in 20 mM ammonium bicarbonate, pH 7.0. The mixtures were incubated for 3 h at 37°C after which time, the digests were spotted and subjected to paper electrophoresis (in the first dimension) followed by paper chromatography (in the second dimension) as described in the Methods section L. Following autoradiography to locate the ^{14}C-peptide spots the corresponding areas on the cellulose plats were scraped and counted using a double-label program for ^{3}H and ^{14}C.

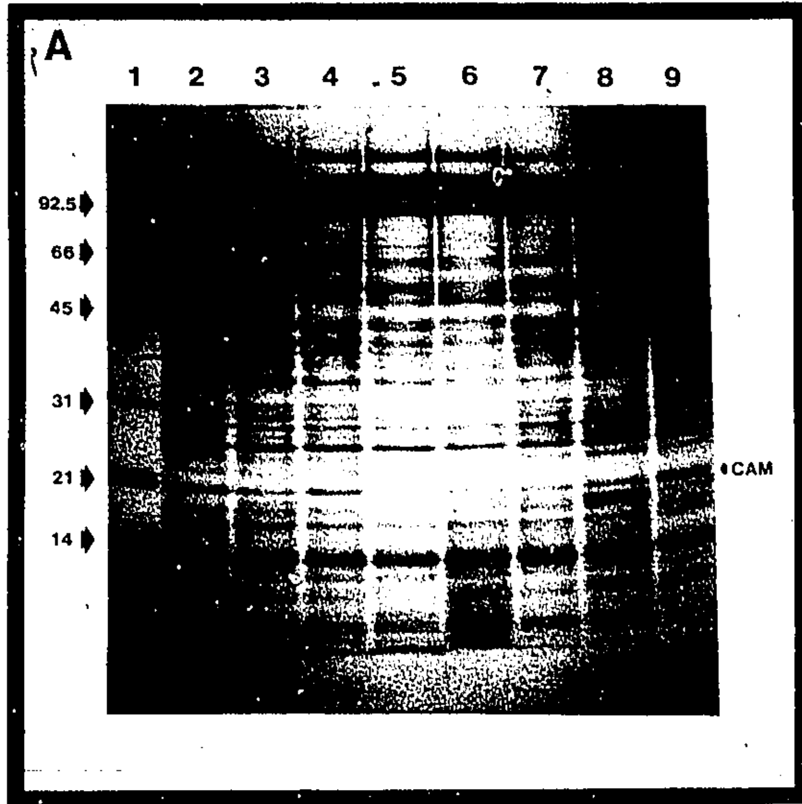
Figure 6-9

A. Extraction of BBMV with detergents.

Several different detergents (1.0%) were added to porcine BBMV (6 mg protein/ml) in 100 mM KCl, 20 mM Hepes/KOH, pH 7.5, and 30 μM PMSF and the mixtures were incubated for 2 h in ice. Following the incubation the samples were centrifuged at 105,000 x g for 60 min and the supernatant was withdrawn and samples were subjected to electrophoresis in 12.5% SDS-PAGE gels as described in Methods section H. The following samples were applied to the gel: lane 1, low molecular weight standards; lane 2, octylglucoside extract; lane 3, Lubrol-PX extract; lane 4, Triton X-100 extract; lane 5, Triton X-114 extract; lane 6, sodium deoxycholate extract; lane 7, sodium cholate extract; lane 8, sodium glycocholate extract; and lane 9, total BBMV protein. Prior to electrophoresis the samples were desalted to remove KCl which would interfere with the migration of the ion front. The molecular weight markers are indicated by the arrows and the molecular weight of each standard is given in kilodaltons.

B. Porcine BBMV extracted with increasing ionic strength.

Pig BBMV (6 mg protein/ml) were incubated with various concentrations of KCl for 2 h in the same buffer as in A, however, in the absence of any detergent. Electrophoresis conditions were identical to those in A. The following samples were applied to the gel: lane 1, low molecular weight standard proteins; lane 2, total BBMV protein; lane 3, no KCl extract; lane 4, 200 mM KCl extract; lane 5, 400 mM KCl extract; lane 6, 800 mM KCl extract; lane 7, 1 M KCl extract; lane 8, 2 M KCl extract; lane 9, high molecular weight standard proteins; and lane 10, total BBMV protein. Molecular weight markers are indicated as in A.


A
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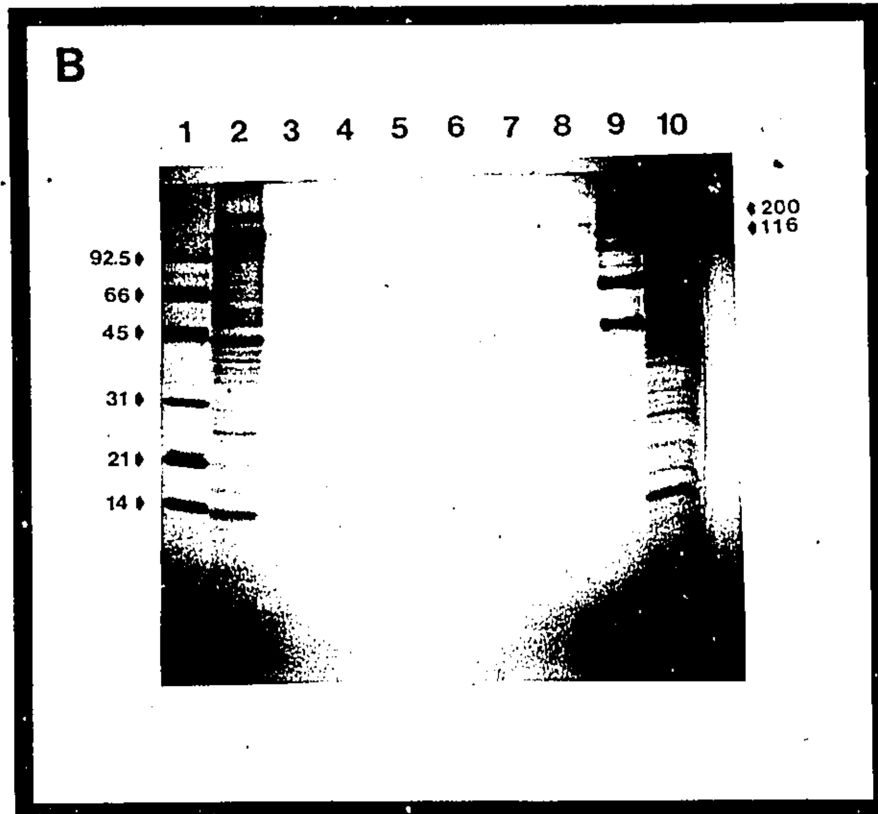
92.5
66
45
31
21
14
CAM


B
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10
200
116
92.5
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14

preparation procedure). Instead, it appeared as though calmodulin was either hydrophobically-associated with the membrane bilayer itself (perhaps in a Ca^{2+}-dependent manner) or very tightly so with an integral membrane protein, which could be functioning as a receptor for calmodulin.

This possibility was tested by employing the phase-separation assay of Bordier (1981), which makes use of the unique property of Triton X-114 to be fully miscible with water at temperatures below 10°C but to dehydrate and subsequently aggregate at higher temperatures resulting in a phase separation of detergent from the aqueous media. Brodier demonstrated that several integral membrane proteins partitioned in the detergent phase (i.e., acetylcholinesterase from the human erythrocyte membrane, bacteriorhodopsin, and cytochrome c oxidase from yeast) and that water-soluble proteins such as serum albumin, catalase, ovalbumin, concanavalin A and myoglobin were located exclusively in the upper aqueous phase in this assay.

Calmodulin, alone did not associate with the detergent phase (background counts 150 cpm, total counts 132,000), but in the presence of solubilized BBMV it was found in the detergent phase (10-11% Triton X-114 as determined by absorbance at 277 nm). This partitioning of the Ca^{2+}-binding protein appeared to be Ca^{2+}-independent (i.e., it occurred in the presence and absence of Ca^{2+}) and happened

