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CALCIUM IN BIOLOGICAL SYSTEMS

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ABBREVIATIONS

ALC	alkali extractable light chain of myosin
amu	atomic mass unit
ANS	8-anilino-1-naphthalenesulfonic acid
ATPase	adenosine triphosphatase
cAMP	adenosine 3′: 5′-monophosphate
CD	circular dichroism
Con-A	concanavalin-A
DTNB	5,5′-dithiobis(2-nitrobenzoic)acid
DTNBLC	DTNB titratable and extractable light chain of myosin
EC	Enzyme Commision, a prefix for their numerical designation of enzymes
EDTA	ethylenediaminetetraacetic acid
EDTALC	EDTA extractable light chain of myosin
EGTA	1,2-bis-(2-dicarboxymethylaminoethoxy)-ethane
ER	endoplasmic reticulum
ESR	electron spin resonance
F-actin	filamentous or polymerized actin
HACBP	high affinity calcium binding protein of the SR
H_2EHDP	dihydrogen ethane-1-hydroxy-1,1-diphosphate
HMM	heavy meromyosin
HMT	hexamethylenetetraamine

K_m	Michaelis—Menten constant
K_d	dissociation constant [A][B]/[AB]
MCBP	muscle calcium binding parvalbumin
MW	molecular weight
NMR	nuclear magnetic resonance
ORD	optical rotatory dispersion
PAR	4-(2-pyridylazo)resorcinol
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
P_i	inorganic phosphate, HPO_4^{-2} or $H_2PO_4^-$
pK_d	$-\log(K_d)$
PMR	proton magnetic resonance
PRE	proton relaxation enhancement
PS	phosphatidyl serine
PVC	polyvinylchloride
ROS	rod outer segment of retinal rod cells
SDS	sodium dodecylsulfate
SR	sarcoplasmic reticulum
T_1	spin—lattice relaxation time
T_2	spin—spin relaxation time
TN-C	troponin, calcium binding component
TN-I	troponin, inhibitory component
TN-T	troponin, tropomyosin binding component

I. INTRODUCTION

This review is oriented toward the research chemist who wants a broad view of calcium in biological systems preparatory to more detailed reading or actual research. Since many advances in the field have been associated with the development and application of chemical techniques, we felt it appropriate to discuss the background and development of several of these procedures, see section III. This approach has led us to consider scores of topics, any one of which would be the subject for an authoritative, detailed review. By choosing breadth we have sacrificed some depth. Our approach has been eclectic. In the outline of various calcium binding proteins and discussion of physiological processes (section IV) we have tried to cite recent review articles and, if not covered in the review, recent papers in the field. Frequently a key paper in the development of a subject is not cited, where a less definitive recent paper is. This practice fails to give perspective, but is hopefully tolerable when one realizes that only partial documentation has generated some 400 references.

In order to appreciate the unique biological role of Ca^{2+} among the metal ions, consider the original observations of Ringer (1882). He found that isolated turtle hearts would continue beating for many hours if, and only if, 10^{-3} M Ca^{2+} were present in the isotonic bathing medium. When exploring

the optimal medium conditions, he initially overlooked the Ca^{2+} requirement because it was present as a contaminant in the London water supply. Subsequently, countless other scientists have overlooked the role of calcium initially without discovering and acknowledging their oversight so quickly and forthrightly as did Ringer. During the next half century a wide variety of seemingly unrelated physiological responses were found to be absolutely dependent on low concentrations of calcium. Heilbrunn (1956) documented many of these responses and made the physiologists well aware of the importance of calcium. Yet at that period his approach was inevitably phenomenological. There were no real unifying concepts.

Quite naturally muscle received much attention. The sliding filament model of Huxley and Hanson (1954) and Huxley and Niedergerke (1954) outlined the basic molecular mechanism which today is still being elaborated. However it was not until Ebashi et al. (1967) isolated troponin that a link was established between the calcium requirement and the contractile mechanism. It has become ever more apparent that myosin and actin are not restricted to muscle cells but are present in nearly all eukaryotic (non bacterial) cells where they participate in a wide variety of functions.

A seemingly unrelated group of responses (secretion by a variety of cells) was suggested by Douglas (1963, 1973) to be effected by a process later termed exocytosis. He realized that Ca^{2+} is a "necessary and sufficient" requirement for stimulus coupled secretion.

Many scientists, particularly Lehninger and his coworkers, see section IV. D.3.b., established that calcium is intimately involved in mitochondrial function.

For many years Sutherland (see Nobel address, 1972) had patiently explored the biochemistry of cyclic-AMP and finally suggested its function as a second messenger. Rasmussen (1970) realized that in many systems involving contraction, exocytosis or enzyme activation, Ca^{2+} functions as a second messenger, often in parallel with cAMP.

As more protein sequences and structures become available it is becoming apparent that many of the targets of the calcium message are proteins which are evolutionarily related. Finally, one of the key areas of contemporary research concerns various cellular membranes — how they establish Ca^{2+} gradients and subsequently how a specific stimulus causes a selective release or passage of calcium ions.

II. ORGANO- AND PROTEIN-CRYSTAL STRUCTURES

A. Organo-calcium crystal structures

In this section we will examine the nature of calcium coordination in small organo-calcium complexes, as determined by high resolution X-ray crystallography. We will place particular emphasis on the following items, which are summarized in table II-1: a. coordination number; b. calcium ligand type;

TABLE II-1

Organo—calcium crystal structure summary

Compound	O No.	$\overline{Ca—O}$	Max.	Min.	O—Om	W	$\overline{Ca—W}$	OH	CO_2	C=O	—O—	PO	(−)	HB	M No.
Thymidylate $Ca(C_{10}H_{13}O_8N_2P) \cdot 6\ H_2O$	7	2.42	2.65	2.29	?	3	2.43	0	0	0	0	4	2	0	3
Hexamethylenetetramine $CaBr_2(C_6H_{12}N_4)_2 \cdot 10\ H_2O$	6	2.33	2.34	2.32	3.13	6	2.33	0	0	0	0	0	0	0	(0)
Ethane-1-hydroxy-1,1-diphosphonate $Ca(C_2H_8O_7P_2) \cdot 2\ H_2O$	8	2.49	2.61	2.42	?	3	2.52	1	0	0	0	4	2	?	3
Blepharisma $Ca(C_{11}H_{10}O_6N)_2 \cdot 3\ H_2O$	7	2.38	2.43	2.34	2.54	2	2.34	2	2	1	0	0	2	?	3
Myoinositol $CaBr_2(C_6H_{12}O_6) \cdot 5\ H_2O$	8	2.45	0.52	2.37	2.62	4	2.41	4	0	0	0	0	0	0	2
α-Galactose $CaBr_2(C_6H_{12}O_6) \cdot 3\ H_2O$	8	2.46	2.55	2.35	2.62	3	2.41	5	0	0	0	0	0	0	3
α-D-Xylose $CaCl_2(C_5H_{10}O_5) \cdot 3\ H_2O$	7	2.40	2.51	2.32	?	3	2.34	4	0	0	0	0	0	?	2
β-D-Mannofuranose $CaCl_2(C_6H_{12}O_6) \cdot 4\ H_2O$	8	?	?	?	?	3	?	5	0	0	0	0	0	0	2
Lactose $CaBr_2(C_{12}H_{22}O_{11}) \cdot 7\ H_2O$	8	2.45	2.54	2.38	2.64	4	2.40	4	0	0	0	0	0	0	2
Lactobionic acid $CaBr(C_{12}H_{21}O_{12}) \cdot 4\ H_2O$	8	2.46	2.52	2.37	2.57	3	2.42	4	1	0	0	0	1	0	2
Garcinia acid $Ca(C_6H_4O_7) \cdot 4\ H_2O$	8	2.45	2.46	2.39	2.57	4	2.47	1	2	1	0	0	1	0	3

TABLE II-1 (continued)

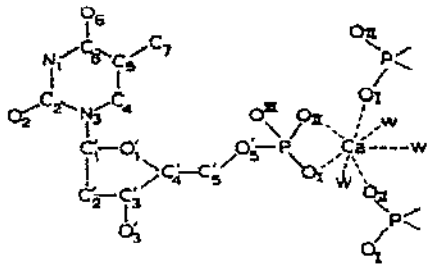
Compound	O No.	$\overline{Ca-O}$	Max.	Min.	O—Om	W	$\overline{Ca-W}$	OH	CO_2	C=O	—O—	PO	(—)	HB	M No.
5-Keto-D-gluconate $Ca(C_6H_9O_7)_2 \cdot 2\ H_2O$	8	2.44	2.47	2.39	2.63	2	2.39	2	2	0	2	0	2	2	2
Glucoisosaccharate $Ca(C_6H_{11}O_6)_2$	8	2.44	2.51	2.38	2.81[h]	0		6	2	0	0	0	2	4	2
Arabonic acid $Ca(C_5H_9O_6)_2 \cdot 5\ H_2O$	8	2.47	2.52	2.44	2.62[h]	2	2.44	4	2	0	0	0	2	4	2
Glutamate $Ca(C_6H_7O_4N) \cdot 3\ H_2O$	8	2.48	2.60	2.38	?	2	2.43	0	5	0	0	0	2	0	3
Glycylglycylglycine $CaCl_2(C_6H_{11}O_4N_3) \cdot 3\ H_2O$	7	2.39	2.50	2.30	?	2	2.37	0	3	2	0	0	2	0	4
D-Glucuronic Acid $CaBr(C_6H_9O_7) \cdot 3\ H_2O$	8	2.468	2.56	2.38	2.603	2	2.476	3	2	0	1	0	1	0	3

"O·No." is the coordination number; only in glutamate is an atom (amino nitrogen) other than oxygen in the primary sphere. The average, maximum and minimum calcium—oxygen distances are listed in the next three columns. "O—O_m" is the closest oxygen—oxygen contact among the calcium ligands. The superscript "h" indicates that the closest contact involves an oxygen—oxygen hydrogen bond. The next series of columns indicate the types of ligands with the average calcium—water oxygen distance listed under "$\overline{Ca-W}$". "OH" is hydroxyl; "CO_2", carboxylate; "C=O", carbonyl; "—O—", oxygen of the sugar ring; and "PO", the phosphorous bonded oxygen. "(—)" is the formal charge of the primary sphere; it may differ from "CO_2" if both oxygen atoms coordinate or if the carboxylate group bridges two calcium ions. "HB" is the number of hydrogen bonds formed between coordinating oxygen atoms. "M No." is the number of different organic molecules coordinating one calcium ion. The last entry is from DeLucas, L., Bugg, C.E., Terzis, A. and Rivest, R. "Calcium Binding to D-Glucuronate Residues: Crystal Structures of a Hydrated Calcium Bromide Salt of D-Glucuronic Acid" Carbohydrate Res. in press. Einspahr, H., Gartland, G.L. and Bugg, C.E. have recently solved a glutamate structure, $Ca^{2+}(C_5H_8O_4N)^-Cl^-$, in which the Ca^{2+} is six coordinate and nitrogen is not a ligand.

c. calcium ligand distance; d. ligand electronegativity; e. ligand distortion; f. primary coordination sphere geometry.

1. Nucleotide complex: Ca^{2+}—thymidylate

Thymine is one of the four bases of deoxyribonucleic acid (DNA), a molecule which occupies the central role in all of molecular biology. Trueblood et al. (1961) found that Ca^{2+} forms a 7-coordinate complex with thymidine monophosphate.



The coordination geometry can best be described in terms of a pentagonal bipyramid, with four phosphate oxygens (from three different molecules) and one water oxygen coordinating in a central pentagonal plane, while two additional water molecules coordinate axially. Calcium coordination is similar to that found in the inorganic complex $CaHPO_4$, where the calcium ion is also 7-coordinated, having an average calcium to oxygen distance of 2.44 Å. In Ca^{2+}—thymidylate the average Ca—O distance is 2.42 Å.

Of the two phosphonate oxygens involved in the coordination of two calcium ions, the O_{II} oxygen is closer to its calcium pair than is the O_I oxygen. This is reflected in a longer phosphorus to oxygen bond length for P—O_{II} (1.514 Å) compared to P—O_I (1.486 Å). Oxygen O_{III}, which is not involved in coordinating any calciums, has the shortest P—O bond length (1.474 Å).

The observed O—P—O bond angle decreases from 118.4° for O_I—P—O_{III}, where only O_I coordinates Ca^{2+}, to 106.0° for the O_I—P—O_{II} angle, where both O_I and O_{II} coordinate the metal ion. The reduction of the O_I—P—O_{II} bond angle is believed to be due to the presence of the bound calcium ion in the median plane of the phosphonate group across the two-fold axis.

2. Diphosphonate complex: Ca^{2+}—dihydrogen ethane-1-hydroxy-1,1-diphosphate

Fleisch et al. (1969) and Francis et al. (1969) have shown that gem-diphosphonates (i.e. compounds with the P—C—P grouping) have the capability of modulating calcium—hydroxyapatite crystal growth, both in vitro and in vivo and the potential pharmacological usefulness of the gem-diphosphonates for the treatment of diseases associated with calcium and phosphate metabolism has been suggested.

Uchtman (1972) solved the crystal structure of the calcium complex with

TABLE II-2

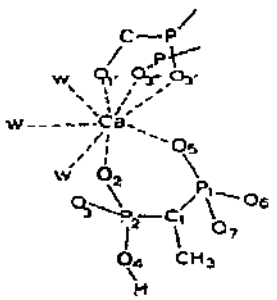
Bond lengths and bond angles in the phosphonate groups of Ca^{2+}—H_2EHDP [a]

Bond length		Bond angle	
P_2—O_4	1.595	O_2—P_2—O_3	116.58
P_1—O_7	1.549	O_5—P_1—O_6	113.25
P_1—O_6	1.530	O_5—P_1—O_7	112.43
P_2—O_3(Ca)	1.505	O_2—P_2—O_4	111.22
P_1—O_5(Ca)	1.507	O_6—P_1—O_7	110.46
P_2—O_2(Ca)	1.496	O_3—P_2—O_4	106.87

[a] Values extracted from the compilation by Uchtman (1972).

dihydrogen ethane-1-hydroxyl-1,1-diphosphate (Ca—H_2EHDP).
Eight oxygen atoms ligate the calcium ion, five from three symmetry-related H_2EHDP molecules and three from water molecules.

In Ca^{2+}—H_2EHDP there are two types of calcium coordination, one strong



and one weak. The stronger calcium binding involves coordination to both phosphonate moeities and results in the formation of a six membered ring. The weaker calcium binding involves one diphosphonate and a methylene un-ionized hydroxyl group, which results in a five membered metal chelate ring.

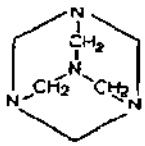
In Table II-2 are listed all the phosphorus—oxygen bond lengths and oxygen—phosphorus —oxygen bond angles for Ca^{2+}—H_2EHDP. Note that the values for the P—O bond distance range from 1.595 Å for the P_2—O_4H bond, which is not ionized and not involved in calcium coordination, to a minimum value of 1.496 Å for the P_2—O_2 bond, which is ionized and is in the primary coordination sphere of the calcium ion. The intermediate values for the P_1—O_7 and P_1—O_6 bonds are related to the partial ionized character of these bonds. Ca^{2+}—H_2EHDP is the first known chelate structure incorporating an un-ionized hydroxyl group of a hydroxy-phosphonic acid into the primary coordination sphere of a metal ion.

More conformationally sensitive to the binding of calcium are the O—P—O bond angles. Both O_2 and O_3 coordinate calcium and a maximum O_2—P—O_3 bond angle of 116.6° is observed. O_5 coordinates calcium but O_7 does not,

and a decreased value of 112.4° is obtained for the O_5—P—O_7 angle. Neither O_3 nor O_4 ligand calcium and a minimum O_3—P_2—O_4 bond angle of 106.9° is observed. Thus the O—P—O bond angle appears to be particularly sensitive to calcium coordination. If calcium is bound to both oxygen atoms, the distance between the ligating oxygen atoms increases.