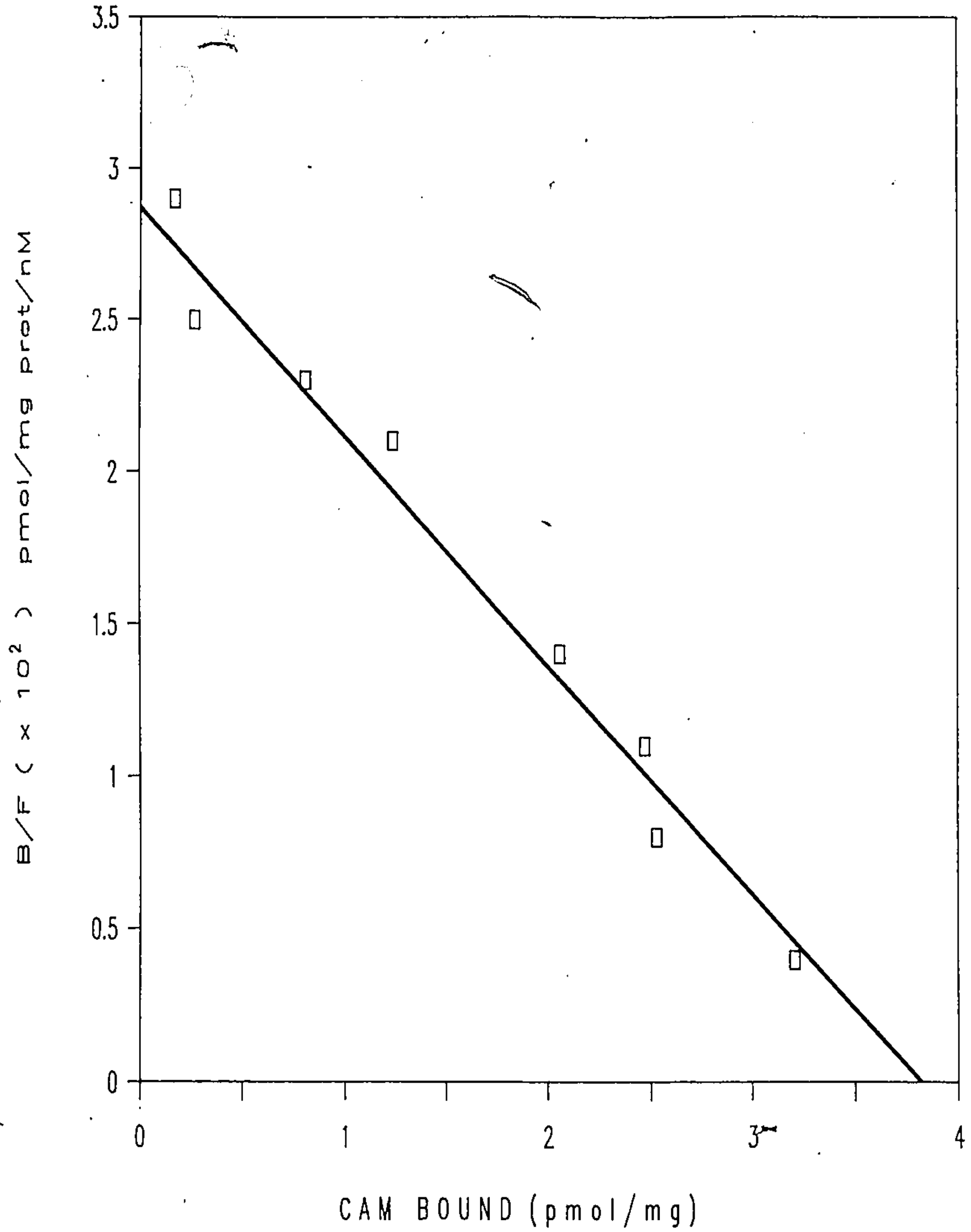
only in the presence of solubilized BBMV; BSA could not mimic the membrane protein extract in this respect.

This binding was found to be specific as defined by the ability of unlabelled calmodulin (several hundred fold excess) to displace the labelled calmodulin from its receptor. A Scatchard curve for the binding of ^{125}I-calmodulin to porcine BBMV is depicted in Figure 6-10. This binding data was analyzed using a computer program as described in Methods (section H.1.) and the apparent K_d was calculated to be 0.14 μM, as compared with the value of 0.13 μM calculated conventionally by treating the infinite dose binding as an exact measure of non-specific binding as described by Klotz (1985). This binding data indicates the presence of a calmodulin-specific receptor in the brush border membrane capable of binding this protein with high affinity which is present at a concentration of 4 pmol per mg protein in the brush border membrane, i.e., the B_{max} value (the x intercept value, Figure 6-10) for ^{125}I-calmodulin binding to BBMV. The identity of this calmodulin-receptor protein of porcine BBMV was sought by using the well-characterized ^{125}I-calmodulin gel overlay procedure as described by Slaughter and Means (1987). Figures 6-11 and 6-12 show the protein pattern of three different amounts of BBMV run on SDS-PAGE followed by the corresponding autoradiograms of two gels after incubation with ^{125}I-calmodulin in the presence of Ca^{2+} (Figure 6-11B)

Figure 6-10

^{125}I-calmodulin binding to Triton X-114 solubilized BBMV.

The amount of calmodulin bound to BBMV protein was determined by the Phase Separation Assay of Brodier (cf. Methods section M). The resulting binding data were corrected for non-specific binding as outlined by Klotz (1985) and the data was best fit by means of a computer program, Ligand (P.J.Munson, NIH), run on an IBM personal computer. The binding data revealed the presence in the solubilized BBMV of one set of high affinity sites with a calculated K_d of 0.14 µM and a concentration of binding sites of approx. 4 pmol/mg BBMV protein.


3.5
3
2.5
2
1.5
1
0.5
0
0
1
2
3
4
B/F (x 10²) pmol/mg prot/nM
CAM BOUND (pmol/mg)

and in its absence (Figure 6-12B). Figure 6-11B reveals the binding of ^{125}I-calmodulin in the presence of Ca^{2+} and Figure 6-12B shows this binding in the absence of Ca^{2+} (i.e., in the presence of EGTA). The binding of calmodulin to the proteins of the brush border occurred in the presence and absence of Ca^{2+}. Careful inspection of the autoradiograms revealed that in both cases ^{125}I-calmodulin bound to a protein with a M_r of 107,000 (calculated from a plot of log MW versus relative mobility for the standard proteins, not shown). This binding appeared to be proportional to the amount of BBMV protein loaded on the gel, for the intensity of the autoradiographic band increased with the amount of protein applied, i.e., compare the band for 10 µg, 20 µg and 50 µg BBMV protein in both Figure 6-11B and Figure 6-12B. However, this comparison did reveal that the binding of calmodulin was greater in the presence of Ca^{2+} than in its absence even though an absolute Ca^{2+} dependency was not found. Conveniently, one of the SDS-standard proteins, phosphorylase b binds calmodulin (Slaughter and Means, 1987) and served as an excellent control for the entire overlay procedure. This protein, M_r 92,500, bound ^{125}I-calmodulin in both the presence and absence of Ca^{2+} (Fig 6-11B and 6-12B).

It was also of interest to try to determine the identity of the other Ca^{2+}-binding activity present in the detergent extract of BBMV seen when passed through Sephadex

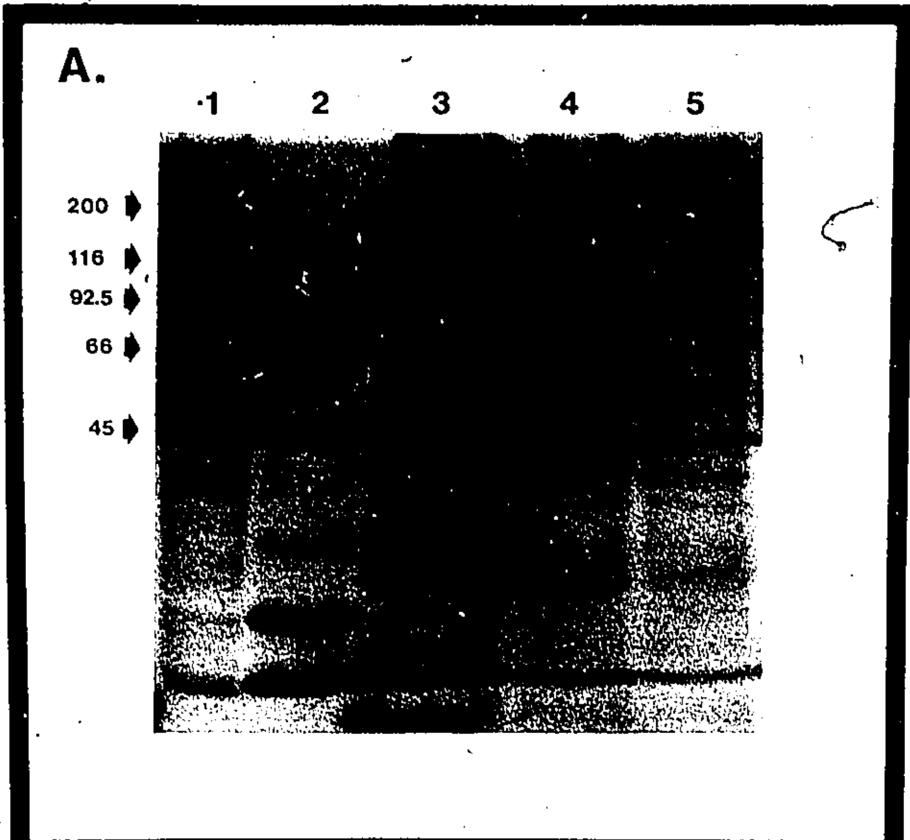
Figure 6-11

A. ^{125}I-calmodulin gel overlay incubated in the presence of 1 mM Ca^{2+}.

The experiment was performed as described in Methods section N. The proteins were stained with Coomassie blue R-250 after running the samples on 10% acrylamide. The following samples were applied to the gel: lane 1, high molecular weight standards; lane 2, low molecular weight standards; lane 3, 50 µg BBMV protein; lane 4, 20 µg BBMV protein; lane 5, 10 µg BBMV protein. The molecular weight markers are indicated by the arrows and the accompanying numbers represent the molecular weight of each in kilodaltons.

B. Autoradiogram of gel A (above).

The stained gel upon drying was exposed to X-ray film for 3 days at -70°C as outlined in Methods section N. The molecular weight standard protein, phosphorylase b and myosin demonstrated specific binding to calmodulin. The molecular weight markers are indicated along the side of the autoradiogram and are expressed in kilodaltons.


A.
1
2
3
4
5
200
116
92.5
66
45

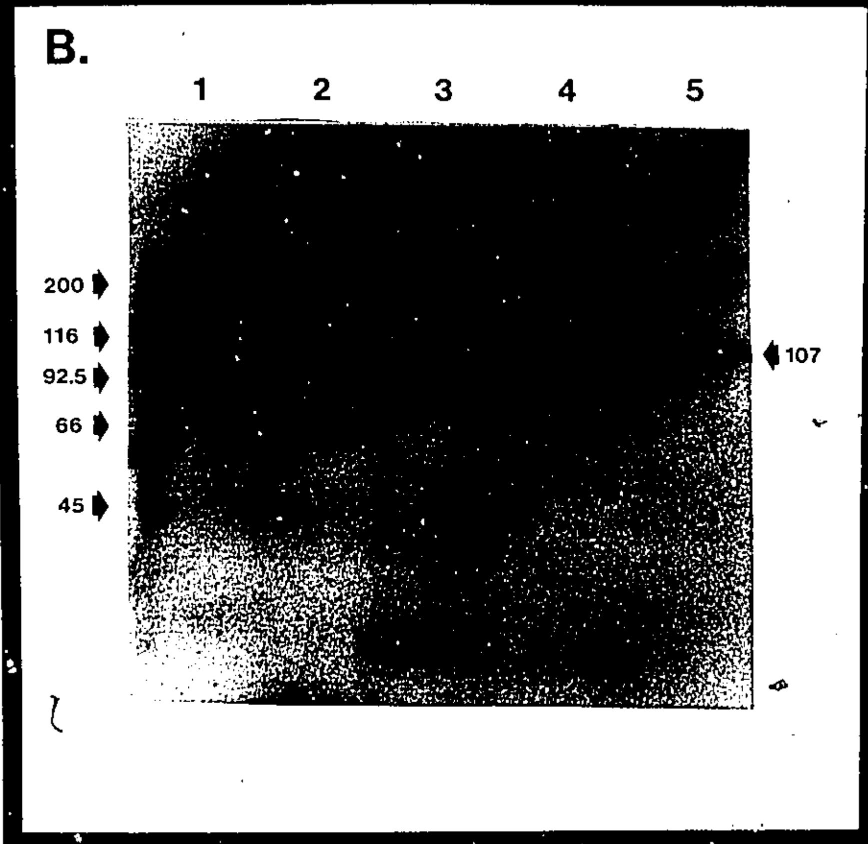

B.
1
2
3
4
5
200
116
92.5
66
45
107

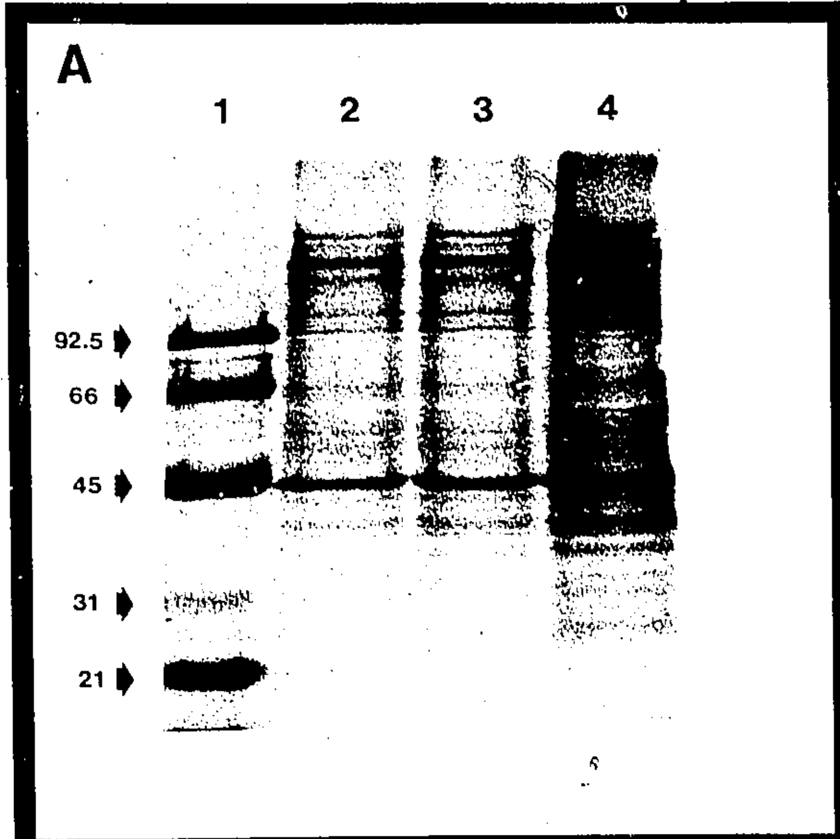
Figure 6-12

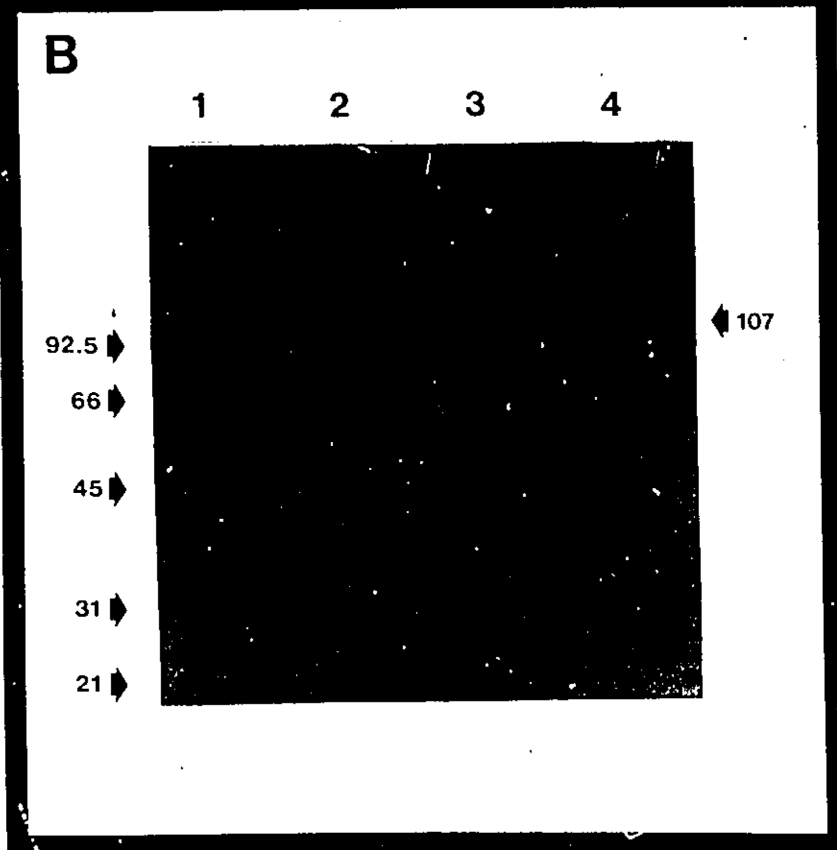
A. ^{125}I-calmodulin gel overlay incubated in the presence of 1 mM EGTA.

This gel was treated identically to the gel in A (above), except that during the incubation of labelled calmodulin with the gel 1 mM EGTA was substituted for 1 mM Ca^{2+}. The gel was stained for the presence of protein with Coomassie blue as described in method section H. The samples run were: lane 1, low molecular weight standards; lane 2, 10 µg BBMV protein; lane 3, 20 µg BBMV protein; 50 µg BBMV protein.

B. Autoradiogram of gel A (above).

The dryed gel after staining for protein was exposed to X-ray film under identical conditions as in Figure 6-11B. The molecular weight marker, phosphorylase b, demonstrated calmodulin-specific binding. The molecular weight markers are indicated along the side of the autoradiogram.