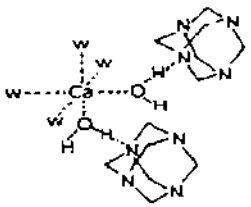
3. Organic base complex: Ca^{2+}—hexamethylenetetramine

Mazzarella et al. (1967) examined the calcium complex of hexamethylenetetramine (HMT).



The objective of the study was to investigate structural features of complexes between inorganic calcium salts and the organic bases. Studies in this area are physiologically relevant since they provide information on the manner in which biological macromolecules in aqueous solution are solvated.

Ca^{2+} in HMT is six coordinated to water, with near octahedral geometry.



The hydrogen atoms of these water molecules are involved in a hydrogen-bond network to the nitrogen atoms of neighboring HMT molecules; however, the hydrogen bonds are not in the primary coordination sphere of calcium, since the nitrogen is not a ligand.

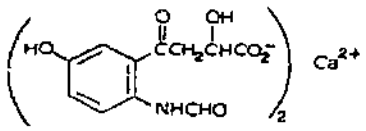
The calcium to water distance is essentially constant, with all values in the range of 2.32—2.34 Å. The formal charge of the primary sphere is zero, since only neutral water molecules are coordinating calcium. The closest contact between two coordinating water molecules is 3.13 Å, which is considerably larger than the sum of the van der Waals' radii (2.8 Å). Thus, there are no geometrical constraints within the primary coordination sphere of Ca^{2+} restricting the number of liganding water molecules. Since each water molecule liganding calcium is also involved in a hydrogen bond to a neighboring HMT molecule, the low coordination number for the calcium ion results simply from the constraints imposed on the crystal packing of the HMT molecules, to which they are linked through coordinated water molecules.

4. Phenolic α-hydroxy acid: Ca^{2+}—blepharismin

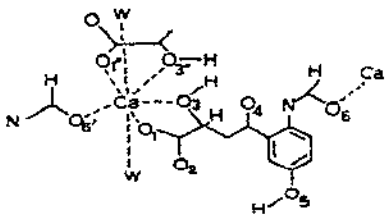
Gamones are chemical substances believed to be involved in the control of

conjugation processes of lower organisms (Sonneborn, 1937). Miyake (1968) isolated and partially characterized a gamone, designated blepharismin, which has induced a particular protozoan mating type (Type I) to conjugate.

Blepharismin, 3-(2′-formamyl-amino-5′-hydroxybenzoyl) lactate, is isolated as the calcium salt.



In the crystal (Kubota et al., 1973) the calcium ion is coordinated in bipyramidal fashion by a total of two carboxyl groups, two hydroxyl groups and a carbonyl group from three separate blepharismin molecules.



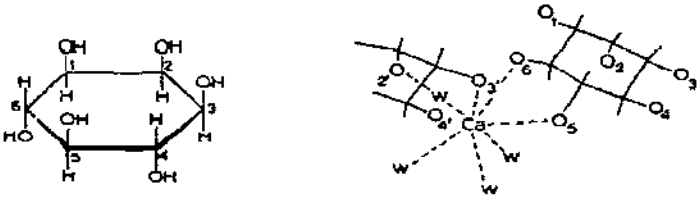
No information has been published on distortions of the blepharismin conformation which might be induced by the coordinated calcium.

5. Monosaccharide complexes

In this and the following two subsections calcium–carbohydrate interactions will be considered. In order, we will present structural information on complexes of calcium with simple monosaccharides, disaccharides and the α-hydroxy sugar acids.

It has been suggested that interactions between calcium and carbohydrates play an important role in a number of physiological processes, including calcification (section IV. B.) and cell adhesion (section IV.D.4.)

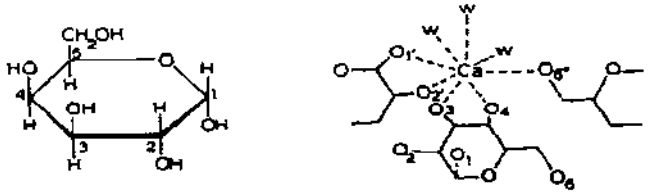
a. Ca^{2+}—myo-inositol. Cook and Bugg (1973b) investigated the calcium complex of myo-inositol.



Four hydroxyl groups, from two-symmetry-related myo-inositol molecules, and four water molecules coordinate the calcium ion.

Cook and Bugg observed that calcium coordination induced distortions in the conformation of the myo-inositol molecule. Deviations in bond angles adjacent to coordinating hydroxyl ligands of up to 5° were noted, while the distance between two adjacent liganding hydroxyl oxygens was found to decrease by about 0.2 Å with calcium binding.

b. Ca^{2+}—galactose. Galactose (Cook and Bugg, 1973a) is the major carbohydrate component associated with bone collagen. Thus, the study of the

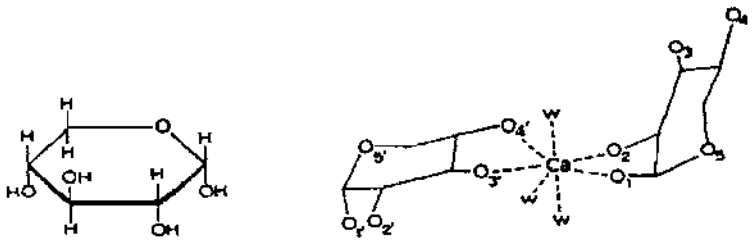


interactions between Ca^{2+} and galactose is relevant to understanding mineralization.

Three water molecules and five hydroxyl groups from three separate galactose molecules coordinate a single calcium ion. The average calcium to oxygen ligand distances are very similar to those of the other calcium carbohydrate complexes considered (Table II-1).

As was the case for myo-inositol, the distance between the oxygen atoms of adjacent hydroxyl groups binding calcium decreases by 0.2 Å.

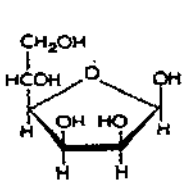
c. Ca^{2+}—α—D-xylose. In a preliminary report, Richards (1973) described the complex of calcium with the monosaccharide, α-D-xylose. Two xylose molecules are coordinated to a single calcium ion, and all oxygen atoms of

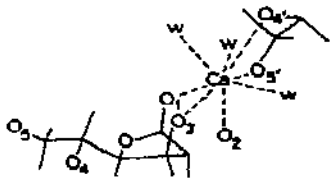


xylose, with the exception of the O_5 ring oxygen, function as Ca^{2+} ligands. No significant alteration of the xylose conformation was observed in the formation of the 7-fold distorted pentagonal bipyramid complex.

d. Ca^{2+}—β-D-mannofuranose. In a preliminary communication, Craig et al. (1972) described the calcium complex of the furanose structure of D-mannose. Each calcium ion is 8 coordinated, with oxygen ligands from two β-D-manno-

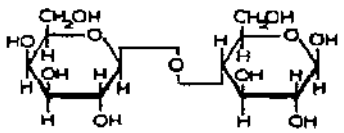
furanose and three water molecules. Possible distortion of the carbohydrate molecule upon calcium complexation was not discussed.



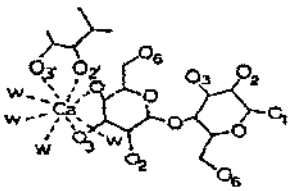


6. Disaccharide complexes

a. Ca^{2+}—lactose. Lactose, accounts for 5% of the dry weight of milk, and has been shown to enhance the rate at which calcium is absorbed from the gastrointestinal tracts of rats and humans.



Bugg (1973) found calcium to be eight coordinated in the Ca^{2+}—lactose complex, with four hydroxyl groups from two lactose molecules and four water molecules binding the calcium ion. Calcium coordination to lactose results in a number of conformational changes in both the glucose and



galactose parts of the molecule. In the glucose moiety, Ca^{2+} induces a 7° decrease in the O_2—C_2—C_3—O_3 torsional angle and a 5° decrease in the C_3—C_2—O_2 bond angle. These angular changes, in turn, result in a 0.2 Å decrease in the distance between the O_2 and O_3 hydroxyl oxygen. In the galactose moiety, a 9° decrease is observed in the O_3—C_3—C_4—O_4 torsional angle and a 5° decrease in the C_4—C_3—O_3 bond angle, which, again, results in a 0.2 Å decrease in the distance between the adjacent hydroxyl oxygen atoms, O_3 and O_4.

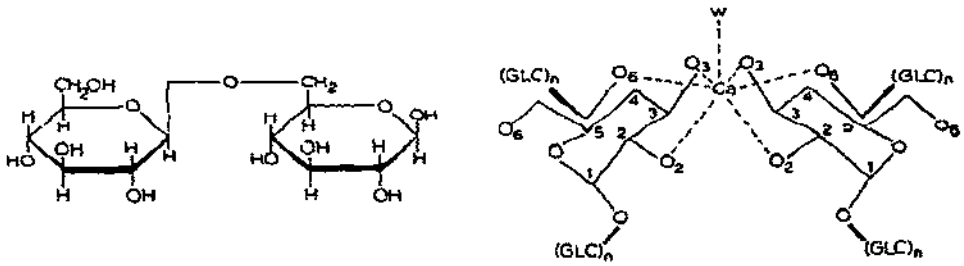
b. Ca^{2+}—α,α-trehalose. The highly branched D-glucans, having α(1 → 6) and α(1 → 3) linkages, are the principal component of dental plaque (Long and Edwards, 1972). Gibbons and Fitzgerald (1969) have suggested that the D-glucans are involved in the agglutination process that leads to plaque formation.

Cook and Bugg (1973d), using the D-glucose-containing oligosaccharide,

α,α-trehalose (α-D-glucopyranosyl α-D-glucopyranoside) as a model for the D-glucans, investigated the nature of calcium—carbohydrate cross-linking.

An examination of the crystal packing in Ca^{2+}—trehalose reveals an interesting cross-linked network of trehalose molecules, with the hydroxyl groups from four symmetry-related D-glucose moieties coordinating a single calcium ion.

The primary coordination sphere is described by a pentagonal bipyramid,



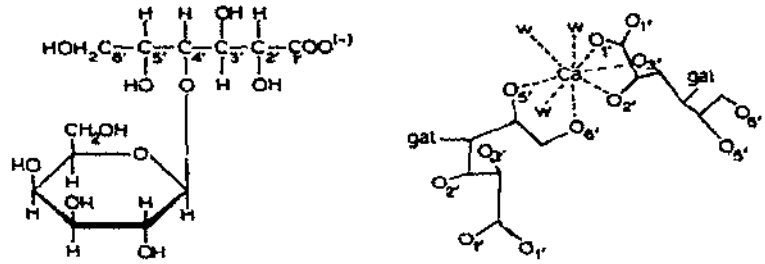
with calcium to oxygen ligand distances slightly shorter than is found for some 8-coordinate calcium carbohydrate complexes (Table II-1). There are one water and six hydroxyl ligands.

Slight distortions of both bond angles and torsional angles of the α,α-trehalose conformation occur with calcium complexation. Bond angles for the hydroxyl groups (e.g. $\angle C_2—C_3—O_2$) involved in calcium coordination are altered up to 7°, while the $O_2—C_2—C_3—O_3$ torsional angle decreases 4 to 5 degrees. These angular variations result in a net decrease in the distance between the O_2 and O_3 hydroxyl oxygens of 0.1 to 0.2 Å.

In Ca^{2+}—trehalose the prime role of hydroxyl groups, particularly hydroxyl group pairs (e.g. the trehalose $O_2—O_3$ hydroxyl pair), in the coordination of calcium ion is firmly established. This binding is favored to such an extent that the carbohydrate conformations frequently submit to induced structural alteration to accommodate calcium coordination.

7. α-Hydroxy sugar acid complexes

a. Ca^{2+}—lactobionic acid. In Ca^{2+}—lactobionate (4-(β-D-galactosido)-D-gluconic acid) the calcium is eight-coordinated through oxygen ligands. The

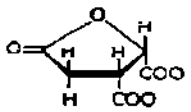


carboxyl oxygen and all four hydroxyl oxygens of the gluconate moiety parti-

cipate in the formation of the primary sphere. The remaining three oxygen ligands are derived from water molecules. There are no calcium—galactose interactions.

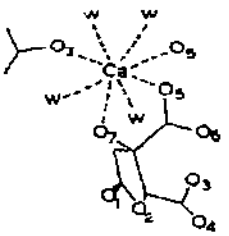
The nature of the calcium ligand interactions in the present example is interpreted by Cook and Bugg (1973c) on the basis of the strong chelating properties of the hydroxy acids. Hydroxy-carboxylic acids are known to have a greater affinity for calcium ions than simple carboxylic acids. Calcium acetate, for example, has a dissociation constant 40 times greater than calcium glycollate (Greenwald, 1938). As the number of hydroxyl groups is increased in a hydroxy acid, calcium binding is further enhanced (e.g. calcium glycollate has a larger dissociation constant than calcium gluconate).

b. Ca^{2+}—garcinia acid. Garcinia acid, the lactone of (—)-hydroxycitric acid, is a substance found to accumulate in plants and has been isolated from the



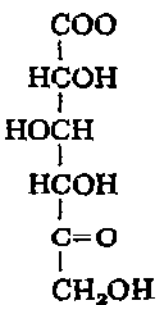
fruits of *Garcinia cambogia* (Lewis and Neelakantan, 1965). Garcinia acid is an enantiomorph of hydroxycitrate, a substrate of isocitrate dehydrogenase in the Krebs cycle, and has been shown to inhibit the activity of the citrate cleavage enzyme (Watson et al., 1969).

In the Ca^{2+}—garcinate complex (Glusker et al., 1971) the calcium ion is 8

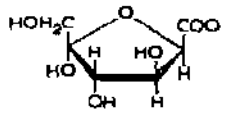


coordinated by oxygen atoms, four of which are derived from water molecules. Two calcium ions share a polyhedron edge in the distorted square antiprismatic complex.

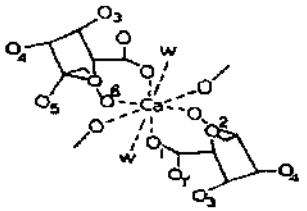
c. Ca^{2+}—5-keto-D-gluconate. The molecule 5-keto-D-gluconate,



an α-hydroxy sugar acid, can undergo ring closure to yield the 4,5 cisdiol furanoid ring structure. Such a cyclization results in more favorable metal ion chelation (Prescott et al., 1953), since oxygen atoms and hydroxyl groups



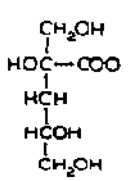
can be brought into closer proximity to the calcium ion. From our previous discussion of the α-hydroxy acid, Ca^{2+}—lactobionate, it would be expected that the calcium—furanoid would be a good calcium ion chelator, particularly in alkaline solution. Complexes between calcium and the ketogluconates have been isolated from soil bacteria and plant rhizospheres, following incubation in a medium of glucose and water-insoluble calcium minerals. This suggested to Duff and Webley (1959) the possible involvement of 2-ketogluconic acid formed by bacteria in the process of solubilization of various soil materials, such as natural silicates. In calcium 5-keto-D-gluconate each calcium ion is bound by two ketogluconate molecules and two water molecules. In the 8-fold coordination sphere, two hydroxyl oxygens, two carboxyl oxygens and two oxygen ring atoms are donated from the two gluconate ions.

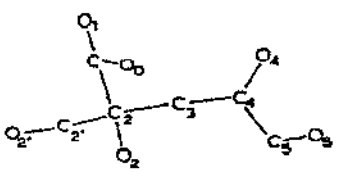


With regard to possible calcium-induced structural changes in the gluconate complex, Balchin and Carlisle (1965) report slightly decreased bond lengths for the C_2—C_1 and C_1—O_1 bonds, measuring values of 1.23 Å and 1.49 Å, respectively.