A
1
2
3
4
92.5
66
45
31
21


B
1
2
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107
92.5
66
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31
21

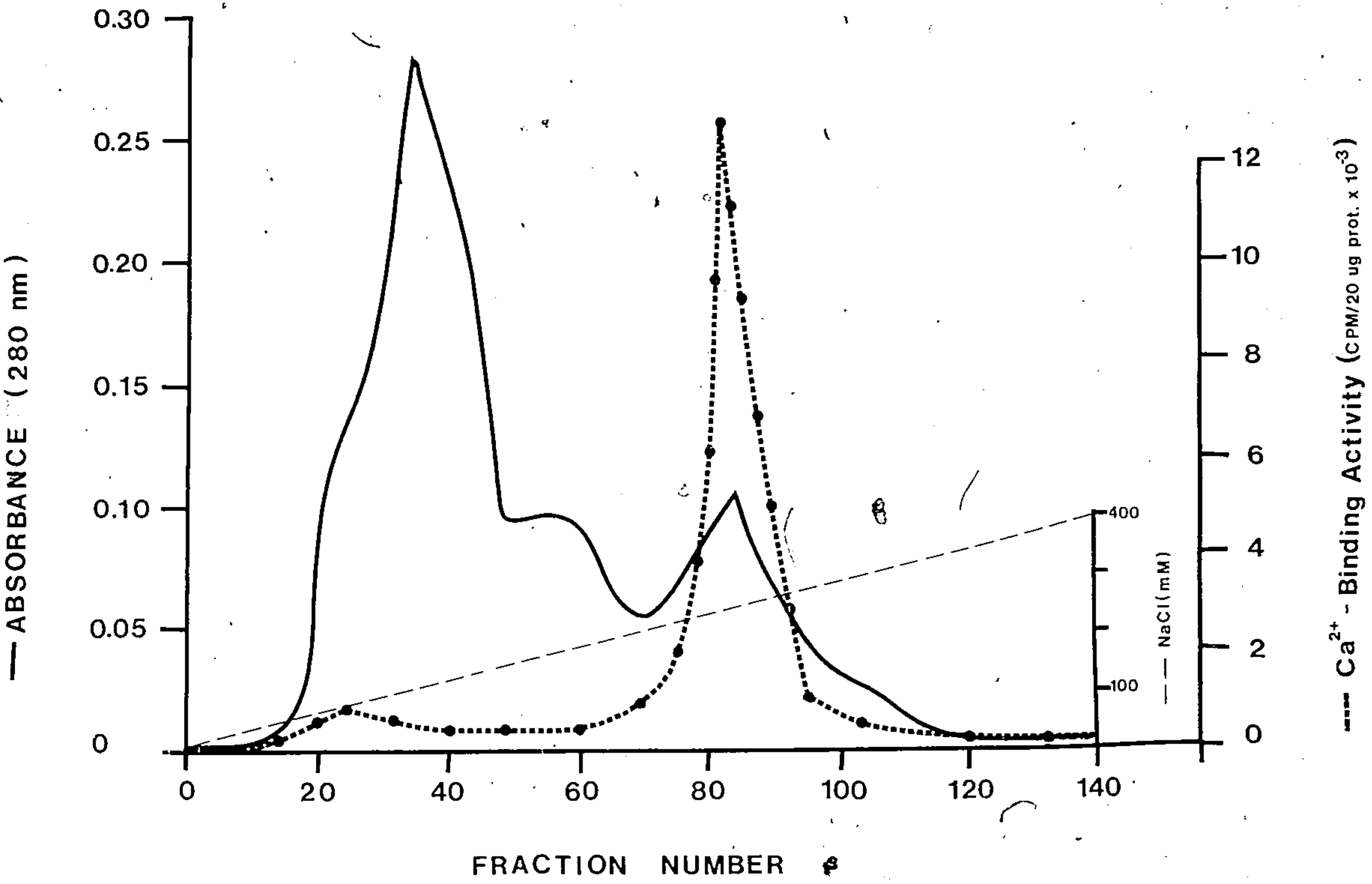
G-150 (peak 2, Figure 6-3). Figure 6-13 is an elution profile of peak 2 activity when applied to DEAE-Sephacel at pH 7.5 and eluted with a linear gradient of NaCl, (0 - 0.4 M). This step resulted in a measure of purification of the activity (225 fold compared with the cell homogenate), the fraction possessing the activity was a composite of five visible bands all having isoelectric points in the 5-7 pH range (Figure 6-14). For this reason, the active fraction from DEAE-Sephacel was taken and applied to a chromatofocussing column (pH 7.1-5.0) attached to the Pharmacia FPLC system for protein separation. The elution profile of this fraction eluted from the chromatofocussing column is presented in Figure 6-15. The individual peptides were resolved exceptionally well, as revealed by SDS-PAGE of the various fractions (Figure 6-16), but the Ca^{2+}-binding activity was lost. This problem could not be overcome despite the addition of protease inhibitors and of accelerating the processing time from the original homogenization of the cells to this final step in the purification procedure.

Figure 6-17 is a flow chart of the partial purification of the two Ca^{2+}-binding activities that were detected in detergent extracts of porcine BBMV. It should be noted that there was the presence also, of a minor binding activity in the void volume of the Sephadex G-150 effluent, which was not further examined.

Figure 6-13

Ion-exchange chromatography of peak 2 (G-150) Ca^{2+}-binding activity.

The chromatography conditions were identical to those in Figure 6-4, except a linear gradient of 0.04 M was run instead.



— ABSORBANCE (280 nm)
0.30
0.25
0.20
0.15
0.10
0.05
0
FRACTION NUMBER
0
20
40
60
80
100
120
140
— — NaCl(mM)
400
100
---- Ca2+ - Binding Activity (CPM/20 ug prot. x 10-3)
12
10
8
6
4
2
0

Figure 6-14

Isoelectric focussing of peak 2 (G-150) activity after further purfication on DEAE-Sephacel.

Isoelectric focussing was carried out as described in Methods section I. The following samples were applied to separate rod gels: 1, bovine brain calmodulin; 2, peak 2 (G-150) activity after elution from DEAE-Sephacel; 3, BBMV protein extract; 4, rabbit muscle aldolase.

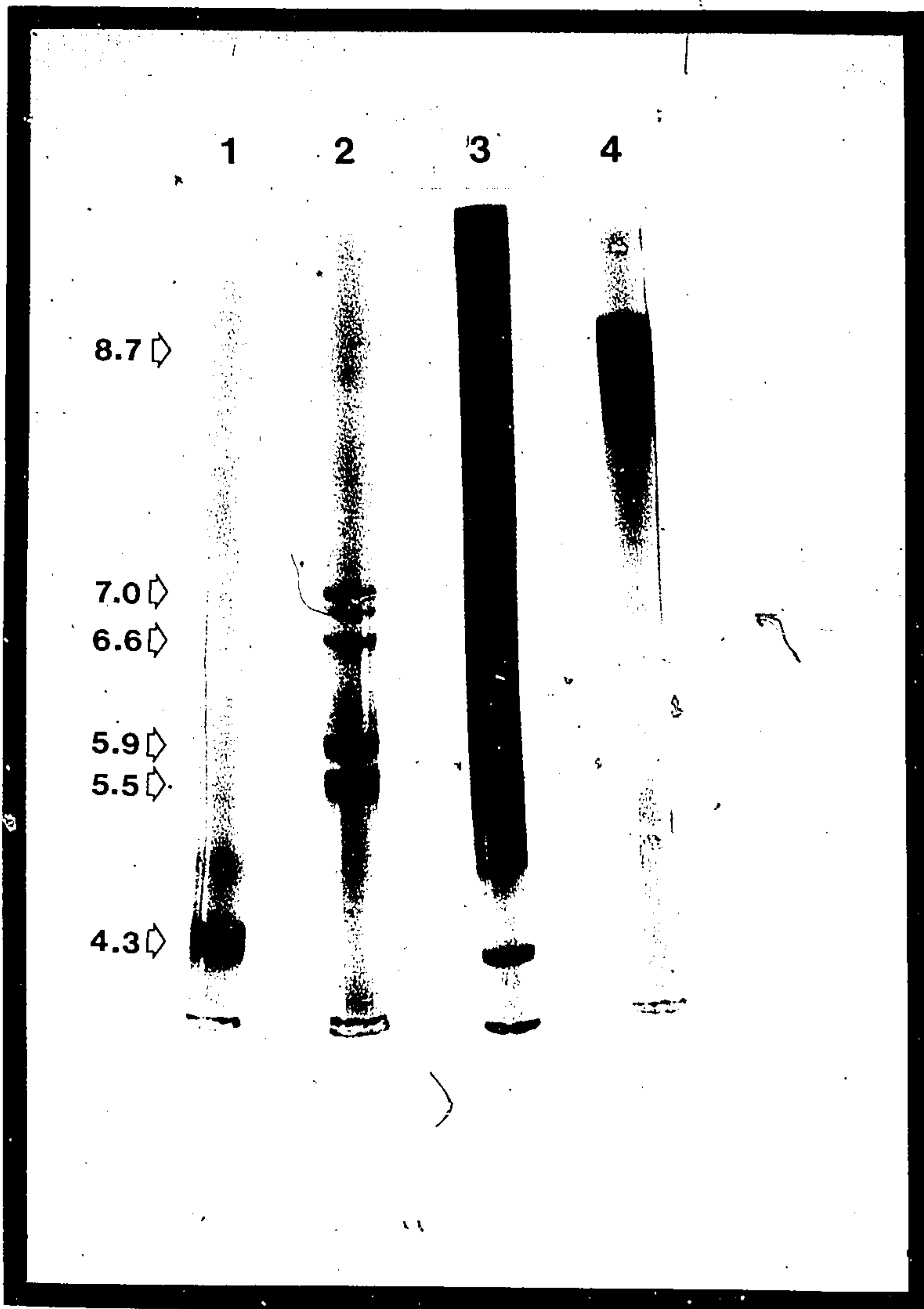
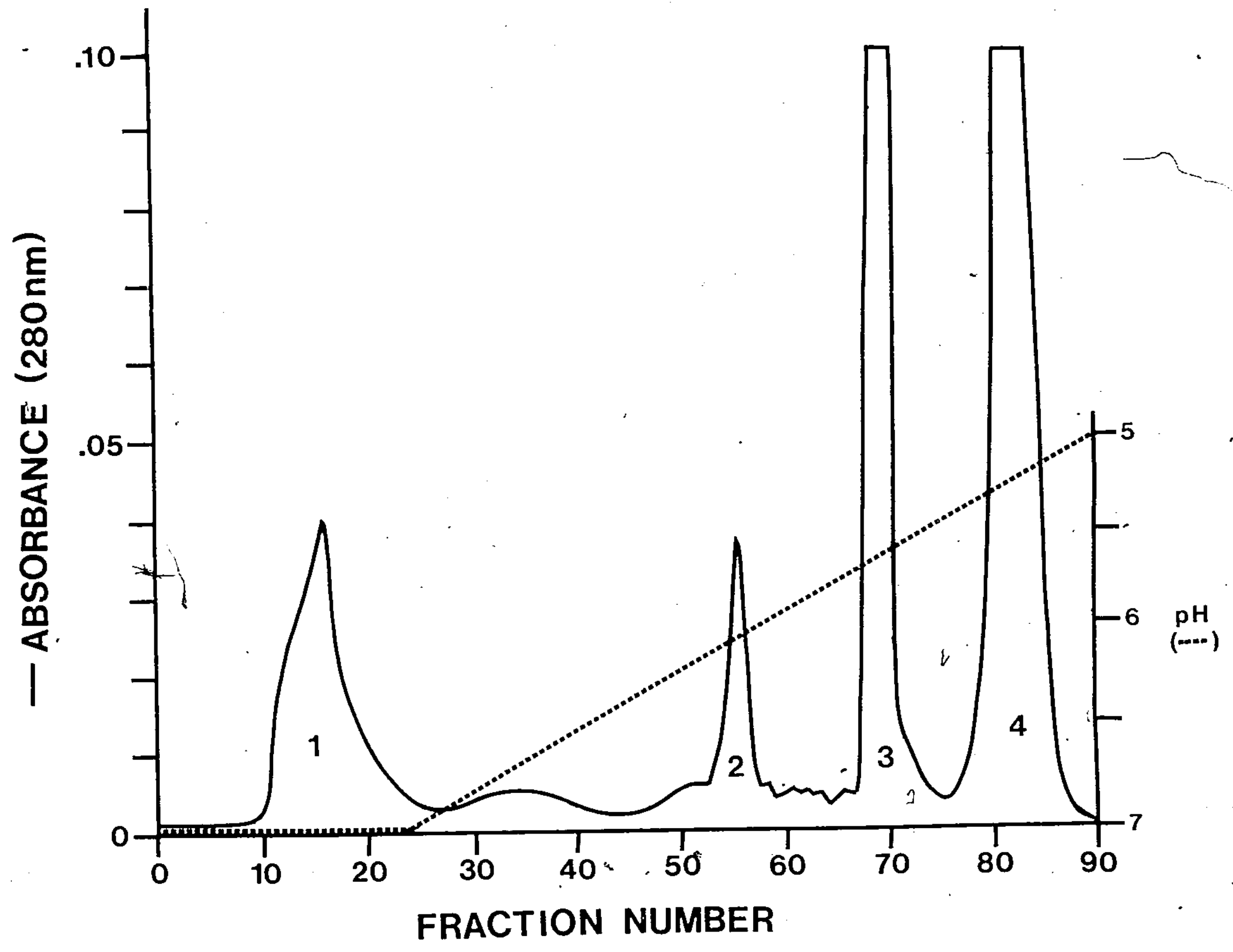