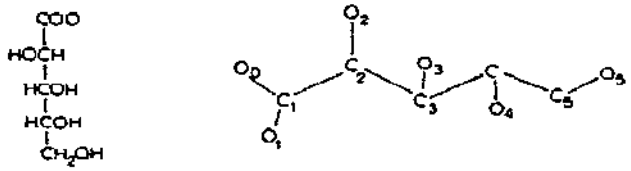
Calcium gluconate represents the first example of a comp'ex containing hydrogen bonds in the primary coordination sphere of the calcium ion.

d. Ca^{2+}—D-glucoisosaccharate and Ca^{2+}—D-arabonate. Two final calcium—sugar acid complexes whose crystal structures have been determined, are calcium—D-glucoisosaccharate (Norrestam et al., 1968), and calcium—D-arabonate (Furberg and Helland, 1962),





Both structures have 8-fold oxygen coordination, with two organic ions bound



to each calcium ion. Carboxyl, hydroxyl, and water oxygen ligands coordinate calcium in the arabonate complex. Ca^{2+} is not coordinated by water in the glucoisosaccharate complex (Table II-1). In a similar way to the calcium—gluconate case, hydrogen bonds (four) are found in the primary coordination sphere of both complexes. The coordination geometries are both a distorted square anti-prism.

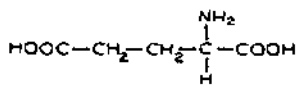
In the Ca^{2+}—glucoisosaccharate complex, the atoms O_0, O_1, C_1 and C_2 are located in a plane, while O_2 deviates from the plane by 0.30 Å. This "puckering" has been shown to be a general feature of the α-hydroxy acids of this type, and is not due to a calcium-induced conformational change. Further, Norrestam et al. observe an unusual "twist" at C_4, which results in a deviation from the regular, planar zig zag chain arrangement. This structural alteration at C_4, however, is not attributable to a calcium-induced conformational change, but rather to the absence of a hydroxy group at carbon-4. Ca^{2+}—arabonate, with oxygen atoms covalently bound to all carbon atoms, shows no deviation from the regular zigzag arrangement.

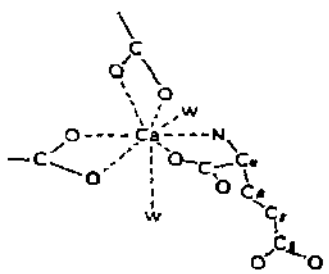
Bond angles and lengths for the Ca^{2+}—arabonate complex were not found to deviate significantly from the normal values. Several of these structures solved in the early 60's were not highly refined and bond distances might have errors of 0.02 Å.

8. Free amino acid and peptide complexes

Protein complexation is the main expression of the physiological roles of calcium (section IV.). The structures of peptide, calcium complexes provide high resolution precedents for the proteins, whose structures can be solved only at much lower resolution.

a. Ca^{2+}—glutamate. The carboxylate group of glutamic acid, and/or of aspartic acid, are involved in the Ca^{2+} coordination by all proteins of known structure (section II.B.).

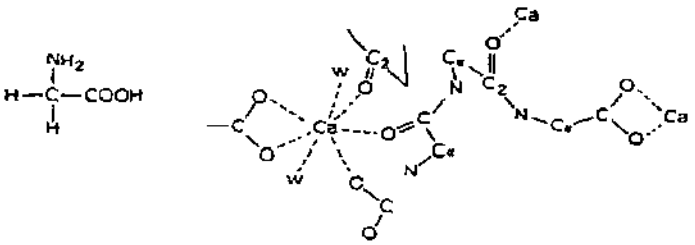




In calcium—glutamate (Einspahr and Bugg, 1974) the calcium ion is eight coordinate, with two water molecules and five carboxyl groups (from three symmetry-related glutamate ions) involved in Ca^{2+} coordination. The average calcium to oxygen ligand distance is 2.48 Å, which is significantly larger than that observed for most of the other small molecule, calcium complexes (Table II-1).

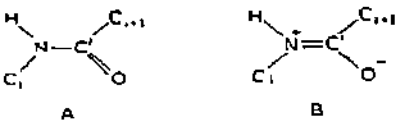
The eighth ligand is the N-terminal amino nitrogen atom, with a calcium to nitrogen ligand distance of 2.56 Å. This is the only reported Ca—N bond in an organo—calcium complex. Einspahr et al. (1975) have recently solved a glutamate structure, $Ca^{2+}(C_5H_8O_4N)^-Cl^-$, in which the calcium ion is six coordinate and nitrogen is not a ligand.

b. Ca^{2+}—glycylglycylglycine. In glycylglycylglycine, (van der Helm and Willoughby, 1969), calcium is seven coordinate. Two ligands are water and



five are carboxyl and carbonyl oxygen atoms of four different glycylglycylglycine molecules. The ligands are arranged in an approximate pentagonal bipyramid, with three carboxyl oxygens, one carbonyl oxygen and one water oxygen forming a pentagonal plane. A carbonyl oxygen and a water molecule are axial. The average calcium to oxygen ligand distance (2.39 Å) is considerably less than the value observed for calcium glutamate (2.48 Å).

The peptide bond can be described as an average of two resonance bond structures. The values of 1.34 Å and 1.35 Å for the two N—C′ bond lengths



A B

obtained by van der Helm and Willoughby are significantly larger than the average value of 1.32 Å expected for non-complexed peptides (Marsh and Donohue, 1967). Further, the length of 1.21 Å measured for both C′—O bonds is significantly smaller than the expected value of 1.24 Å. These differences appear to be real and were the basis of the authors' suggestion that resonance form A contributes relatively more in the calcium complex of glycylglycylglycine than it does in the noncomplexed form. This is interesting, since transition metals bound to peptide oxygen atoms apparently favor resonance form B, with N—C′ bond lengths of about 1.30 Å and C′—O bond

lengths of 1.26 Å (Freeman, 1966). Chemically, this suggests that upon coordination, the calcium ion decreases the polarity of the C—O bond, thus increasing the bond order. This would result in both a decrease in the stretching frequency of the C—O bond and an increase in the electronic shielding at the carbonyl carbon atom. This polarity-related shielding effect would be reflected as an upfield chemical shift of the carbonyl carbon in a ^{13}C magnetic resonance experiment. In fact, significant upfield chemical shifts have been observed for both carbonyl carbon atoms found in the primary coordination spheres of muscle calcium binding parvalbumin (Opella et al., 1974).

9. General features of small molecule calcium coordination

From the preceding discussion of the seventeen organo-calcium complexes it can be concluded that oxygen is the preferred coordinating ligand for calcium ion. Even though five of the structures examined have nitrogen atoms available for coordination, only in calcium glutamate do we find nitrogen as a ligand for Ca^{2+}. In the eight structures containing chloride or bromide as the counterion, the halide does not enter the primary coordination sphere of calcium.

The coordination number is usually eight, but in five of the seventeen complexes it is seven. In the case of hexamethylenetetramine the coordination number is six, with all oxygen ligands derived from water molecules. For 8-fold coordination the geometry of the primary sphere can best be described as a distorted square anti-prism; however, for calcium gluconate it is described best as a triakistetrahedron. All examples with 7-fold coordination are pentagonal bipyramid. The 6-fold coordinate example is a near regular octahedron. For all complexes, the primary coordination sphere is well defined with calcium to oxygen distances ranging from 2.29 to 2.65 Å. Other potential ligands are all over 3.0 Å away from the calcium ion.

Calcium—oxygen bond distances can be correlated with the coordination number. In the nine complexes in which the calcium is eight-coordinate (excluding calcium—dihydrogen ethane-1-hydroxy-1, 1-diphosphate) the average calcium to oxygen distance is 2.452 Å. In the four 7-fold coordinate compounds (excluding calcium glutamate) the average distance is 2.396 Å and in the 6-fold coordinate hexahydrate complex the distance is 2.330 Å.

In contrast, the coordination number shows no discernible relationship to the nature or electronegativity of the oxygen atom ligands. The calcium ion is usually hydrated; however, there is no water in the glucoisosaccharate crystal.

The calcium ion is only weakly electropositive. In most of the complexes examined the organic phosphate or the carboxylate anion is involved in calcium coordination; however, in the calcium—garciniate complex the 2′ carboxylate group is not in the primary coordination sphere. On average the calcium—carboxylate oxygen bond distance is no shorter than the other calcium—oxygen distances in that particular complex. In fact the calcium to water oxygen distance is on average 0.028 Å shorter than the other distances in the complex.

Oxygen—oxygen distances as close as 2.54 Å are seen in the 7 coordinate calcium—blepharismin. In three structures, coordinating oxygen atoms are

hydrogen bonded to one another as close as 2.62 Å. These distances are shorter than the canonical 2.80 Å oxygen—oxygen van der Waals' contact distance or the 2.90 Å of a strong oxygen, oxygen hydrogen bond.

In a regular square anti-prism with an oxygen—calcium distance as short as 2.40 Å the oxygen—oxygen distance is 2.77 Å. Thus calcium might be expected to be nine coordinate in some organic complexes, as it is occasionally found in the inorganic complexes.

Usually the multidentate ligands form five member rings, except in those instances where both oxygen atoms of a carboxylate group coordinate. In glucoisosaccharate, lactobionic acid and mannofuranose, six member rings are formed as a result of forming two five member rings. In ethane hydroxy diphosphonate there is a five member ring formed in coordinating one calcium, and a six member ring in coordinating another calcium ion.

The calcium ion can induce changes in the structure of a coordinating ligand. In the series of calcium—carbohydrate complexes there is good comparative data. Cook and Bugg (1973a,b,c) have observed that "calcium binding to adjacent hydroxyl groups results in a decrease of about 0.2 Å in the intermolecular spacing between the hydroxyl oxygen atoms". In contrast, in the preliminary descriptions of the cyclic xylose and mannofuranose structures there is no indication of the hydroxyl groups being drawn together, nor is there any evidence in the structures of linear arabonate and glucoisosaccharate.

Carboxylate groups coordinate oxygen with either one or with both oxygen atoms (e.g. glycylglycylglycine or glutamate). Of particular relevance to the protein thermolysin, with a double calcium site, and to concanavalin A, with a calcium—manganese site, four of the structures examined, garciniate, glycylglycylglycine, thymidylate and glutamate, have calcium—calcium distances ranging from 3.624 to 4.792 Å. In each of these double calcium structures a single oxygen atom of either a carboxylate or a phosphate group, coordinates both calcium ions.

Calcium itself can function to link together the coordinating ligands. In glucoisosaccharate, arabonate and gluconate, two molecules coordinate calcium without forming chains. Due to the shape of the sugar rings not all of the oxygen atoms of one molecule can chelate a single calcium ion. Usually one dimensional chains or two dimensional sheets are formed of the alternate pattern: sugar, calcium, sugar, etc. In the glycylglycylglycine complex, four different tripeptides coordinate a single calcium ion and conversely a single tripeptide coordinates four different calcium ions, thereby building up a three dimensional network.

B. Protein calcium complexes

1. Muscle calcium binding parvalbumin

MCBP is found in the white muscle of chordates, has pI about 4.5, MW about 11500 and binds two calcium ions, pK_d 6.7. Although its specific function remains unknown, it appears to be modulated by Ca^{2+}. The crystal structure of

carp MCBP, component 3, has been determined and refined to 1.9 Å resolution by Moews and Kretsinger (1975a). The structure consists of six α-helical regions, A—F. A calcium ion is bound in the loop between helices C and D. A second Ca^{2+} is bound in the EF region, referred to as the "EF-hand".

Six ligands coordinate the CD calcium ion. The ligands can be assigned to the vertices of an octahedron (Fig. II-1), where the +X and +Y vertices are defined as being first and second in linear sequence. The EF calcium ligands can also be assigned octahedral vertices; however the EF calcium is formally eight coordinate because the carboxylate groups of Asp-92 and Glu-101 coordinate with both oxygen atoms. This octahedral representation, Fig. II-1, shows that:

(i) The register of ligands is the same in both loops.

(ii) The CD and EF octahedra are related by an intramolecular approximate two fold axis.

(iii) All ligands are oxygen atoms of side groups except at the —Y vertex where the peptide oxygen of Phe-57 and Lys-96 coordinate calcium.

(iv) All ligands of the CD calcium come from the protein; the —X ligand at the EF site is H_2O; residue 98 is glycine.

These characteristics are summarized in Table II-3 as are the coordination geometries for the other calcium binding proteins of known or predicted structure. Note that there are four carboxylate groups in both primary coordination spheres with no additional cations in the immediate vicinity. Moews and Kretsinger (1975b) showed by difference Fourier techniques that Tb^{3+} could completely replace the EF calcium under nondenaturing conditions without replacing any of the CD calcium.

MCBP amino acid sequences have been determined for carp (Coffee and

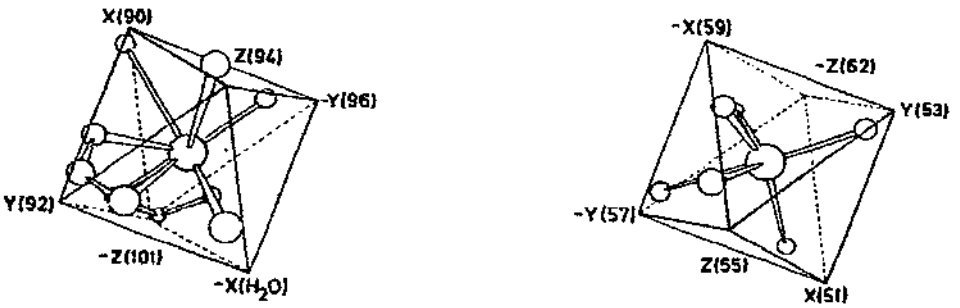


Fig. II-1. Calcium coordination octahedra of MCBP. The CD calcium ion is coordinated by oxygen atoms from six amino acids (Asp-51, Asp-53, Ser-55, Phe-57, Glu-59 and Glu-62) and EF by five amino acids (Asp-90, Asp-92, Asp-94, Lys-96 and Glu-101) and by water. Residues 92 and 101 coordinate with both oxygen atoms of their carboxylate groups. The first and second residues in sequence define the +X and the +Y vertices of the two octahedra. The octahedra are related by the intramolecular two fold axis; they are viewed from the outside of the molecule. The register of amino acids is the same in both octahedra. At the —Y vertices (Phe-57 and Lys-96) peptide oxygen atoms coordinate calcium. The CD calcium is not accessible to solvent, while the —X vertex of the EF octahedron is water (residue 98 is glycine).

TABLE II-3

Protein calcium crystal structure summary

Protein		X	Y	Z	−Y	−X	−Z	$-CO_2$	No.	C=O	H_2O	pK_d
MCBP	CD	D-51	D-53	S-55	F-57	E-59	E-62	4	6	1	0	~6.7
	EF	D-90	[D-92]	D-94	K-96	H_2O	[E-101]	4	8	1	1	~6.7
TN-C	1	D-27	D-29	H_2O	D-33	S-35	E-38	3	6	1	1	5.5, 7.5
	2	D-63	D-65	S-67	T-69	D-71	E-74	4	6	1	0	5.5, 7.5
	3	D-103	N-105	D-107	Y-109	D-111	E-114	4	6	1	0	5.5, 7.5
	4	D-139	N-141	D-143	R-145	D-147	E-150	4	6	1	0	5.5, 7.5
ALC	α	D-59	T-61	D-63	K-65	T-67	Q-70	2	6	1	0	?
	γ	D-136	E-138	H_2O	V-142	(M-144)	E-147	3	6	1	0	?
	σ	Q-171	D-173	N-175	C-177	N-179	(H_2O)	1	(6)	1	(1)	?
Con-A		D-10*	Y-12	D-19*	H_2O	N-14	H_2O	1	6	1	2	3.3
Nuclease		D-19	D-21	H_2O	E-43	D-40	T-41	4	6	1	1	~3
Thermo-	1	D-138	E-177*	H_2O	E-187	D-185*	E-190*	2.5	6	1	1	4.7
lysin	2	E-177*	N-183	D-185*	E-190*	H_2O	H_2O	1.5	6	1	2	4.7
	3	D-57	D-59	Q-61	H_2O	H_2O	H_2O	2	6	1	3	>6
	4	Y-193	T-194	T-194	$2H_2O$	I-197	D-200	1	7	3	2	>6

As discussed in the text, Ca^{2+} is six coordinate in most proteins of known (or inferred) structure. The MCBP EF calcium is eight coordinate since D-92 and E-101 coordinate with both oxygen atoms of their carboxylate groups. Thermolysin calcium 4 is seven coordinate; there are two water ligands approximately in the −Y direction. Underlined amino acids coordinate with peptide oxygen atoms. Octahedral vertices X and Y are assigned to the first two residues in sequence. An "*" indicates that the two oxygen atoms of a carboxylate group coordinate two different metal ions. C Cys, D Asp, E Glu, F Phe, I Ilu, K Lys, M Met, N Asn, Q Gln, R Arg, S Ser, T Thr, V Val, Y Tyr.

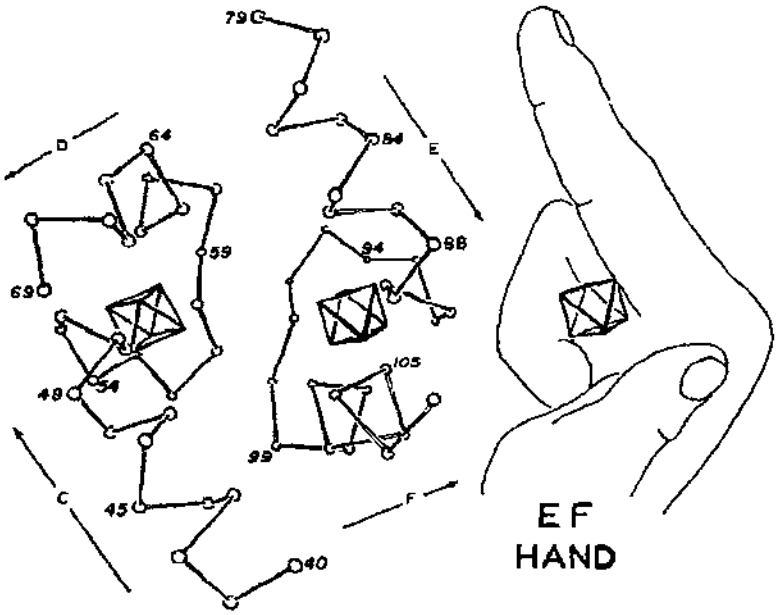


Fig. II-2. CD and EF regions of MCBP. The C helix, the CD calcium binding loop and the D helix of carp MCBP are related by an intramolecular approximate two-fold axis to the EF region. The loops are in β-antiparallel sheet conformation and are connected by one hydrogen bond, residues 58-97. To the right is drawn a symbolic "EF hand". The hands are viewed from the inside of the molecule.

Bradshaw, 1973), hake (Capony et al., 1973), pike (Frankenne et al., 1973), frog and *Coelocanth* (Demaille et al., 1974) and rabbit (Enfield et al., 1975). The calcium binding loops are invariant. Residues Arg-75 and Glu-81 are invariant and form an internal polar hydrogen bond. Gosselin-Rey et al. (1973) reacted Arg-75 with 1,2 cyclohexandione and observed loss of two antigenic determinants, reduced α-helix content and a loss of half of the Ca^{2+} binding capacity of MCBP. Consistent with this Donato and Martin (1974) found that the EGTA removal of both calcium ions made the sole —SH group, which is only 5 Å from the Arg-75, Glu-81 hydrogen bond, much more reactive to DTNB. Nelson et al. (1975) attached a trifluoroacetonyl ^{19}F-NMR label to this —SH group and showed that it was sensitive to Ca^{2+} release. Even though the Arg-75, Glu-81 salt bridge is 25 Å from the calcium ions its conformation is significantly changed upon Ca^{2+} binding.

MCBP evolved from a "primitive" EF hand (Fig. II-2) by repeated gene duplication and fusion. The AB region subsequently had two amino acids deleted and lost its Ca^{2+} binding ability. Given the basic characteristics of the EF hand (helix, Ca^{2+} binding loop, helix), Collins et al. (1973) easily identified four EF hand regions within TN-C (the calcium binding component of troponin, see

TABLE II-4

Amino acid sequences of MCBP, TN-C and ALC [a]

MCBPAB	$_{6}$L N D A D I A A A L E A C K A A D S F D H K A F F A K V_{33}
MCBPCD	$_{38}$K S A D D V K K A F A I I D Q D K S G F I E E D E L K L F L Q N F K A D A R A_{70}
MCBPEF	$_{77}$L T D G E T K T F L K A G D S D G D G K I G V D E F T A L V K A_{108}
TN-C 1	$_{14}$E M I A E F K A A F D M F D A D G G G D I S V K E L G T V M R M L G Q T_{49}
TN-C 2	$_{50}$P T K E E L D A I I E E V D E D G S G T I D F E E F L V M M V R Q M K E D A K G_{89}
TN-C 3	$_{90}$K S E E E L A E C F R I F D R N A D G Y I D A E E L A E I F R A S G E H_{125}
TN-C 4	$_{126}$V T D E E I E S L M K D G D K N N D G R I D F D E F L K M M E G V Q_{159}
ALC α	$_{46}$E Q Q D E F K E A F L L Y D R T G D S K I T L S Q V G D V L R A L G T_{80}
	G S K
ALC β	$_{81}$N P T N A E V K K V L N P D E Q M N A K I E F E Q F L P M L Q A I S N N K D Q_{122}
ALC γ	$_{123}$G T Y E D F V E G L R V F D K E D G T V G M G A E L R H V L A T L G E_{157}
ALC δ	$_{158}$K M K E E E V E A L M A G Q E D S N G C I N Y E A F V K H I M S I_{190}
LIGANDS	X Y Z −Y −X −Z

[a] The sequences are aligned to show the homologous EF hand region. There has apparently been a deletion of two amino acids in the AB region of MCBP and an insertion of three in the β region of ALC. The underlined residues are (supposedly) involved in calcium coordination; the coordination octahedra are shown in Fig. II-1. The vertical lines indicate the (supposed) inner aspects of the α-helices. (Ala A, Arg R, Asn N, Asp D, Cys C, Gln Q, Glu E, Gly G, His H, Ile I, Leu L, Lys K, Met M, Phe F, Pro P, Ser S, Thr T, Trp W, Tyr Y, Val V)

section II.B.2.). Kretsinger (1974) predicted that the TN-C structure consists of two pairs of EF-hands.

Considering partial sequence data, Collins (1974) identified one EF hand in the alkali extractable myosin light chain (see section IV.C.2.a). Subsequently considering complete data, Weeds and McLachlan (1974) and Tufty and Kretsinger (1975) identified four EF hand regions in ALC (Table II-4). Its tertiary structure is very probably similar to that of TN-C. It seems apparent that these evolutionary considerations will yield further insights into the structures and functions of calcium control proteins.

2. Troponin, calcium binding component

The troponin complex of muscle (see section IV.C.2.a.) is a "trimer" of three separate proteins, one of which, TN-C (MW $\simeq$ 17846), binds four calcium ions (Potter and Gergely, 1974), with pK_d values in the range 5.5 to 7.5. In the predicted structure of TN-C (Kretsinger and Barry, 1975), the four calcium ions of TN-C are coordinated in two pairs of octahedra as illustrated for MCBP in Fig. II-1. The "hands" of each pair are related by a two fold axis as are the CD hand and EF hand of MCBP. Further, the two fold axis of the TN-C hands 1 and 2 is colinear with the axis of hands 3 and 4, but the senses of the axes are opposite. That is, the entire molecule possesses 222 symmetry with the 1,2 and 3,4 calcium binding regions some 40 Å from one another (see Fig. II-3). Note in Table II-3 that the same register of ligands is maintained in all four octahedra and that the —Y vertex apparently employs a peptide oxygen atom and not a side group oxygen as is found at the other vertices.

The main ligating oxygens of calcium in TN-C are derived from the carboxyl groups of glutamic and aspartic acid residues; however, two hydroxyl oxygens of serine (Ser-35 and Ser-67) and the amide oxygen of asparagine-104, as well as the four main chain carbonyl groups (Asp-33, Thr-69, Tyr-108 and Arg-145), also participate in calcium coordination. A water molecule also coordinates calcium, along the +Z vertex, at site 1.

3. Staphylococcal nuclease

The rather nonspecific, extracellular nuclease from *Staphylococcal aureus*, functions physiologically to hydrolyze both DNA and RNA to 3'-mononucleotides. Nuclease binds a single calcium ion with an apparent pK_d of 3. This calcium is essential both for substrate binding and for enzymatic catalysis (see review by Anfinsen, 1971).

The crystal structure of nuclease has been determined by Cotton et al. (1971) to nearly 2 Å resolution. Calcium coordination in the protein (Fig. II-4) is roughly octahedral in nature, with coordinating ligands from carboxyl groups of glutamate (Glu-43) and aspartate (Asp-19, Asp-21 and Asp-40) residues, and the main chain carbonyl oxygen of threonine-41. Both oxygen atoms of the Asp-21 carboxylate group may be involved in calcium coordination. A sixth liganding group, possibly a water molecule, directed toward the +Z octahedral vertex (Table II-3), has not been identified due to the noise level in this

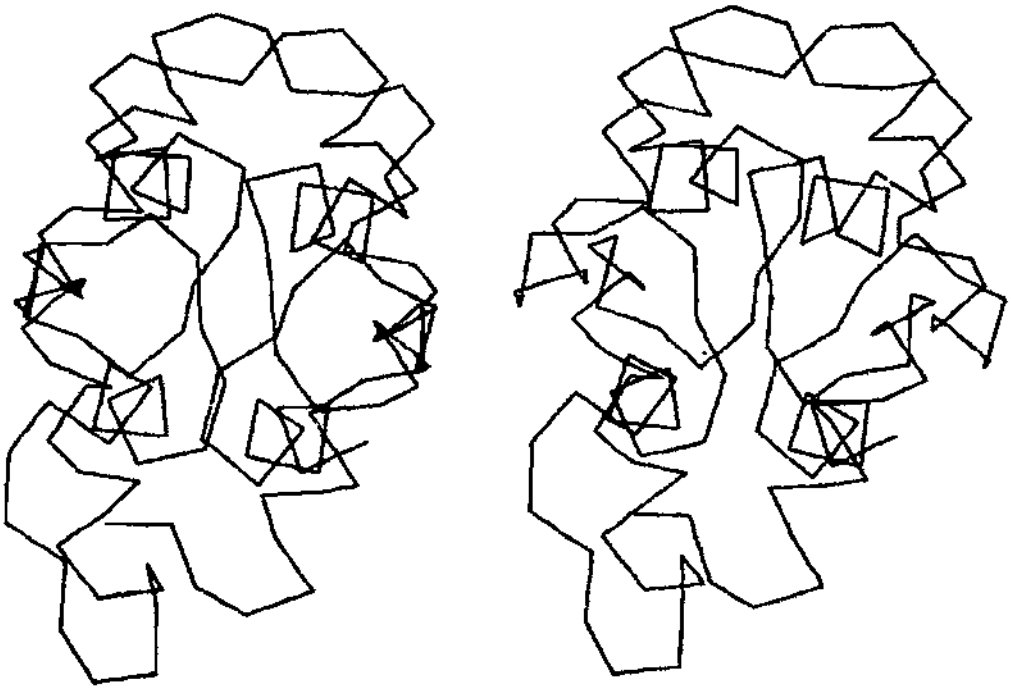


Fig. II-3. Predicted structure of TN-C. The assumptions underlying the prediction (Kretsinger and Barry, 1975) are: (1) TN-C contains four EF hands (see figure II-2) as indicated by the amino acid sequence (Table II-4). (2) The hands are arranged in pairs as are the CD and the EF regions of MCBP. (3) The pairs of hands interact at their hydrophobic surfaces with their respective two-fold axes coinciding but in opposite senses. Interactive graphics model building was used to get the indicated fit.

region of the electron density map. In this regard, it should be noted that the calcium ion does not bind directly to the substrate nucleotide phosphate group, but appears to coordinate through the postulated bridging water at the +Y vertex.

4. Concanavalin-A

Con-A is a lectin product of the jack bean, *Canavalia ensiformis*. While the function of this lectin is not established, Con-A has found practical utility as an agglutinating agent for cells transformed by oncogenic viruses (Inbar and Sachs, 1969) and as an inhibitor of cellular growth in malignant tumors (Powell and Leon, 1970). Con-A binds carbohydrates, and its biological effects are probably associated with its sugar binding properties. The protein binds both calcium and manganous ions, at sites over 20 Å removed from the carbohydrate binding site. The metal ions are essential for sugar binding, (Kalb and Levitzki, 1968).

Con-A (sub-unit MW 25200) exists as a dimer between pH 3.5 and 5.6 and

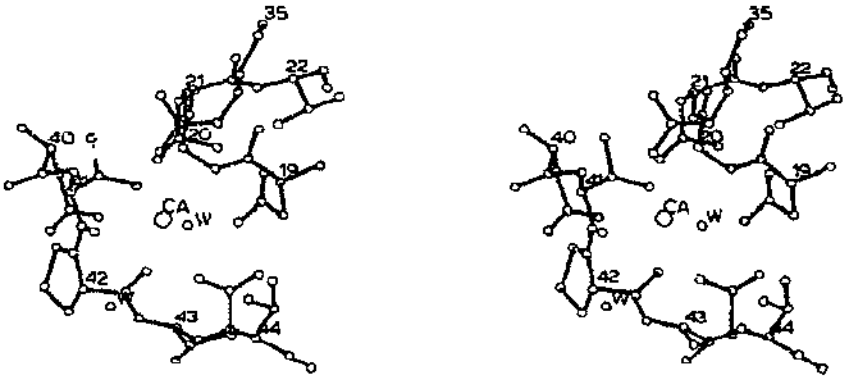


Fig. II-4. Calcium coordination in Staph. nuclease. Refer to Table II-3 for assignment of ligands. Reprinted by permission of Cotton et al. (1971).

as a tetramer at higher pH. Each sub-unit of Con-A has a single calcium ion binding site ($pK_d = 3.3$) and a transition metal (Mn^{2+}) binding site. An electron density map of Con-A at 2 Å resolution has been reported by Edelman et al. (1972) and Becker et al. (1975) and one at 2.4 Å resolution by Hardman and Ainsworth (1972). The environment of the calcium and manganous ions is shown in Fig. II-5. The calcium ion is octahedrally coordinated by carboxyl groups from glutamate (Glu-202) and aspartate (Asp-10 and Asp-19) residues, the amide oxygen of asparagine-14 and the main chain carbonyl oxygen of tyrosine-12. A sixth calcium liganding group, possibly a water molecule directed toward the —Y octahedral vertex (Table II-3), has not been confirmed. The carboxyl groups of Asp-10 and Asp-19 also coordinate the manganous ion, which is located 4.3 Å from the calcium. The second oxygen of the carboxylate binds Mn^{2+}.

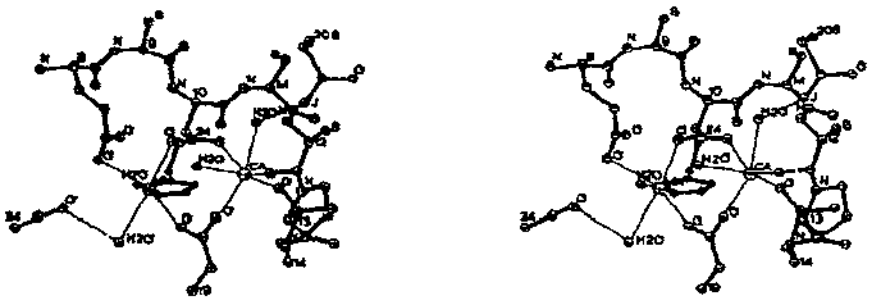


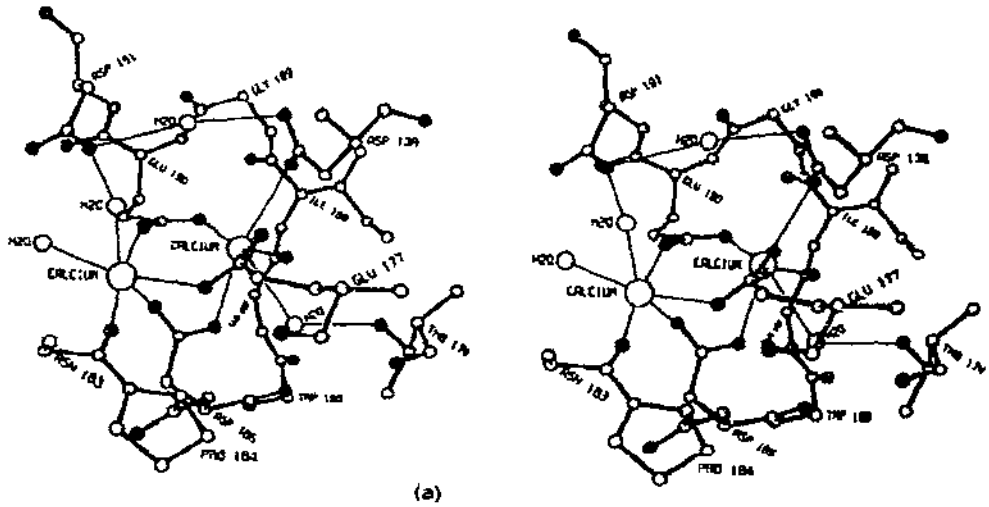
Fig. II-5. Calcium coordination in Concanavalin-A. Refer to Table II-3 for assignment of ligands. Reprinted by permission of Hardman (1973).