1
2
3
4
8.7
7.0
6.6
5.9
5.5
4.3

Figure 6-15

Chromatofocussing of DEAE-Sephacel Ca^{2+}-binding activity (peak 2, G-150).

Chromatofocussing of the Ca^{2+}-binding activity eluted from DEAE-Sephacel (G-150 peak 2 activity) was performed as described in Methods section G. To the column was applied 2.0 ml of 3 mg/ml protein solution in 0.1% Lubrol-PX, 25 mM bis-Tris/HCl buffer, pH 7.1 and the column was developed with Polybuffer-74, titrated to pH 5.0 with HCl. Ninety, 0.3 ml fractions were collected with the ultraviolet absorbance being read and recorded during the chromatography. The pH gradient was read by measuring directly the pH of every third fraction with a pH meter. Immediately following the determination of the pH gradient, 50 µl of 100 mM Tris buffer, pH 7.5 was added to each tube to avoid possible protein precipitation problems.

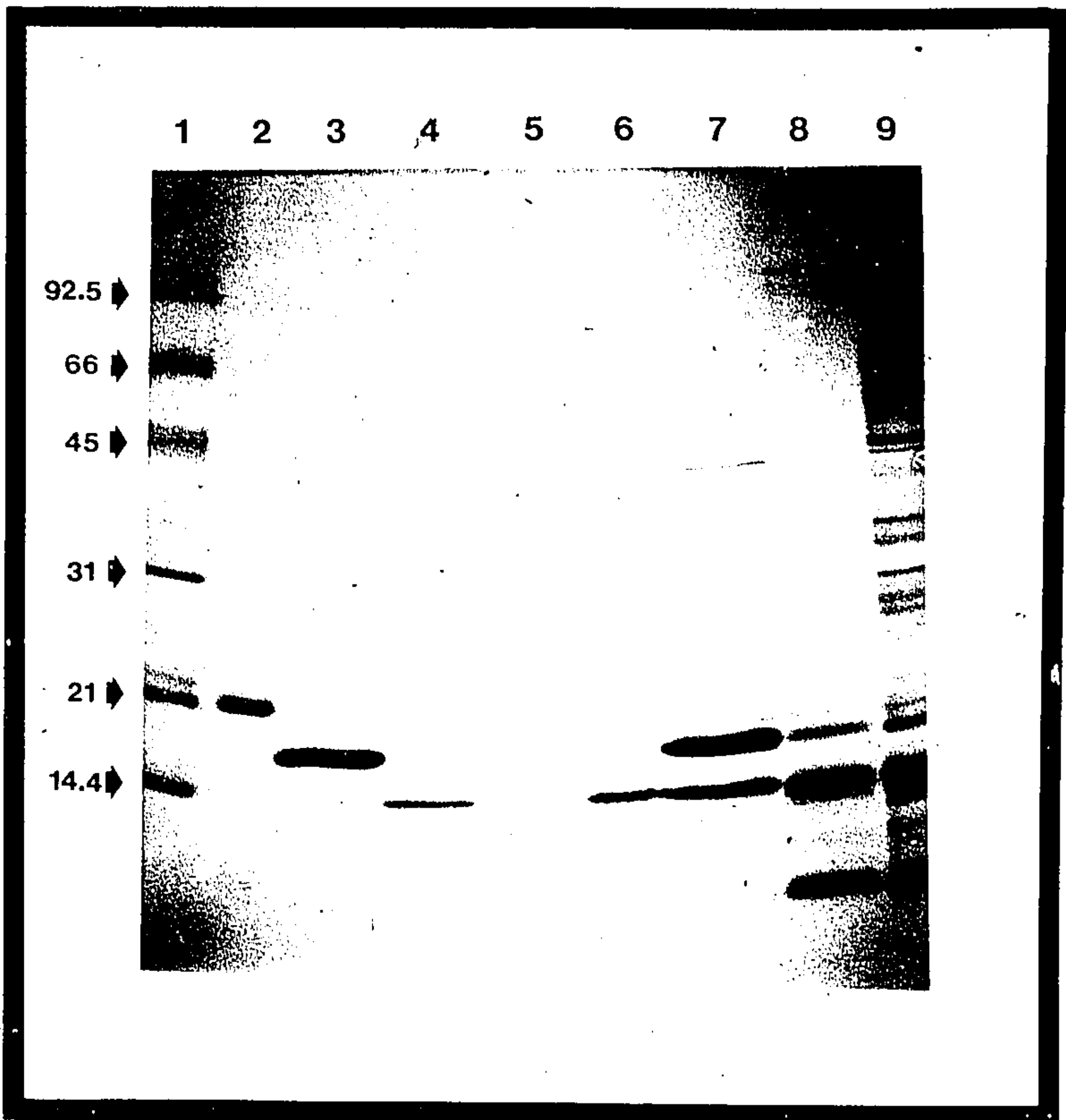


— ABSORBANCE (280nm)
.10
.05
0
pH
(----)
5
6
7
1
2
3
4
0
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

Figure 6-16

Electrophoretic protein pattern of various fractions eluted from the FPLC chromatofocussing column (Figure 6-15).