5. Thermolysin

Thermolysin is a heat stable, neutral protease, secreted by *Bacillus thermoproteolyticus*. Thermolysin (MW 37500) binds four calcium ions with high affinity; two calciums, Ca_1 and Ca_2, bind with a pK_d of 4.7 and a Hill coefficient of 2.0, while C_3 and C_4 apparently bind with $pK_d > 6$ (Voordouw and Roche, 1974). The protein also binds a single zinc ion. If three or four calcium ions are removed, catalytic activity is not greatly altered, but the protein becomes quite heat labile (Feder et al., 1971).

The X-ray structure of thermolysin has been determined to 2.3 Å resolution (Matthews et al., 1974) and lanthanide ions (section III.C.5.) have been used to replace calcium in a number of crystallographic studies (see Matthews and Weaver, 1974 and references therein). The four calcium ion binding sites of thermolysin are shown in Fig. II-6a.,b. and c. For Ca_1, Ca_2 and Ca_3, the primary protein ligands are the carboxyl groups of glutamate and aspartate residues (Table II-3). One to three water molecules and a single main chain carbonyl oxygen atom also coordinate the calcium at each of these sites. Site 4 is somewhat unusual since, apparently, only one carboxyl group is involved in calcium binding, the other ligands being derived from one water molecule, the hydroxyl oxygen of threonine-194 and the main chain carbonyl oxygens of Tyr-193, Thr-194, and Ilu-197. The Zn^{2+} ion is at the active site of protein, 13.7 Å from the nearest calcium ion, Ca_1.

Calcium ions at sites 1 and 2 are only 3.8 Å apart, and form what is termed a "double binding site". Ca_3 and Ca_4, in contrast, bind at single sites, far removed from each other and from the Ca_1—Ca_2 double site. The Ca_1—Ca_2 double site is illustrated in Fig. II-6c., where it is seen that three carboxyl groups (Asp-185, Glu-177 and Glu-190) bridge Ca_1 and Ca_2. The primary sphere for



(a)

Fig. II-6a.

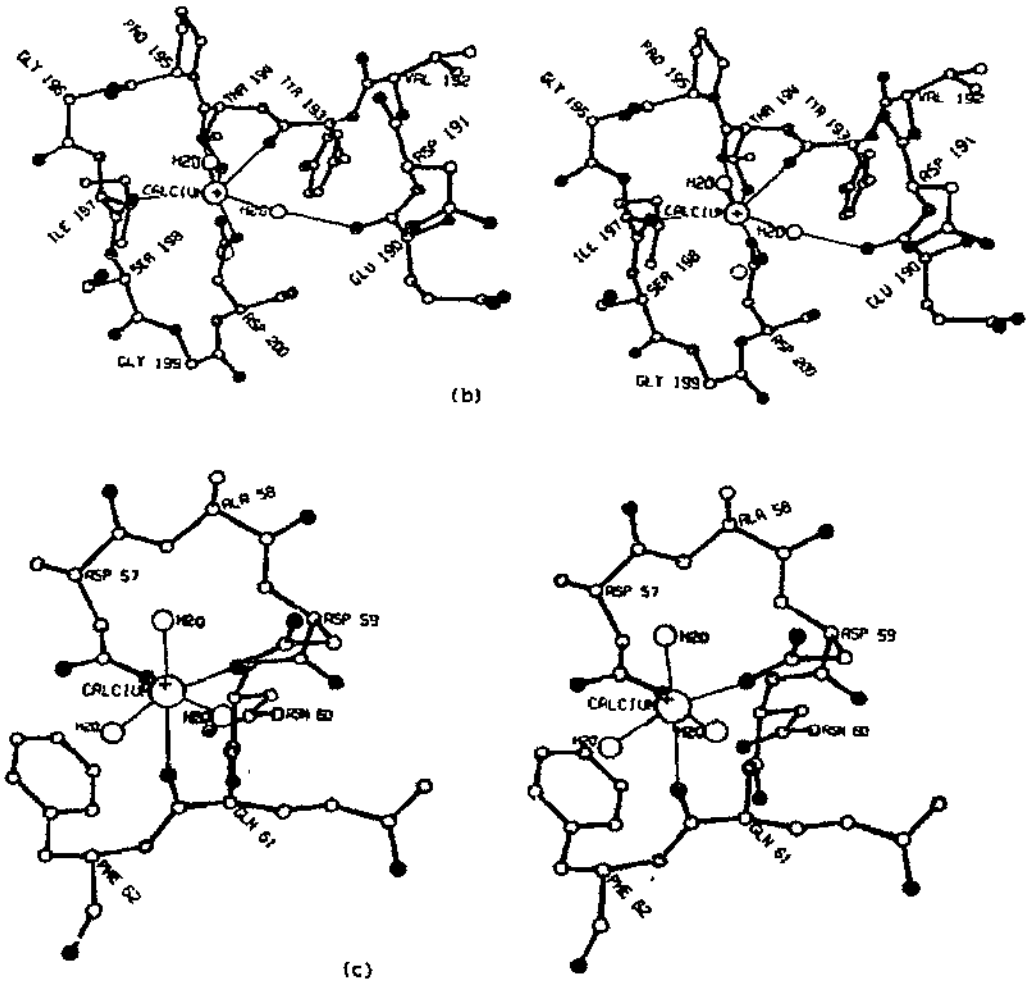


Fig. II-6. Calcium coordination in thermolysin. Refer to Table II-3 for assignment of ligands. In Fig. II-6(a) the double site with calcium ions 1 and 2 is sbown. Calcium 3 is shown in II-6(b) and calcium 4 in II-6(c). Reprinted by permission of Matthews and Weaver (1974).

both Ca_1 and Ca_2 can best be described as an octahedron, with a common octahedral face between the two calciums. It is the nature of this metal ion binding arrangement, which essentially bridges two lobes of the molecule, that imparts structural reinforcement to thermolysin, most clearly manifested in its heat resistant properties.

6. General features of protein calcium coordination

The coordination of calcium in the four proteins of known structure, as well as the postulated coordination by TN-C, is summarized in table II-3. All

of the geometries can be represented in terms of the octahedral vertices described for MCBP. As noted, in MCBP the carboxylates of Asp-92 and Glu-101 apparently coordinate calcium with both oxygen atoms, as does Asp-21 of nuclease. However, at the resolution used in protein studies it is usually impossible to define precisely the orientation of these carboxylate groups, unless the crystal structure has been refined.

Two of the carboxylate groups in Con-A bridge calcium and manganous ions separated by 5.3 Å. In thermolysin Ca_1 and Ca_2 are 3.8 Å apart, bridged by three carboxylate groups. In neither of these bridge structures does it appear, however, that a single oxygen atom coordinates both metal ions.

The net charge on the primary coordination sphere ranges from −4 at several sites to −1 at thermolysin site 4. As seen in the small molecule complexes (Table II-1) the coordination charge can be as low as zero. It does seem probable that the −4 charge for the coordination spheres found in some protein complexes binds the calcium ion more strongly. Although one might expect the calcium binding affinity to correlate with the number of coordinating carboxylate groups, such a correlation is not in fact revealed in the published pK_d values.

The X-ray data suggest that in all of the calcium binding sites, except thermolysin site 4, one carbonyl oxygen coordinates calcium. In each case the coordinate system has been defined by assigning the +X octahedral vertex to the first liganding amino acid in sequence and +Y to the second, and so forth. The trace of the main chains of nuclease, Con-A, and thermolysin are all different from that observed in MCBP. No redefinition of the axes, and in particular, no common assignment of the liganding carbonyl oxygen to the −Y vertex, improves the polypeptide main chain fit. Nature has evolved many different ways of wrapping a protein around a calcium ion.

In the context of the present discussion, the most important points for calcium coordination in proteins are:

i. Coordination numbers in protein complexes with calcium are usually six, whereas in the small organo complexes calcium is usually eight or seven coordinate.

ii. The proteins tend to use approximate octahedral geometry.

iii. No nitrogen ligands have been described; it is possible that the β-region of myosin light chain employs a methionine sulfur to coordinate Ca^{2+}. Over half of all oxygen ligands come from carboxylate groups. Water ligands range from zero to three.

iv. In all known calcium—protein complexes, the protein main chain (i.e. carbonyl group oxygen atom) is also involved in calcium coordination.

v. There is no obvious correlation between the pK_d for Ca^{2+} and the number of carboxylate ligands.

III. DETERMINATION OF CALCIUM IN BIOLOGICAL SYSTEMS

A. Introduction

As will be elaborated in section IV on "Physiological Processes Involving Calcium", research progress has been limited by the seemingly mundane obstacle of calcium determination. If we are to understand the key informational role played by the calcium ion in biological systems, it is imperative to quantify calcium ion levels in the system. Since calcium cannot be measured or monitored directly by either optical or magnetic spectroscopy, workers have had to resort to more subtle, indirect approaches. In most eukaryotic organisms the concentration of free calcium ion in the cytosol is 10^{-7} M. Hence many in vitro studies must be conducted at very low calcium concentration, often in the presence of 10^{-3} M Mg^{2+} or Na^{+}, concentrations which may potentially interfere with attempts to measure calcium by some techniques. Finally the calcium concentration within individual cells is precisely controlled. One must be able to measure the free ion concentration, as opposed to total calcium, within individual cells.

We feel it is important to present a brief survey of the various analytical tools which are currently available for measuring calcium ion levels and interactions in biological systems, along with their drawbacks and possible developments.

B. Calcium concentration determination

1. Calcium ion selective electrodes

Ion selective electrodes function satisfactorily in determining calcium ion levels in the concentration range 10^{-1} M to 10^{-5} M (see reviews by Carr, 1972, and Buck, 1974), and have been employed in a number of biological studies (Chance and Yoshioko, 1966, Perris and Whitfield, 1967, Woodward and Davidson, 1968, Moore, 1970 and Wells, 1972). The usefulness of calcium determinations with Ca^{2+} ion-specific electrodes is related to both the pCa range of interest and the pH of the system. The reliability of the measurements is strongly dependent on careful calibration of the electrode with standard calcium solutions, temperature control and suitable precautions against possible interferring ions, such as Mg^{2+}, Ba^{2+} and Ni^{2+}.

2. Emission spectroscopy

Standard emission spectroscopy procedures, such as flame emission, helium glow photometry, d.c. and high voltage a.c. arc emission and condensed spark emission spectroscopy, can be employed to obtain at least a semi-quantitative analysis for the calcium content of a sample (see review by Grove, 1972, Mika and Török, 1973, Barnes, 1974 and Winefordner and Vickers, 1974). Haljamäe

and Wood (1971) described an ultramicro emission flame photometer, suitable for the analysis of picogram quantities of Ca^{2+} in biological systems. Shemet et al. (1972) reported a sensitivity of 5×10^{-6} M Ca^{2+} for microliter sample volumes. An example of a biological application of flame emission can be found in the work of Chuang et al. (1973) in a comparative study of Na^+, K^+ and Ca^{2+} levels in blood serum.

3. Atomic absorption spectroscopy

Calcium ion levels have most commonly been determined by atomic absorption spectroscopy (Willis, 1963 and Winefordner and Vickers, 1974). Atomic absorption is more sensitive than flame photometry, since it is an absorption phenomenon with irradiation at a single wavelength. A detection limit of 10^{-7} M Ca^{2+} is possible with an irradiation wavelength of 4227 Å. One must be cautious, however, of possible quenching from other metals that may be present. The technique is as easy to use as flame photometry although the equipment is considerably more expensive.

Flame atomic absorption spectroscopy has been employed routinely to assay calcium concentration in numerous biological systems (Cohen and Selinger, 1969, Feder et al., 1971, Bowers and Pybus, 1972 and Benzonana et al., 1972 and the review by Bek and Sychra, 1971). Kuntziger et al. (1974) described a new flameless atomic absorption apparatus, particularly suited to determining picomole amounts of calcium in the nanoliter volume range. The authors applied the instrument in an investigation of calcium ion levels in late proximal renal tubular fluid and plasma ultrafiltrate samples from normal and parathyroidectomized rats. The procedures outlined by Kuntziger et al. yielded satisfactory reproducibility, with no apparent interference from Na^+, K^+, Mg^{2+}, and inorganic phosphate present at biological levels. This procedure might be applied to single cells.

4. Calcium ion buffers

In many biological studies involving calcium both in vitro and in vivo, it is essential to distinguish total calcium from the free ion. Further, in many systems of physiological interest the $[Ca^{2+}]_{free}$ is in the range of 10^{-5}—10^{-8} M. Since it is extremely difficult to measure $[Ca^{2+}]_{free}$ as opposed to $[Ca^{2+}]_{total}$ in this range without disturbing the Ca^{2+}—protein equilibrium, one can often "set" the $[Ca^{2+}]_{free}$ with a Ca^{2+} buffer just as one uses a H^+ buffer. Again by analogy with pH buffers, one can control the pCa of a reaction mixture with a calcium buffer. This is important not only because of the difficulty of measuring pCa in the range 5—8, but also because so many reagents, and even plastics and of course glassware, are inevitable sources of contaminating Ca^{2+}. The general utility of "metal ion buffers" in maintaining desired levels of free metal ion has been discussed by Raaflaub (1960). Calcium—EGTA buffers (see Figs. III-1 and III-2) have been employed by Weber et al. (1966) to study calcium fluxes

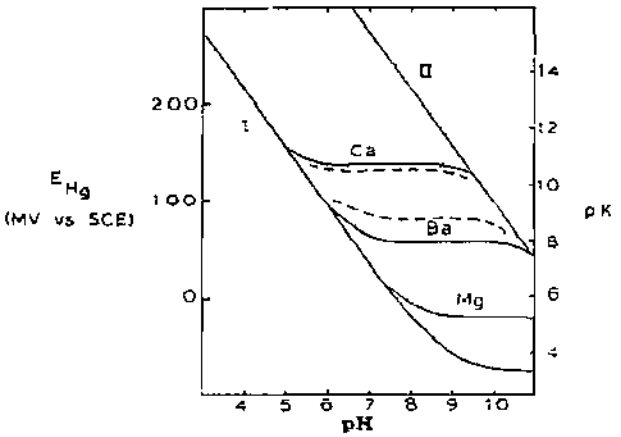


Fig. III-1. Cation selectivity of EGTA. Potential-pH diagram from a potentiometric titration for ethylene glycol bis-(β-amino-ethyl ether)-N, N'-tetraacetic acid (Z) system.

I. Lower limit of electrode potential in certain pH ranges according to

$$HgZ^{2-} + nH^{+} + 2e^{-} \rightarrow Hg^{0} + H_nZ^{n-4}$$

(0.001 M) (0.001 M)

II. Electrode reaction

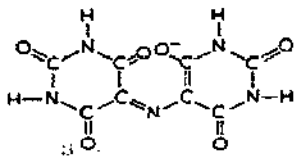
$$HgO + H_2O + 2e^{-} \rightarrow Hg^{0} + 2OH^{-}$$

For the solid curves labeled Ca^{2+}, Mg^{2+}, and Ba^{2+}, the potential corresponds to solutions containing 0.001 M Me^{2+}, 0.001 M HgZ^{2-} 0.001 M HgZ^{2+}, and 0.1 M $NaClO_4$. For comparison, the dotted curves illustrated correspond to the potential curves for EDTA with calcium and magnesium. Redrawn by permission of Schmid and Reilley (1957).

in rabbit sarcoplasmic reticulum and by Portzehl et al. (1964) to investigate the influence of free calcium ions on the contractile cycle of muscle fiber from the crab *Maia squinado*.

5. Calcium by spectrophotometric assay

Various reagents have been found to react colorimetrically with calcium ion, making feasible a relatively simple spectrophotometric assay for Ca^{2+} concentration. Perhaps the most commonly used colorimetric reagent for Ca^{2+} is murexide, a metallochromic indicator with high sensitivity for calcium ion.



Direct calcium determination with murexide has the advantages of speed, accuracy and general dependability; however, it has the disadvantages of most

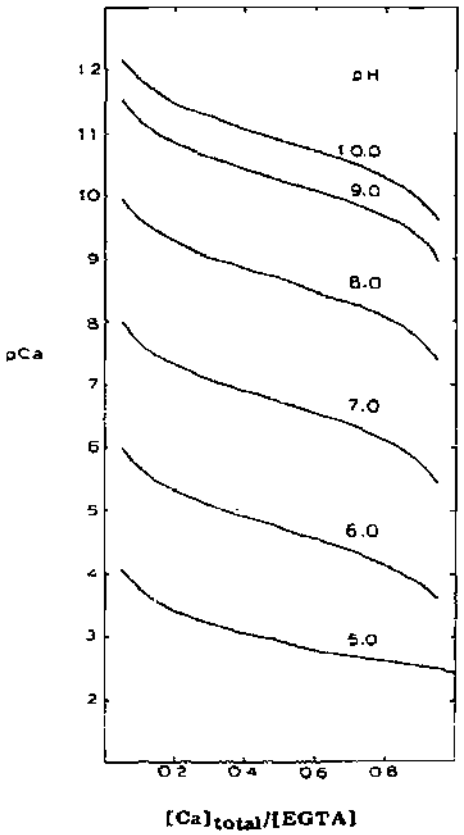


Fig. III-2. EGTA titration with calcium. EGTA as a calcium-buffer and the dependence of pCa on hydrogen ion concentration.

of the colorimetric methods, i.e. some instability of the reagent color as well as interference from most divalent (except Mg^{2+}) and trivalent cations. At low ionic strengths (ca. 2 mM), the murexide Ca^{2+} assay has a maximum sensitivity of 2×10^{-7} M (Ohnishi et al., 1972). Ohnishi et al. (1972) employed dual wavelength spectrophotometry and murexide in an elegant study of the multiple Ca^{2+} binding sites in a protein fraction (Fr-2) from rat skeletal muscle sarcotubular membranes.

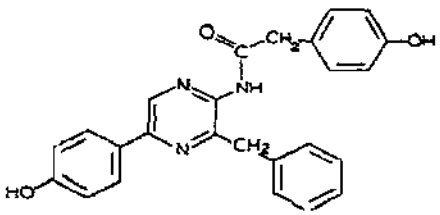
Murexide has also been used in biological systems to study kinetic processes, such as the rate and extent of calcium ion movement across membranes. Scarpa (1972) and Rottenberg and Scarpa (1974) have used the technique to study calcium uptake (and release) across mitochondria membranes. Entman et al. (1973) studied calcium uptake in sarcoplasmic reticulum. Other potentially useful calcium complexons include the glyoxal bis (2-hydroxyanil) class of reagents (Milligan and Lindstrom, 1971), sodium rhodizonate (Feigel and

Gentil, 1954 and Prokopov, 1973), chlorophosphonazo III (Howell et al., 1966) naphthalhydroxamic acid (Trinder, 1960), picrolonic acid (Mackereth, 1951), arzenazo (Polyak, 1962), and the phthaleinazo calcium complexons (Kobatake et al., 1967).

Funahashi et al. (1971) used a kinetic method to quantitatively determine 10^{-5}—10^{-3} M calcium in the presence of magnesium. In this technique, the effect of calcium on the rate constant of the ligand substitution reaction of Cu(II)—EGTA with 4-(2-pyridylazo) resorcinol is followed photometrically. This kinetic, ligand substitution technique has not been used for biological studies.

6. Aequorin spectroscopy

It is often critical to distinguish between $[Ca^{2+}]_{free}$ and $[Ca^{2+}]_{total}$ inside a cell. Ca^{2+} electrodes have not yet been miniturized; furthermore, they are not sensitive at concentrations below pCa 5. None of the colorimetric reagents are adequately sensitive and specific. Some experiments can be designed in such a way that the pCa is not "measured" but "set", by micro-injection of an EDTA buffer. However, to date the only really successful method of measuring intracellular $[Ca^{2+}]$ in living cells involves the micro-injection of the photoprotein aequorin, followed by microspectrophotometry. Aequorin is a bioluminescent photoprotein (Shimomura et al., 1974) obtained from the jellyfish *Aequorea*. When isolated, the pure photoprotein luminesces blue light (λ = 470 nm) in the presence of calcium. The molecular weight of the protein is about 30000 (Kohama et al., 1971). The light-emitting chromophore of aequorin has been shown to be 2-(p-hydroxy-phenylacetyl)-amino-3-benzyl-5-(p-hydroxyphenyl) pyrazine (Shimomura and Johnson, 1972 and 1973, Kishi et al., 1972).



The ionic calcium assay developed by Shimomura et al. (1963) is both highly sensitive (i.e. calcium concentration as low as 10^{-7} M in 2 μl sample volumes can be determined) and highly selective for Ca^{2+} (i.e. biological levels of Na^+, K^+, Mg^{2+} and inorganic phosphate produce no interference). Sr^{2+} also luminesces with aequorin, but this is of little consequence in systems of biological interest. Aequorin spectroscopy has been employed to investigate the rapid calcium fluxes within a single muscle fiber during a contractile cycle (Ashley and Ridgway, 1970 and Ashley, 1970), calcium levels in saliva and serum (Izutsu et al., 1974) and in a study measuring the rise in intracellular

calcium following illumination of the ventral eye of *Limulus* (Brown and Blinks, 1974).

7. ^{45}Ca radioactivity measurements

There is one common radioactive isotope of calcium, ^{45}Ca. It is an easy isotope to use because of its long half life (165 days) and its β emission. Further, it is inexpensive and can be made with high specific activity. $^{45}Ca^{2+}$ can be incorporated into the metal ion binding sites (s) of calcium—binding proteins by a number of techniques, including equilibrium dialysis (see below), and bound calcium determined in a scintillation spectrometer. References to the more routine applications of $^{45}Ca^{2+}$ in biological systems will be deferred to the appropriate parts of sections III. C. 1., 2. and 3., where we discuss various experimental procedures involved in the determination of protein-bound calcium.

A particularly novel application of $^{45}Ca^{2+}$ involves the measurement of calcium exchange in a single layer of rat cardiac cells (Langer et al., 1969, Langer and Frank, 1972 and Langer et al., 1974). The prime objective of the study was to exploit the sensitivity (and convenience) of radioactive tracer techni-

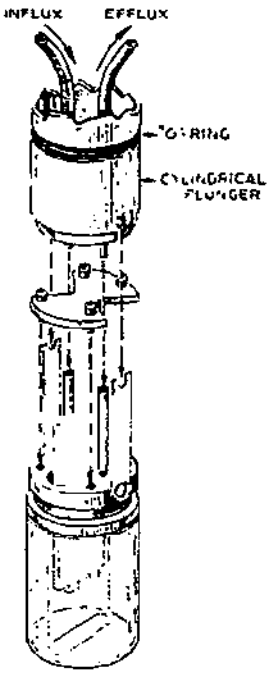


Fig. III-3. Scintillation vial flow cell. Flow cell and scintillator slide. The slide is shown during insertion into the flow cell. Following insertion, the cap (with four screws) is tightened in place, the tubular fittings on the plunger are inserted in the flow cell tubes and the plunger is attached to metal strap-fittings on each side of the cell. The flow cell is then ready for insertion into the spectrometer well. Reprinted by permission of Langer et al. (1969).

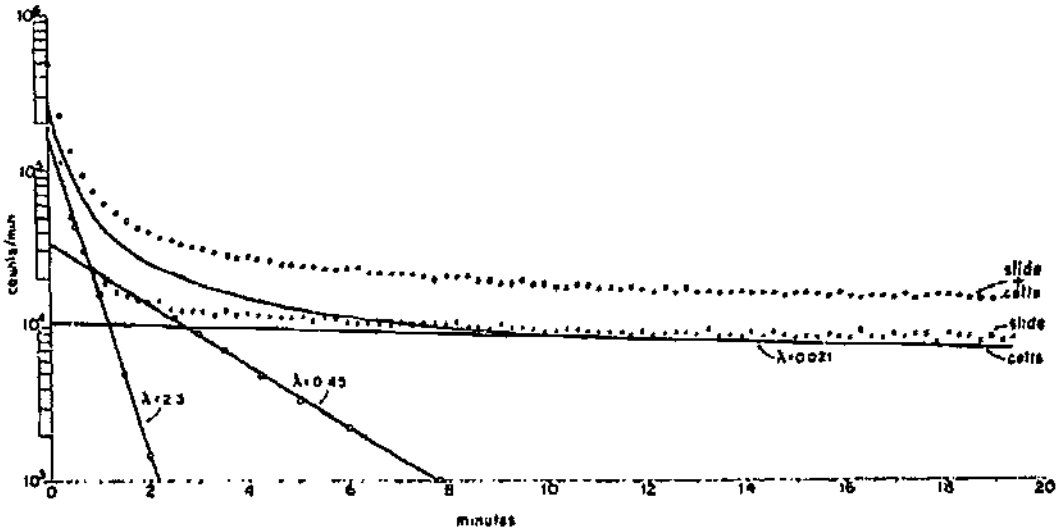


Fig. III-4. Calcium release from the scintillation flow cell. ^{45}Ca washout (plotted semilogarithmically) from scintillator slide with and without cells. The slide, with cells attached, was labeled with ^{45}Ca for 30 min, then washed out for 20 min (solid circles). Cells were then removed, and the slide was rinsed with EDTA and then relabeled with ^{45}Ca for 30 min. It was then washed out for 20 min (X). The second washout curve (X) was then subtracted from the first (solid circles) to obtain the washout curve for the cells alone (solid line). This is resolved into three exponential components with rate constants (λ) of 2.3, 0.45 and 0.021 min^{-1}. Reprinted by permission of Langer et al. (1969).

ques in the study of a complete physiological process, in this case, a single contractile cycle. The procedure involves attachment and growth of a layer of contractile cardiac cells on a slide made of scintillator material. The slide is then mounted in a scintillation vial-flow cell (Fig. III-3). The flow cell, through which $^{45}Ca^{2+}$ can be introduced or "washed out", is next placed into the well of a scintillation spectrometer, prior to the measurement of isotopic activity of the cellular layer during calcium uptake or release. Representative results of a calcium release experiment from the study by Langer et al. (1969) are shown in Fig. III-4).

8. Electron microscopy

Glutaraldehyde and osmium tetraoxide have been employed for many years in the routine fixation of tissue samples prior to electron microscopy. The osmium, which greatly enhances visual contrast upon staining, has been shown, however, to "mask" certain cellular components. When Oschman and Wall (1972) examined intestinal epithelial tissue which had been fixed with glutaraldehyde but not osmium stained, they did, in fact, observe dense, electron opaque deposits adjacent to membraneous structures (Fig. III-5). These electron opaque regions correlate with the expected localization of calcium

Fig. III-5. Calcium deposition in insect intestine. Unstained section of apical surface of a caecal cell fixed in glutaraldehyde plus 5 mM l^{-1} calcium chloride. Unosmicated tissue. Electron opaque deposits occur within microvilli (MV) and along apical (APM) and lateral (LPM) membranes. Magnification x 56000. Reprinted by permission of Oschman and Wall (1972).

within living tissues, as physiological data indicate that one of the primary sites of calcium localization is the cell membrane. Further, Oliveira-Castro and Barcinski (1974) observed that high intracellular concentrations of calcium inhibited cell to cell coupling, and suggested sites of calcium binding on the cytoplasmic surface of junctional membranes, consistent with the electron microscopy studies of Oschman and Wall on insect midgut calca. Oschman and Wall found no electron dense deposits near the membranes of the nuclear envelope, mitochondria, endoplasmic reticulum or muscle sarcoplasmic reticulum.

Oschman et al. (1974) applied X-ray microprobe analyses to squid axons which had been fixed in glutaraldehyde containing $5 \cdot 10^{-3}$ M $CaCl_2$ and later post-fixed with OsO_4. They used a column voltage set at 40 kV with spot size $2 \cdot 10^3$ Å and counted Ca and P K_α X-radiation. Distinct Ca and P plaques were found along the inside of the plasma membrane as well as in the mitochondria. Using the Oschman and Wall technique, Hillman and Llinás (1974) have located calcium binding sites on synaptic membranes and vesicles (see section IV. D.1.d.). We emphasize that since the material is fixed in the presence of calcium, the technique identifies potential calcium binding sites, not calcium distribution in the living tissue. Nonetheless the technique will soon be refined in terms of spatial resolution, sensitivity and sample preparation.

9. Polarography

General applications of polarography (Barnard and Chayen, 1965) to medicine, biochemistry and pharmacy can be found in the text by Brezina and Zuman (1958) and the reviews by Homolka (1971), Harrison (1972) and Pietrzyk (1974). Direct polarography has been used to study interactions between serum albumin and various metal ions, including zinc, mercury, cadmium and copper (Tanford, 1952, Saroff and Mark, 1953, and Kacena, 1954). Riboflavine—metal ion interactions (Sawyer and McGreery, 1972) and thiouracil metal complexes (Garrett and Weber, 1972) have also been studied by polarography. Calcium is more difficult to determine by polarographic techniques and indirect methods must be employed. Nakagawa and Tanaka (1962) described procedures for the quantitative determination of calcium ion by indirect selective polarography involving exchange reactions with EGTA.

C. Determination of Protein-bound calcium

In this section we summarize four classes of techniques for examining the reaction:

$$Pr + nCa^{2+} \rightleftharpoons PrCa_n^{2+} \qquad (Pr = \text{Protein}) \quad (1)$$

In the dialysis, filtration and ion exchange procedures one usually cannot distinguish directly between Pr and $PrCa_n$. One measures total protein, $[\Sigma Pr]$. Further one cannot distinguish directly free Ca^{2+} and protein bound Ca^{2+}. One measures total Ca^{2+}, ($[\Sigma Ca] = [Ca] + n[PrCa_n]$). However, by making

these measurements under appropriate conditions and subsequently analyzing the data by various plots (van Holde, 1971 and Tanford, 1961) one can determine:

i. the number of Ca^{2+} binding sites per protein molecule, n;
ii. the dissociation constants, K_d, of the various binding sites;
iii. the degree of cooperativity (Hill coefficient) for multiple Ca^{2+} binding sites.

In the various spectral techniques one cannot measure calcium directly. Instead, one measures some difference in the protein spectrum with and without calcium; [Pr] and [$PrCa_n$] are directly and instantaneously measured. If one is fortunate enough to find such a spectral difference, one can determine not only n, K_d and the Hill coefficient but also obtain kinetic information.