Electrophoresis conditions were as described in Figure 6-5 A. The samples applied to the gel were the following: lane 1, low molecular weight standards; lane 2, bovine brain calmodulin; lane 3, peak 4, FPLC; lane 4, peak 3, FPLC; lane 5, peak 2, FPLC; lane 6, peak 1 (FPLC); lane 7, Ca^{2+}-binding activity from DEAE-Sephacel of peak 2 Ca^{2+}-binding activity (G-150); lane 8, Ca^{2+}-binding activity, peak 2 (G-150); and lane 9, BBMV protein extract. The molecular weight markers are indicated by arrows and the corresponding number represent molecular weights in kilodaltons.


1
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9
92.5
66
45
31
21
14.4

Figure 6-17

Flow chart summary of the purification steps for the two major Ca^{2+}-binding activities in porcine BBMV.

The time required for the completion of the outlined procedure was routinely 8-9 days. All procedures were conducted either in ice or in a cold-room at 4°C.

PURIFICATION SCHEME FOR PORCINE INTESTINAL MEMBRANE CALCIUM-BINDING PROTEINS

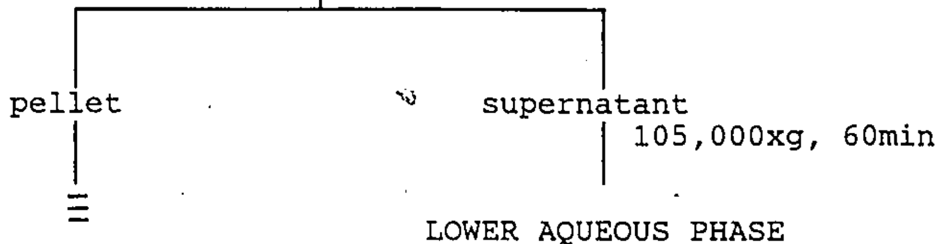
BBMV

homogenize (6mg/ml) | in 1% Lubrol-PX 40mM Tris, 100mM mannitol pH 7.4

STIR 4°C, 18 h

| 30% 1-butanol, stir vigor. 30 min at 4°C

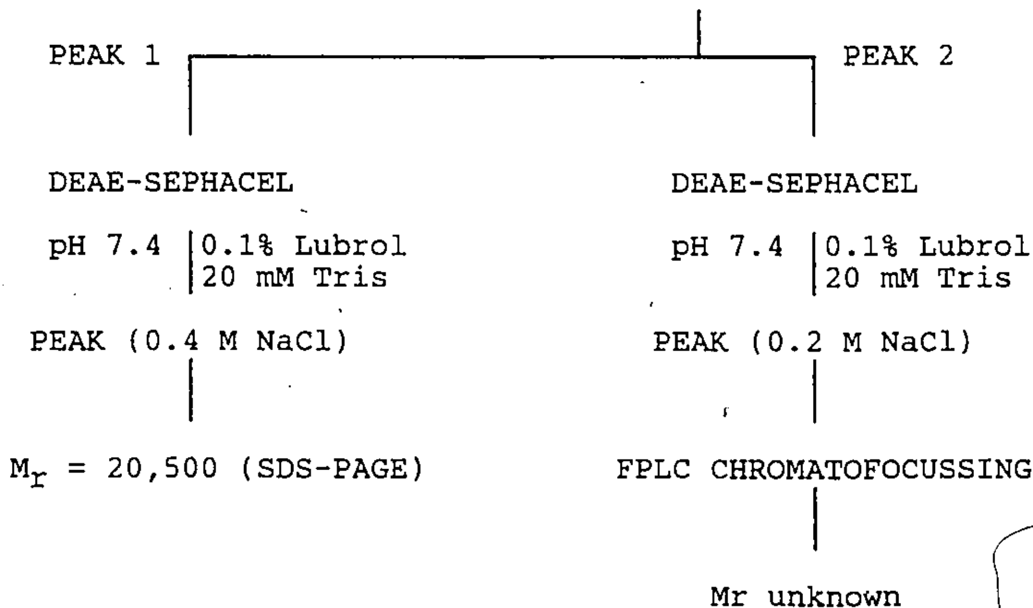
| centrifuge 10,000xg, 30min



| conc. ultrafilt. dialyze 12h

| 105,000xg, 60min

SEPHADEX G-150



V. DISCUSSION

The present studies demonstrated that there were two major Ca^{2+}-binding activities in purified porcine BBMV that could be extracted with non-ionic detergents. There was also a third minor activity detected that was associated with the void volume fractions of the Sephadex G-150 column and hence this activity must exist in the form of larger (greater than 300,000 MW) protein complexes or aggregates. The two major activities eluted later than the third, but nonetheless eluted earlier than would be expected on the basis of the molecular weight of the active components characterized (Figure 6-3). This suggested the possibility that these Ca^{2+}-binding proteins exist as part of a high molecular weight protein complex in the brush border membrane. It must be noted that although only three activities were detected with respect to Ca^{2+}-binding activity the presence of other components of the Ca^{2+}-uptake system could be precluded. This was because the presence of Ca^{2+} channel proteins that were associated with Ca^{2+} binding per se was not examined.

The idea that these Ca^{2+}-binding proteins may be part of a protein complex in the brush border membrane received further support from a preliminary finding (results not shown), which involved freezing and thawing of the brush border membrane extract. In this approach, the butanol

extract was run through Sephadex G-150 as described in Methods section E. The fractions that eluted in the void volume of the column were then subjected to quick-freezing (in a methanol-dry ice bath) and thawed in 10°C water. This freeze and thaw procedure was completed a total of three times in the presence and absence of three protease inhibitors, PMSF, aprotinin, and leupeptin. The treated extract was then re-chromatographed on Sephadex G-150 and it was found that more of both peak 1 and peak 2 activities were generated by this freeze and thaw procedure. No difference was seen between the extracts treated in this manner in either the presence or the absence of the protease inhibitors. This finding indicated that these Ca^{2+}-binding proteins were associated with other proteins in a complex. However, whether or not such complexes exist in the intact brush border membrane requires further experiments.

Calcium-binding activities of the BBMV could best be extracted by the non-ionic detergents Triton X-100 and Lubrol-PX at an optimum concentration of 1.0% and at a BBMV protein concentration of 6 mg per ml. Several other detergents were tried but none gave the results obtained with these two detergents. Delipidation of the detergent-solubilized extract with the mild organic solvent, 1-butanol, led to a slight increase in the specific activity of the extract (Table 6-1) and also to a stabilization of

the extract in the solubilized state (as revealed by centrifugation at 105,000 x g for 60 min).

The identification of G-150 peak 1 activity as calmodulin or calmodulin-like was unexpected, but concluded on the basis of the similarity of this protein and bovine brain calmodulin with respect to molecular weight, isoelectric point, purification properties, activation of the calmodulin-sensitive enzyme, phosphodiesterase, and susceptibility to protease activity.

A criterion for the existence of an integral membrane protein is whether the protein and its activity can be extracted with detergent. If so, this would provide evidence for the presence of hydrophobic interactions anchoring the protein to the membrane. Also, by the same logic, an integral membrane protein should not be extracted in high ionic strength medium since this condition increases hydrophobic interactions. This was exactly what was found for porcine intestinal calmodulin (cf. Figure 6-9 A and B). Considering the similarity of porcine intestinal calmodulin (identified presently) with bovine brain calmodulin (a water-soluble protein) the likelihood of the former protein being associated with the lipid bilayer of the membrane by itself did not seem great. However, there was the distinct possibility that porcine intestinal calmodulin was associated very strongly with another protein or proteins in the membrane. Bikle and Munson (1985) proposed that

calmodulin was associated with an integral membrane protein in the rat brush border membrane and suggested that calmodulin associated with this complex was part of the Ca^{2+} transport machinery in this plasma membrane.

This suggestion that pig intestinal calmodulin associated with an integral membrane protein in the intestine was supported by the observation herein that ^{125}I-calmodulin preferentially partitioned into the hydrophobic (detergent) phase when mixed with protein extract from the brush border membrane. In the absence of protein extract the ^{125}I-calmodulin was found exclusively in the aqueous phase. The calmodulin-binding protein from the brush border membrane, which seemed to act as a receptor for calmodulin, was revealed in the gel overlay experiment (Figures 6-11 and 6-12) and its M_r was calculated to be 107,000.

This protein was similar in size, although slightly smaller, than the membrane protein isolated from rat intestine by Bikle and Munson (1985) and was slightly larger than the protein found in chicken intestinal brush borders (Glenney and Glenney, 1984; Moosekar and Conzelman, 1984). The three distinct molecular weights reported likely represent species differences for the same functional protein, since the differences reported were not great. However, Glenney and Glenney (1984) used as their starting material intestinal brush borders and not purified brush border membrane vesicles. Their conclusion was that the

110K protein was part of the cytoskeletal apparatus of the epithelial cell and that this protein linked the cytoskeleton to the microvillus membrane. To date there is no evidence from studies conducted in this laboratory that the 107K-protein identified in pig BBMV is connected with the cytoskeleton. However, no attempt was made to establish whether or not such a relationship exists and such a correlation would require further experimentation similar to that conducted by Glenney and Glenney.