1. Dialysis procedures

One of the most widely used techniques for measuring Ca^{2+} binding to proteins is that of equilibrium dialysis, employing $^{45}Ca^{2+}$. Let Pr_i and Ca_i refer, respectively, to the protein and calcium species inside the dialysis sac, while Ca_o corresponds to the calcium in the dialysate, outside the sac. If a protein species, Pr, has n binding sites, the equilibrium between Pr_i and $PrCa_{n_i}$ can be expressed as follows.

$$\begin{array}{l} \quad Ca_o \\ \quad \Downarrow \\ Pr_i + n \cdot Ca_i \rightleftharpoons PrCa_{n_i} \end{array} \tag{2}$$

For the total protein concentration, $[\Sigma Pr_i]$, we can write

$$[\Sigma Pr_i] = [Pr_i] + [PrCa_{n_i}] \tag{3}$$

and for the total calcium concentration, inside the sac

$$[\Sigma Ca_i] = [Ca_i] + n \cdot [PrCa_{n_i}] \tag{4}$$

The quantity of interest for measuring the extent of calcium binding is the concentration of $PrCa_{n_i}$

$$[PrCa_{n_i}] = ([\Sigma Ca_i] - [Ca_o])/n \tag{5}$$

It should be emphasized that eqn. (5) is valid only if the system is at equilibrium and if the internal concentration of uncomplexed calcium, $[Ca_i]$, can be approximated by $[Ca_o]$. It cannot generally be assumed that both of these conditions are satisfied, and therefore, their validity should be independently tested. Even if both conditions are satisfied, there is still the problem that $[PrCa_{n_i}]$ is calculated from the difference between two large measured quantities, the $^{45}Ca^{2+}$ counts inside and outside the dialysis sac, corresponding to $[\Sigma Ca_i]$ and $[Ca_o]$. Once reliable values for $[PrCa_n]$ are available, $\bar{\nu}_{Ca^{2+}}/n$ can be calculated.

$$\frac{[PrCa_n]}{[Pr_i]} = \frac{\bar{\nu}_{Ca^{2+}}}{n}$$

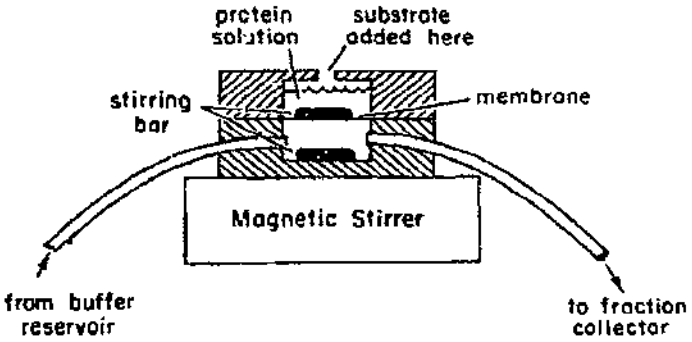


Fig. III-6. Flow dialysis apparatus. Diagram of the apparatus for measuring small molecule binding to proteins from rates of dialysis. Reprinted by permission of Colowick and Womack (1969).

Equilibrium dialysis techniques have been employed to investigate calcium binding in numerous biological systems (Calissano et al., 1969, Ingersoll and Wasserman, 1971, MacLennan and Wong, 1971, Chevallier and Butow, 1971, Benzoriana et al., 1972, Sottocasa et al., 1972, Grizzuti and Perlmann, 1973 and Bredderman and Wasserman, 1974).

The flow dialysis technique of Colowick and Womack (1969), in contrast to equilibrium dialysis procedures, is a relatively rapid method for determining calcium binding to proteins. Flow dialysis is applied practically by measuring the rate of dialysis of $^{45}Ca^{2+}$, for example, from a $^{45}Ca^{2+}$—protein equilibrium mixture (Fig. III-6). One important advantage of flow dialysis procedures is that equilibrium measurements at a number of concentrations of unlabeled calcium can be made from a single $^{45}Ca^{2+}$—protein mixture, since only negligible amounts of protein are withdrawn in any one measurement. Thus all the data required for determining the number of calcium ion binding sites, n, and the dissociation constant, K_d, can be collected within 10 to 20 minutes.

Flow dialysis is mainly applicable to metal ion—protein complexes having dissociation constants in the range of 10^{-3} to 10^{-6} M. The reliability of the flow dialysis procedure is also dependent upon the added calcium ion not inducing any changes in membrane permeability or in solution viscosity. Finally, if the metal binding is extremely slow, equilibrium might not be achieved during the normal measurement period, leading to erroneous binding data.

Colowick and Womack applied their procedures to the binding of sugars and nucleotides to one of the yeast hexokinases. Shami et al. (1974) employed flow dialysis to measure calcium binding to placental plasma membranes.

2. Filtration and Sedimentation Procedures

The gel filtration, or Hummel and Dreyer technique (1962), involves equilibrating a cellulose gel (e.g. Sephadex G-25) in a buffer containing a known concentration of $^{45}Ca^{2+}$. In a typical experiment an aliquot of the protein, equilibrated against $^{40}Ca^{2+}$, is applied to the G-25 column and eluted with the

$^{45}Ca^{2+}$ buffer. If $^{45}Ca^{2+}$ binds to the protein there will be a peak of radioactivity (with an intensity above that of the baseline, buffer counts) co-eluting with the protein. A trough of low counts will elute shortly after the protein, corresponding to the amount of $^{45}Ca^{2+}$ removed from the buffer and bound to the protein. The technique has two important built in controls. The number of counts in the protein peak should equal those in the trough. If the protein is pure and if equilibrium has been reached, the protein to $^{45}Ca^{2+}$ ratio should be constant across the protein peak. The Hummel and Dreyer method has been employed in a number of recent calcium binding studies (Teo and Wang, 1973, Grizzuti and Perlmann, 1973 and Pieterson et al., 1974).

In the Millipore filtration technique (Harigaya and Schwartz, 1969) one incubates the calcium-binding protein of membrane preparation in a suitable buffer containing $^{45}Ca^{2+}$. At selected times after the initial mixing of reactants, aliquots are removed from the reaction mixture and passed through a Millipore filter. The filter is then washed with the buffer solution and the ^{45}Ca content determined. The main source of error in the Millipore filtration procedure is potential binding of $^{45}Ca^{2+}$ directly by the filter; this would lead to larger values for $\bar{\nu}_{Ca^{2+}}$. Biological application of the Millipore method can be found in the work of Sulakhe et al. (1973) and Entman et al. (1973).

Paulus (1969) has described an ultrafiltration method for measuring the binding of radioactively-labeled small molecules and ions to proteins. Since the technique is relatively rapid and requires small amounts of protein, two drawbacks of the equilibrium dialysis and Sephadex gel filtration procedures are circumvented. Protein concentrations as low as 0.5 μM, with metal ion concentrations in the range of 10—100 μM, are suitable for a typical ultrafiltration experiment, of perhaps an hour or less duration. In experiments on the binding of ^{14}C-L-threonine to aspartokinase, Paulus demonstrated that the ultrafiltration technique was two orders of magnitude more sensitive than equilibrium dialysis. The main sources of error in the method arise from droplets of solution (containing both protein and $^{45}Ca^{2+}$) adhering to the walls of the sample chamber, and $^{45}Ca^{2+}$ being partially excluded or bound by the Diaflo membrane employed in the apparatus. Applications involving calcium binding to biological material can be found in the work of Martonosi and Feretos (1974), Wolff and Siegel (1972) and Brooks and Siegel (1973).

In the sedimentation method (Cohen and Selinger, 1969), the preparation is incubated in a buffer containing variable amounts of $^{45}Ca^{2+}$. The sediment recovered following an ultracentrifugation is then washed in a calcium-free buffer and radioactivity is determined. Errors in the measured calcium content derive mainly from the sediment washing step. If washing is incomplete, "trapped" calcium will yield erroneously high values for $\bar{\nu}_{Ca^{2+}}$; however, one must be cautious of exhaustive washing, since these procedures may drive the equilibrium toward dissociation, yielding low $\bar{\nu}_{Ca^{2+}}$ values. The technique is suitable only for readily sedimented complexes such as membrane fragments.

3. Ion exchange resin (CHELEX) method

In the ion exchange method of Briggs and Fleischman (1965) and Wasser-

man et al. (1968), calcium binding is assayed on the basis of competition between the calcium binding protein and an exchange resin, (e.g. CHELEX 100) for variable amounts of added $^{45}Ca^{2+}$ in the buffer. Following incubation of the protein in a suspension of the resin, the mixture is centrifuged, and the amount of $^{45}Ca^{2+}$ present in both the resin and supernatant phases is measured. If a linear relation is obtained between the concentration of calcium-binding protein and the percentage of $^{45}Ca^{2+}$ in the supernatant phase, Ca^{2+} binding activity can be quantified. Following Schubert (1948) the dissociation constant, K_d, can be written

$$K_d = \frac{[Pr] \cdot m}{[(K_f^0/K_f) - 1]}$$

Where [Pr] is the concentration of the calcium binding protein, and m is the molar ratio of calcium to calcium binding protein in the calcium—protein complex. K_f, the distribution coefficient in the presence of protein, is defined as follows

$$K_f = \frac{\% - {}^{45}Ca^{2+} \text{ in resin (mg)} \times \text{Vol. of soln. (ml)}}{\% - {}^{45}Ca^{2+} \text{ in soln. (ml)} \times \text{Mass of resin (mg)}}$$

A log—log plot of $([K_f^0/K_f] - 1)$ versus the concentration of calcium binding protein yielding a straight line would indicate a single class of calcium binding sites. The slope of the curve, m, would give the number of moles of Ca^{2+} bound per mole of protein. Biological applications of the ion exchange technique can be found in the work of Briggs and Fleischman (1965), Wasserman et al. (1968) and Sottocasa et al. (1972).

4. Spectral methods

A particularly convenient way to characterize metal ion binding to a protein involves the measurement of changes in some characteristic spectral property of a protein induced by the metal ion binding. The spectral property may arise from natural reporter groups on the protein (e.g. ultraviolet absorbance from protein aromatic residues), from attached, spectrally active labels (e.g. fluorescence labels, covalently or non-covalently attached to sites on the protein), or from incorporated metal ions which possess favorable spectral and/or magnetic properties (e.g. calcium replacement by the magnetic resonance probe gadolinium or the fluorescence probe terbium, see section III.C.5). Characterization of metal ion binding by induced spectral changes provides a convenient, accurate and nondestructive method of determining such parameters as metal ion dissociation constants and number of equivalent metal ion binding sites on the protein.

Halfman and Nishida (1972) have developed mathematical procedures for determining the binding of ions and small molecules to proteins, providing such binding causes an alteration in some readily measurable spectral property of the protein. Pieterson et al. (1974) have employed the techniques of Halfman and Nishida using ultraviolet and fluorescence difference spectro-

scopy to investigate the binding of calcium ions to pancreatic phospholipase A_2. The non-covalently bound compound ANS, 8-anilino-1-naphthalenesulfonic acid, was used as the fluorescence probe. The following relations can be written for defining the quantity $\bar{\nu}_{Ca^{2+}}$ in such a system.

$$\bar{\nu}_{Ca^{2+}} = \frac{\Delta A}{\Delta A_{max}} \text{ and } \bar{\nu}_{Ca^{2+}} = \frac{\Delta F}{\Delta F_{max}}$$

ΔA and ΔF are the difference ultraviolet and fluorescence intensities, respectively, at a single Ca^{2+} ion concentration and ΔA_{max} and ΔF_{max} are the maximal ultraviolet and fluorescence intensity values obtainable with added Ca^{2+} ion. From plots of ΔA (or ΔF) versus $[Ca^{2+}]$, ΔA_{max} (or ΔF_{max}) and the molar difference extinction coefficient can be evaluated. One usually assumes that a sharp isosbestic point indicates that only two protein conformations are involved, most often suggestive of, but not proof of, a single metal ion binding site. If more than one Ca^{2+} binding site is present per protein molecule and if the multiple binding sites are of different affinities, possessing different optical properties, the determination of binding parameters becomes somewhat more complex (see Halfman and Nishida, 1972). Täljedal (1974) has recently employed the fluorescence probe chlorotetracycline, in an investigation of the interaction of Na^+ and Mg^{2+} with Ca^{2+} in pancreatic islets. The characterization of the conformational environment of calcium ion binding sites in proteins with the use of paramagnetic lanthanide ions and nuclear magnetic resonance spectroscopy will be deferred to the following section.

5. Lanthanide replacement of calcium

The potential utility of lanthanide ions as replacements for Ca^{2+} in biological systems was first suggested by Lettvin et al. (1964) in their theory of passive ion flux through axon membranes. They predicted that La^{3+}, which has an ionic radius (Table III-1) similar to that of Ca^{2+} might function similarly to Ca^{2+} in "locking" the membrane calcium channel, and that such locking would be ennanced due to the higher charge density on La^{3+}. Subsequently lanthanides have been used in various in vivo studies to provide an electron dense stain for electron microscopy. The assumption that La^{3+} selectively marks Ca^{2+} sites is reasonable but difficult to prove. In other in vivo studies La^{3+} is assumed to resemble Ca^{2+} closely enough to bind to enzymes, like membrane CaATPase, yet be dissimilar enough to block the function of the enzyme.

Chemical studies using lanthanide replacement have had two "biological" goals. First one wants to know to what extent lanthanides do in fact compete for Ca^{2+} binding sites, and then how functional the replacement is. Secondly, as we will discuss in this section, the optical properties of the lanthanides can be exploited to study the cation—protein interaction. Unfortunately the two classes of experiments are seldom combined, so it is often difficult to interpret the functional significance of elegant spectroscopic or crystallographic studies.

TABLE III-1

Ionic radii

Ion	Atomic number	Ionic radius (Å)
Ca^{2+}	20	0.99
Mg^{2+}	12	0.65
Na^{+}	11	0.95
K^{+}	19	1.33
Mn^{2+}	25	0.91
Cd^{2+}	48	0.92
Cu^{2+}	29	0.69
Ni^{2+}	28	0.72
La^{3+}	57	1.061 [a], 1.15 [b]
Ce^{3+}	58	1.034, 1.11
Pr^{3+}	59	1.013, 1.09
Nd^{3+}	60	0.995, 1.08
Pm^{3+}	61	0.979, 1.06
Sm^{3+}	62	0.964, 1.04
Eu^{3+}	63	0.950, 1.03
Gd^{3+}	64	0.938, 1.02
Tb^{3+}	65	0.923, 1.00
Dy^{3+}	66	0.908, 0.99
Ho^{3+}	67	0.894, 0.97
Er^{3+}	68	0.881, 0.96
Tm^{3+}	69	0.869, 0.95
Yb^{3+}	70	0.858, 0.94
Lu^{3+}	71	0.848, 0.93

[a] Values obtained from crystal structure study of D.H. Templeton and C.H. Dauben, (J. Amer. Chem. Soc., 76 (1954) 5237).
[b] Values obtained from empirical crystal data by L. Pauling in The Nature of the Chemical Bond, Cornell University Press, New York, 1961, p. 518. Values employed by B.W. Matthews and L.H. Weaver, Biochemistry, 13 (1974) 1719.

Levitzki and Reuben (1973) and Darnall and Birnbaum (1973) found that various lanthanides, and particularly those close to 0.99 Å ionic radius (Table III-1), could fully activate the Ca^{2+}-free α-amylase of *B. subtilus*. Replacing Ca^{2+} by various lanthanides has little effect on the enzymatic activity of porcine trypsin (Epstein et al., 1974). Eu^{3+}-thermolysin has normal proteolytic activity (cited by Matthews and Weaver, 1974). In contrast Nieboer et al. (1973) and Furie et al. (1973) found that several lanthanides competitively inhibited *Staph.* nuclease (crystal structure, section II.B.3.) with apparent pK_d 5.7—6.0 (cf. $pK_d(Ca^{2+}) = 3.0$). This is interesting because the Ca^{2+} in the nuclease is at the active site and possibly involved in the enzymatic mechanism whereas the calciums in trypsin and the thermolysin are not near the active sites.

Matthews and Weaver (1974) studied the replacement of Ca^{2+} in thermolysin (section II.B.5., crystal structure) by difference Fourier projections. At the surface sites, 3 and 4, the lanthanides bind at the Ca^{2+} coordinates within 0.4 Å. At the more internal double site (1,2) where four carboxyl groups co-

ordinate the two calcium ions, only a single lanthanide is bound. Moews and Kretsinger (1975b) showed by difference Fourier techniques that terbium could completely replace the EF calcium under non-denaturing conditions without replacing any of the CD calcium in MCBP.