The present study (Figure 6-10) includes the only report of the affinity of the binding between calmodulin and the brush border membrane (although high affinity complexation has been implied through the use of the ^{125}I-calmodulin gel overlay technique, Bikle and Munson, 1986). Purification of the 107K-protein from porcine BBMV and determination of its affinity for calmodulin would verify whether the calculated K_d (0.14 μM, Figure 6-10) for calmodulin-BBMV complex was an accurate measurement of the binding of calmodulin with this protein.

The function of the other Ca^{2+}-binding activity (peak 2, G-150) and its potential role in Ca^{2+} movement across the brush border membrane must await its purification to homogeneity and subsequent characterization. Its similarity to other Ca^{2+}-binding proteins of the brush border membrane such as IMCal remains uncertain. Attempts to isolate the IMCal protein using the method described by Schachter and

Kowarski were not successful. There may be a high molecular weight complex present in porcine BBMV which resembles that described by these authors. However, the low molecular weight protein that dissociated from one such complex in porcine BBMV was calmodulin-like (G-150 peak 1). The possibility that the low molecular weight fragment isolated by Schachter is indeed calmodulin has not been ruled out sufficiently. However, it does seem clear that the peak 2 activity is not a proteolytic fragment of calmodulin because this Ca^{2+}-binding fraction did not activate phosphodiesterase as did peak 1 activity. Also, in the two-step column assay for calmodulin (Table 6-2), peak 2 activity did not co-purify with bovine brain calmodulin indicating that if this protein represented a proteolytic fragment of calmodulin it must have lost its ability to bind both phenyl-Sepharose and DEAE-Sephacel as well as its ability to bind to phosphodiesterase, while keeping its ability to bind Ca^{2+}. Why the protein lost activity after chromatofocussing is still unclear.

The finding of calmodulin bound to a protein of the brush border membrane raised the obvious question of the physiological relevance of such a complex and the role that this protein may have in the translocation of Ca^{2+} across the microvillus membrane and/or the binding of this cation to the membrane. The finding that calmodulin binds to this receptor in a Ca^{2+}-independent manner (Figures 6-11 B and 6-

12B) provides the foundation for the formulation of a model. A model could be envisioned which designates as being one part of the Ca^{2+} translocating machinery of the brush border membrane, the calmodulin-107K complex. Although evidence to date is scanty it may be suggested that calmodulin was bound to the 107K-protein at low Ca^{2+} concentrations (since calmodulin binding to the 107K-protein was independent of Ca^{2+}), when little or no transport was occurring. However, when the Ca^{2+} concentration increases, the cation could be allowed to flow across the membrane via the 107K-calmodulin complex with the result being that calmodulin becomes saturated and conformational changes induced in the complex result in the dissociation of calmodulin from the complex. The 107K-protein could be functioning as a Ca^{2+} channel protein to help regulate the movement of Ca^{2+} across the membrane in response to hormonal or other cell-mediated stimuli. Bikle and Munson (1985) have found that vitamin D increased the amount of calmodulin that was associated with the brush border membrane. The presence of this Ca^{2+}-binding protein bound to the complex in the membrane could serve to act as a buffer of Ca^{2+} as it moved from the extracellular medium to the cytosolic face of the microvillus membrane. This concept is corroborated by the finding that Ca^{2+} appears to be bound, after movement across the membrane, to internal sites of BBMV as discussed at length in Chapter 2. However, there is no evidence provided

thus far suggesting how the interaction of calmodulin with this protein is controlled or regulated. It may be that a vitamin D-metabolite or cellular feedback messenger is involved with influencing the degree of association of calmodulin with the membrane. It must be left to a more detailed analysis of this interaction and eventually of the <u>in vivo</u> mechanism as it occurs in the cell.

The candidacy of this complex for involvement in Ca^{2+} transport across the brush border membrane cannot adequately be assessed however, using the approach taken in the present study. As mentioned previously, to correctly assay for the presence of a Ca^{2+}-channel protein, reconstitution techniques would need to be applied to this problem. This study has been concerned only with identification of binding proteins of the brush border membrane and results from such studies can, at best, be expected to determine the presence of proteins capable of binding Ca^{2+} in the brush border membrane. Nonetheless, at one stage, it will become imperative that an approach be taken similar to that which has been adopted for the characterization of the glucose transporter of the brush border membrane (Semenza et al., 1984) if success is to be found for the isolation and characterization of Ca^{2+} channel or transport proteins.

CONCLUSIONS

The following conclusions can be drawn from the study presently described:

(1) The use of $CaCl_2$ as the precipitant for preparing rabbit BBMV resulted in the preparation of a vesicle preparation that was sufficiently pure, as determined by marker enzyme and lipid content, for transport and binding studies. This preparation was superior to one obtained by the addition of $MgCl_2$ as the precipitant and did not display a high content of lysophospholipids.

(2) This characterized rabbit BBMV preparation was used to study the uptake of Ca^{2+} *in vitro*. This process was found to be similar in some respects to that which occurred in other species, namely rat and chick. However, Ca^{2+} uptake by rabbit BBMV was characterized by the complete binding of the cation to apparent internal sites of the vesicles. The interior location of the sites were deduced on the basis that the binding process occurred after translocation of this cation across the membrane bilayer as was evidenced by studies with the Ca^{2+} ionophore, A23187. Also, the inability of chelators to remove Ca^{2+} from the BBMV, after a brief wash, indicated that the

cation was internalized within the vesicle structures.

(3) Incubation of various fatty acids and the methyl ester of oleic acid with rabbit BBMV, in vitro, resulted in the uptake of these lipids by the membrane vesicles. The uptake of fatty acids was found to be dependent upon the chain length of the fatty acid, the longer the acyl chain the greater the amount of fatty acid incorporated. This incorporation of fatty acids into the brush border membrane was determined to be hydrophobic in nature because the uptake of these acids was unaffected by high ionic strength. Also, the rate of incorporation of oleic acid into BBMV was considerably faster than methyl oleate incorporation under similar conditions.

The uptake of Ca^{2+} by rabbit BBMV was stimulated by treatment of the membranes with low concentrations of unsaturated fatty acids (0.05 mM) as well as with various concentrations of octanoic acid (0.1 - 3.0 mM) and inhibited by treatment with higher concentrations of unsaturated fatty acids (0.20 - 0.60 mM). Saturated fatty acids had no effect or were slightly inhibitory on this Ca^{2+} uptake process. On the other hand, incorporation of methyl oleate

resulted in a concentration-dependent stimulation of this process. Interestingly, the stimulatory concentrations of unsaturated fatty acids did not change the Ca^{2+}-binding characteristics of the membranes, whereas the higher concentrations decreased the equilibrium binding of the cation and very probably the number of high-affinity binding sites.

(4) The incorporation of these lipids into BBMV caused an alteration in the structure of the membrane as reported by fluorescence anisotropy using DPH as the hydrophobic membrane probe. It was established that oleic acid, linoleic acid, and methyl oleate decreased the fluorescence anisotropy of the membranes in a dose-dependent manner. In contrast, palmitic acid had little or no effect on the fluorescence anisotropy within the range of concentrations used. In addition, the results obtained with lipid extracts of the BBMV suggested that the proteins influence the membrane fluidity. This was especially evident with oleic acid-treated membranes, the extracts of which displayed reduced anisotropy values throughout the lower temperature range. All in all, these results demonstrated an effect of exogenous lipid incorporation on the structure of

the brush border membrane.

(5) Attempts to identify and characterize the protein(s) responsible for the binding of Ca^{2+} by BBMV led to the resolution of two Ca^{2+}-binding activities from porcine intestine. One activity was characterized as intestinal calmodulin while the other was not completely identified. It appeared from the available data that both activities reside as parts of complexes extracted from the brush border membrane. In fact, the former activity was found to bind to a receptor protein, which could also be extracted from BBMV with detergents. This calmodulin-binding protein was identified by a gel overlay procedure and found to possess a M_r of 107,000.

Further investigations into the nature of the Ca^{2+} uptake system in BBMV would require the identity of the as yet uncharacterized Ca^{2+}-binding protein, which to date could be purified only some 250 fold. This would make possible a study of this protein's role in the Ca^{2+} uptake process in the brush border membrane through the use of reconstitution methods. The use of reconstitution techniques would facilitate our understanding of the role that this protein as well as the calmodulin-107K complex may have in

the binding and transport of Ca^{2+} by the brush border membrane. In addition, the specificity of the lipid-protein interactions shown to affect Ca^{2+} uptake in BBMV could be ascertained in much more precise terms with such an approach. This procedure, together with the study of the effects of various other lipids, commonly found in the diet, i.e. cholesterol, phospholipids etc., on Ca^{2+} uptake by BBMV would provide further insight into the structure-function relationships existing in this unique plasma membrane.

APPENDIX

TABLE A-1

Calculation of Vesicular Volume and Equilibrium Uptake Maxima

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A. D-Glucose Uptake

Equilibrium Uptake .... 90 pmole/mg prot.

[D-glucose] in uptake medium .... 100 μM

$$\frac{1000\ \text{ml}}{100\ \mu\text{moles}} \times \frac{9 \cdot 10^{-5}\ \mu\text{moles}}{\text{mg protein}} = 9.0 \cdot 10^{-4}/\text{mg prot.}$$

$$= 0.9\ \mu\text{l/mg prot.}$$

B. Calcium Uptake

(1) Theoretical

At $[Ca^{2+}] = 0.36$ mM:

$$\frac{0.36\ \text{mmole}}{1000\ \text{ml}} \times 9.0 \cdot 10^{-4}\ \text{ml/mg prot.} =$$

$$3.24 \cdot 10^{-7}\ \text{mmol/mg prot.}$$

or

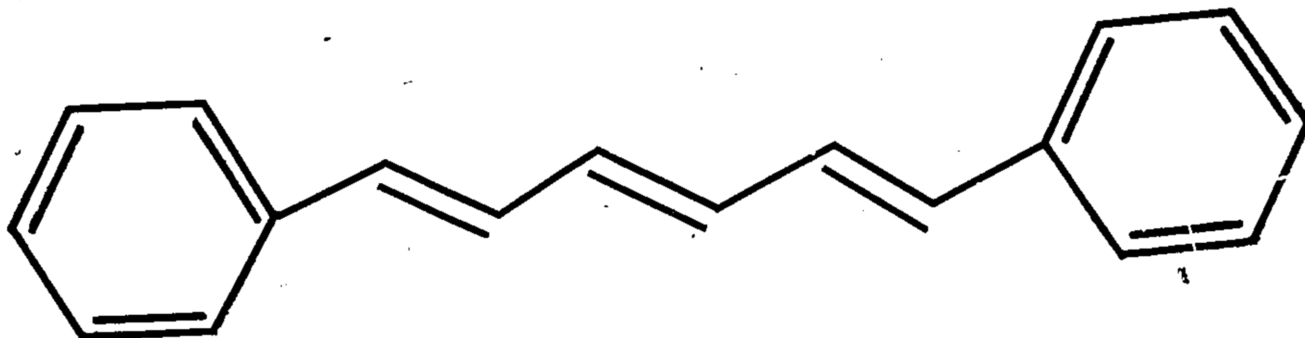
$$3.24 \cdot 10^{-1}\ \text{nmol/mg prot.}$$

(2) Empirical equilibrium value ... 18.0 ± 4 nmol/mg prot.

$$\frac{18.0\ \text{nmol/mg prot.}}{0.32\ \text{nmol/mg prot.}} = 56.3$$

Thus, the actual equilibrium value for Ca^{2+} uptake is 56 fold higher than the theoretical value if no binding of the cation to the vesicles occurred.

Figure A-1



Structure of the fluorescent probe, 1,3,5-diphenylhexatriene. This molecule has a rod-like shape and intercalates into the hydrophobic regions of membrane bilayers. It is highly fluorescent with an excitation wavelength maximum at 350 nm and an emission wavelength maximum at 430 nm. It possesses a high molar extinction coefficient, 80,000 L/moles · cm.

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BIRTHDAY: August 3, 1956

MARITAL STATUS: Married, 3 children

EDUCATION

1974
- Graduated from Cardston High School
Cardston, Alberta
- Honors in Chemistry and Physics
- Matriculation/diploma

1981
- Graduated from University of Lethbridge
Lethbridge, Alberta
- BSc. major in Chemistry
- Minor in Biology

(1987)
- PhD. Biochemistry from University of Ottawa
Ottawa, Ontario (thesis in progress)

RESEARCH EXPERIENCE

Sept. 1983 to present
- University of Ottawa
Department of Biochemistry
School of Medicine
Ottawa, Ontario
- conducted graduate research work in the laboratory of Dr. Pierre Proulx (PhD., McGill), Professor of Biochemistry, University of Ottawa and in the laboratory of Dr. Arthur Szabo (PhD.,Toronto), Senior Research Scientist, National Research Council, Ottawa and adjunct professor, Department of Biochemistry, University of Ottawa
- National Research Council
100 Sussex Drive
Ottawa, Ontario
Guest worker Sept. 1984 to present
- acquired knowledge of the biophysical technique

of fluorescence anisotropy and applied it to the study of biological membranes, in particular, the study of membrane-bound calcium binding proteins.

Feb. 1982 to Sept. 1983	-	Agriculture Canada Lethbridge Research Station Lethbridge, Alberta
	-	Research Technician (EG-ESS-4)
	-	worked with a Physiologist in the Department of Animal Science in Reproductive Physiology
	-	involved in the taking and processing of biological samples from large research animals
	-	techniques included radioimmunoassay, histology, blood chemistry, microscopy and sperm cell evaluation
Aug. 1981 to Feb. 1982	-	Gemini Biochemical Limited 3650 - 21 Street N.E. Calgary, Alberta
	-	Research Technician
	-	worked closely with a Research Chemist on a biomass conversion project involving the production of useable fuel from agricultural plant wastes.
April 1980 to Sept. 1980	-	Agriculture Canada Lethbridge Research Station Lethbridge, Alberta
	-	summer student in Soil Science Section
	-	assisted research technicians in soil studies and related experiments
April 1979 to Sept. 1979	-	Agriculture Canada Lethbridge Research Station Lethbridge, Alberta
	-	summer student in Soil Science Section
	-	assisted research technicians in soil studies and related experiments

MEMBERSHIPS

1986 to present	-	Member of the Canadian Biochemical Society

PhD. PROJECT:

My project has involved the elucidation of some of the properties of the Ca^{2+} uptake system in rabbit intestinal brush border

membranes and the effect that exogenous fatty acids have on the activity of this system. Changes in Ca^{2+} uptake have been correlated with alterations in the "fluidity" of the brush border membrane as monitored by steady state fluorescence anisotropy using primarily the rod-like hydrophobic probe, diphenylhexatriene. Additionally, a study was undertaken which helped to further define the nature of the calcium-binding protein entities found in the native, intact membrane by employing a fluorescent lanthanide, terbium, which can substitute for Ca^{2+} in Ca^{2+}-binding proteins. Attempts at isolating and purifying the Ca^{2+}-binding proteins responsible for the uptake of this cation by the brush border membrane have resulted in the finding of calmodulin bound to a receptor in this membrane. In addition to calmodulin another Ca^{2+}-binding activity has been identified but the protein responsible has only been partially purified.

RESEARCH INTERESTS AND CAREER GOALS

I am very much interested in protein-lipid interactions in general with particular interest in membrane-bound proteins and their interactions in the lipid bilayer. I feel that a biophysical approach to biochemical problems is essential to the elucidation of the complex reactions occurring in the cell. My background is one of chemistry and as a result I am keenly interested in using a molecular approach to study membrane cellular processes.

PUBLICATIONS

1. *Merrill, A.R., Proulx, P., and Szabo, A.G. (1985) Meeting of the Canadian Biochemical Society, Toronto, Ontario, June 17-21, 1985 Volume 28:110

2. Merrill, A.R., Proulx, P., and Szabo, A.G. (1986) Effect of exogenous fatty acids on calcium uptake by brush border membrane vesicles from rabbit small intestine. Biochim. Biophys. Acta 855, 337-344

3. *Merrill, A.R., Proulx, P., and Szabo, A.G. (1986) Meeting of the Canadian Biochemical Society, Guelph, Ontario, June 16-20, 1986 Volume 29:290

4. Aubry, H., Merrill, A.R., and Proulx, P. (1986) A comparison of brush border membranes prepared from rabbit small intestine by procedures involving Ca^{2+} and Mg^{2+} precipitation. Biochim. Biophys. Acta 856, 610-614

5. Merrill, A.R., Proulx, P., and Szabo, A.G. (1986) Studies on calcium binding to brush border membranes from rabbit

small intestine. Biochim. Biophys. Acta 859, 237-245

6. *Proulx, P., Merrill, A.R., and Szabo, A.G. (1986) A study of the effect of unsaturated fatty acids on calcium uptake in the gut. Symposium Lipids and Membranes June 23-25, 1986 Utrecht, The Netherlands

7. *Proulx, P., Merrill, A.R., and Szabo, A.G. (1986) Calcium transport in the small intestine. Symposium Lipids and Membranes June 23-25, 1986 Utrecht, The Netherlands

8. Merrill, A.R., Aubry, H., Proulx, P., and Szabo, A.G. (1987) Relation between calcium and fluidity of brush border membranes isolated from rabbit small intestine and incubated with fatty acids and methyloleate. Biochim. Biophys. Acta. 896, 89-95

9. *Merrill, A.R., Szabo, A.G., and Proulx, P. (1987) Purification of a Ca^{2+}-binding complex from porcine intestinal brush border membranes. Cell Calcium Metabolism '87, Seventh International Washington Spring Symposium, Washington, D.C. (May 19 - 22, 1987)

10. Merrill, A.R., Proulx, P., and Szabo, A.G. Probing for calcium binding sites in rabbit small intestine using a fluorescent lanthanide. (submitted to Journ. of Membr. Biol.)

11. Merrill, A.R., Proulx, P., Anderson, P.J., and Szabo, A.G. Purification and characterization of part of the machinery involved in Ca^{2+} uptake by porcine intestinal brush border membrane vesicles. (manuscript)

12. Merrill, A.R., Anderson, P.J., Szabo, A.G., and Proulx, P. Identification of a novel calmodulin-binding protein from the intestinal brush border membrane. (in preparation)

*abstracts

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