The fluorescence properties of some trivalent lanthanides, particularly terbium and europium, provide a convenient probe for studying metal ion—protein interactions. Epstein et al. (1974) have replaced the single Ca^{2+} of trypsin with Tb^{3+} in a fluorescence study of the metal ion binding site. Maximum fluorescence enhancement (10^5 fold over free Tb^{3+} in solution in the absence of protein) was observed at an excitation wavelength of 295 nm, consistent with energy transfer from one or more tryptophans to the bound terbium. From the pH dependence of the fluorescence ($pK \simeq 5.2$) it was concluded that glutamic acid residues coordinate the bound terbium. Fluorescence spectroscopy has also been used to study the metal binding sites of MCBP and TN-C (Donato and Martin, 1974). Maximum fluorescence emission for the Tb^{3+}—MCBP complex occurs at an excitation wavelength of 259 nm, consistent with energy transfer from phenylalanine, while for the Tb^{3+}—TN-C complex maximum emission occurs at an excitation wavelength of 280 nm, indicating a predominant energy transfer from a tyrosyl residue to the bound terbium. Sherry and Cottam (1973) observed that Tb^{3+} added to a solution of concanavalin A produces a slight quenching of the protein fluorescence but a greatly enhanced Tb^{3+} fluorescence at both 492 and 545 nm. These results were interpreted on the basis of intramolecular energy transferred from one or more aromatic residues of the protein to the bound Tb^{3+}. Sherry and Cottam did not observe enhanced fluorescence with bound Eu^{3+}.

In a recent application to a biomembrane system, Mikkelsen and Wallach (1974) employed terbium as a fluorescent probe of high affinity calcium binding sites on erythrocyte membrane proteins. When Tb^{3+} is bound to erythrocyte ghosts, terbium fluorescence is greatly enhanced while tryptophan fluorescence is quenched. Concomitant to the enhanced terbium fluorescence is the emergence of a new excitation peak at 295 nm. The authors interpreted these observations on the basis of intermolecular energy transfer, resulting from the chelation of tyrosine hydroxyl group(s) to the bound terbium ion.

The general utility of the paramagnetic lanthanides as magnetic resonance probes (LaMar et al., 1973) in systems of biological interest, has been the topic of a number of recent reviews (Dwek, 1973, Mayo, 1973, Reuben, 1973, and Reuben, 1975). A lanthanide ion bound to a protein can interact with other magnetic nuclei on the protein (e.g. protons or ^{13}C nuclei) in a number of ways. A resonance signal from the protein may be "chemically shifted", the extent of such shifting being proportional to $1/r_i^6$, where r_i is the distance between the lanthanide ion and target nucleus, i, on the protein. All of the lanthanides, with the exception of La^{3+}, Gd^{3+} and Lu^{3+}, can be employed as chemical shift reagents, due to the anisotropy of their magnetic susceptibility. Further, a resonance signal may be "broadened". The extent of broadening is proportional to $1/r_i^3$ and is a function of the angle between the vector connecting

the bound lanthanide to the target protein nucleus and the g tensor of the lanthanide ion complex (Jesson, 1967). The lanthanide ions most frequently employed as line broadening reagents are Gd^{3+} and Nd^{3+}; both possess relatively long electron relaxation times. These "shifting" and "broadening" effects are just beginning to be exploited to characterize the cation coordination spheres of proteins.

The best example of the utility of the paramagnetic lanthanides and magnetic resonance spectroscopy as applied to a calcium binding protein concerns *Staphylococcal* nucleus. Histidine-46 of nuclease was implicated as being involved in metal ion binding when Williams (1971) reported that addition of neodymium leads to selective, progressive line broadening of an imidazole ring proton resonance. In a later, more extensive study of the histidine residues of nuclease (Nieboer et al., 1973), distance ratios were calculated from Ca^{2+} binding sites to all four imidazole groups on the basis of europium-induced chemical shifts. The resulting values were found to agree to within 6% with the same ratios calculated from the crystal structure. Morallee et al. (1970) employed both europium and the line broadening lanthanide, gadolinium, in an investigation of the binding of a substrate analogue, β-methyl-N-acetyl glucosamine (β-Me-NAG), to lysozyme. Distances between the metal ion binding site and the acetamido and glycosidic methyl groups were calculated to be 6.7 Å and 5.6 Å, respectively, in close agreement with the crystal structure. The utility of lanthanide-induced chemical shifts for the study of model membrane systems has been demonstrated in the work of Michaelson et al. (1973), Andrews et al. (1973), Huang et al. (1974) and Assmann et al. (1974) and is further discussed in the review by Reuben (1975).

Certain transition metal paramagnetic ions, such as Mn^{2+}, have been employed to study the environment of metal ion binding sites in proteins by the technique of proton relaxation enhancement, PRE (Mildvan and Cohn, 1970). The d-transition metals are unsatisfactory, however, as probes of calcium ion binding sites, due mainly to the predominant covalent nature of the metal complex formed and the d-orbital steric direction of the protein ligands, both of which tend to alter the coordination geometry of metal binding. In contrast, paramagnetic lanthanide ions have been shown to bind similarly to calcium ions, and if the bound lanthanide ion (e.g. Gd^{3+}) has a long electron spin relaxation time, the PRE method can prove valuable in characterizing the metal ion binding sites. Dissociation constants and values for the number of binding sites have been determined by PRE methods, employing gadolinium, for trypsin (Epstein et al., 1974), α-amylase (Levitzki and Reuben, 1973), lysozyme (Dwek et al., 1971) bovine serum albumin (Reuben, 1971), concanavalin A (Sherry and Cottam, 1973), inorganic pyrophosphatase (Cooperman and Chin, 1973), and pyruvate kinase (Valentine and Cottam, 1973). These results have been reviewed by Reuben (1975).

IV. PHYSIOLOGICAL PROCESSES INVOLVING CALCIUM

A. General view of calcium distribution

In Table IV-1 we list a few selected values of calcium ion concentrations and activities. The main point is that for nearly all organisms the Ca^{2+} concentration in the extracellular fluid is rather near that of ocean water. In marked contrast the intracellular concentration of free Ca^{2+} (as opposed to total calcium) is in the range of pCa 6—7.5. Also, the inside of most cells is 50—100 mV negative relative to the outside. According to the Nernst equation

$$E_{Ca} = (RT/2F)\ln(a_{Ca_{out}}/a_{Ca_{in}})$$

the distribution of Ca^{2+} is about 10^4—10^5 lower inside the cell than anticipated in the "equilibrium" situation. Apparently all eukaryotic organisms have calcium pumping mechanisms in their cell membranes (as well as in their mitochondria and endoplasmic reticulum). The idea which will be developed in section IV.C. is that this outside, inside Ca^{2+} gradient is maintained by the cell not as a potential source of energy (as energy is stored in ATP), but as a potential source of information. In section IV.D. we will discuss the membranes which must maintain and monitor this Ca^{2+} gradient. Although the process of calcification (section IV.B.) at first appears unrelated to informational role of calcium, certain of the mechanisms involved are related.

TABLE IV-1

Metal ion concentrations in various tissues

Tissue	(free) Ion	(total)	Concentration (M)
Human blood plasma	Ca^{2+}		0.0012
Human blood plasma	Ca^{2+}		0.0024
Human blood plasma	Ca^{2+}		0.00036 (activity)
Human blood plasma	Na^{+}		0.145
Human blood plasma	Na^{+}		0.11 (activity)
Squid hemolymph	Ca^{2+}		0.004
Squid hemolymph	Ca^{2+}		0.00093 (activity)
Squid hemolymph	Na^{+}		0.437
Squid hemolymph	Na^{+}		0.30 (activity)
Vertebrate muscle	Ca^{2+}		0.0000002
Mollusc muscle	Ca^{2+}		0.000001
Squid axoplasm	Ca^{2+}		0.000001
Squid axoplasm	Mg^{2+}		0.01
Squid axoplasm		Ca^{2+}	0.0001
Human erythrocyte		Ca^{2+}	0.000016
Human erythrocyte		K^{+}	0.11
Human milk		Ca^{2+}	0.008
Cow milk		Ca^{2+}	0.030
Various plants	(of dry weight 0.01 to 2.0%)		

Of these physiological processes which have a molecular interpretation, nearly all of them involve calcium protein interactions. We discuss a few of these in terms of their calcium coordination (section II.B.) or in the context of contraction (IV.C.2.) or membrane transport (IV.D.2. and 3.). Over sixty calcium binding proteins are discussed in the review by Kretsinger (1976). In section IV.C.3. some of the main characteristics of these proteins are tabulated.

B. Calcification

1. Scope

Many eukaryotic tissues, in addition to bone, can form solid deposits of calcium salts. The list includes egg shells, exoskeletons of molluscs and crustaceans and parts of certain plant stalk. In fact one might consider any tissue potentially able to undergo calcification. For instance human tendon, muscle, kidney, liver and vascular tissue undergo calcification under certain pathological conditions. Certainly the details vary in different tissues of different organisms; nonetheless the following generalizations seem valid.

The mineral phase of bone and most calcified tissues consists of a mixture of amorphous calcium phosphate $Ca_3(PO_4)_2$ and microcrystalline hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ (see review by Posner, 1973). In the bones of young animals two thirds of the mineral is highly hydrated $Ca_3(PO_4)_2$ while at the other extreme the enamel of adult teeth is almost 100% apatite with large crystal domains. Physiologically it is unknown what prevents $Ca_3(PO_4)_2$ from dissolving and what controls the thermodynamically favored transition from $Ca_3(PO_4)_2$ to apatite. The selective removal of various inhibitors, Mg^{2+} or polyglutamic acid, is a reasonable possibility. Most bone apatites have some 3% by weight HCO_3^- and 1% citrate adsorbed to the crystal surface as well as traces of Fe, Cu, Pb, Mg, Sm, Al, Sr and B.

2. Mitochondria

As is further discussed in section IV.D.3.b. mitochondria can accumulate up to 0.2 mg of calcium and phosphate per mg of mitochondrial protein. Metabolically this calcium phosphate accumulation assumes precedence over ATP generation (see review by Lehninger, 1970). In many tissues, particularly those involved in calcification (or resorption), there are amorphous or even crystalline (Becker et al., 1974) deposits of $Ca_3(PO_4)_2$ within the mitochondrial matrix. Lehninger (1970) and Harris et al. (1974) have suggested that these $Ca_3(PO_4)_2$ microparticles might be transported through the mitochondrial membranes, the cytoplasm and the cell membrane to be deposited at the site of ossification. During transport the particle would have to be protected from dissolution by some still unidentified factor.

Schraer et al. (1973) reviewed the research on the involvement of the mitochondria of hen egg shell gland in the deposition of $CaCO_3$ (calcite) crystals. The gland mitochondria have twice the calcium accumulation in vivo as do the liver mitochondria. In vitro the gland mitochondria can accumulate cal-

cium at four times the rate of the liver mitochondria. Further, this additional calcium is released from the gland mitochondria at the time of shell formation. At precisely this time the calcium content of the endoplasmic reticulum increases. This suggests that $CaCO_3$ "precipitates" might be transported from the mitochondria to the extracellular space.

3. Protein involvement

Urry (1971) suggested that the initial event in ossification might be Ca^{2+} coordination by peptide carbonyl oxygen atoms of residues in a β-bend conformation. This interesting proposal is certainly unproven. The actual capacity (one calcium ion per 5×10^4 a.m.u.) of insoluble elastin is rather low and has been ascribed to carboxylate groups (6 carboxylates per Ca^{2+} bound). If the elastin is alkali solubilized, the binding capacity increases to one Ca^{2+} per 2.5×10^4 a.m.u. (Abatangelo and Daga-Gordini, 1974). The involvement of elastin, or of collagen, is still only inferential. It is certainly possible that the glycoproteins (cf. section IV.D.4.) of the acidic ground substance play a role in hydroxyapatite nucleation.

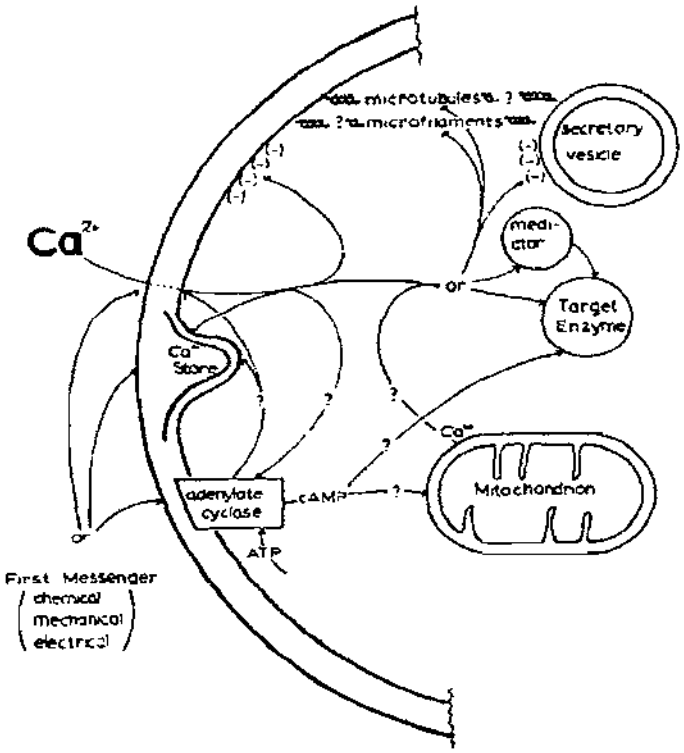


Fig. IV-1 Diagram representation of the second messenger pathways. The various potential sources of calcium and of cAMP, their possible interactions and ultimate targets are discussed in the text.

C. Calcium as a second messenger

1. Concept

One of the more rewarding ways of analyzing an organism, or an organ, or an entire ecosystem is in terms of its information content and means of information transfer. Different cells communicate with one another by hormones or nervous impulses. In the same sense cellular organelles exchange information. From the early 50's Sutherland (1972) investigated cAMP and definitely established that it served as a "second messenger" in many different systems, see Table IV-2. Douglas (1963) and his colleagues showed that in

TABLE IV-2

Systems in which calcium and/or cAMP function as second messengers

System	Stimulus	Ca^{2+}		cAMP produced	Response
		required extracellular	increase cytoplasm		
Synapse	electrical	x	x	x	transmitter release
Neuromuscular junction	electrical	x	(x)	x	transmitter release
Coelenterates	electrical	x	x	(—)	light emission (aequorin)
Vorticellids	(mechanical)	(—)	x	—	spasmoneme contraction
Paramecium	mechanical	(—)	x	—	ciliary beat
Rod outer segment	photon	(—)	x	x cGMP	block dark current
Muscle, cardiac	adrenaline	x	x	x	contraction, glycogenolysis
Muscle, skeletal	acetylcholine	(—)	x	(x)	contraction, glycogenolysis
Adrenal medulla	acetylcholine	x	(x)	—	adrenaline release
Posterior pituitary	(acetylcholine)	x	(x)	?	vasopressin release
Exocrine pancreas	acetylcholine	x	(x)	—	amylase release
Salivary gland	adrenaline	x	(x)	x	amylase release
Adipocyte	adrenaline	x	(x)	x	lipolysis
Anterior pituitary	growth hormone releasing factor	x	(x)	x	growth hormone release
Anterior pituitary	LH releasing factor	x	(x)	x	luteinizing hormone release
Anterior pituitary	T releasing factor	x	(x)	x	thyrotropin release
Beta cell, pancreas	glucose	x	(x)	x	insulin release
Adrenal cortex	adrenocorticotropic hormone	x	(x)	x	steroid release
Liver	glucagon	?	?	x	glucose synthesis and release
Thyroid	thyroid simulating hormone	x	(x)	x	thyroxine release
Corpus luteum	luteinizing hormone	x	(x)	x	progesterone release
Stomach	histamine	x	(x)	x	HCl secretion
Toad bladder	vasopressin	?	?	x	Na and H_2O transport
Kidney tubule	parathyroid hormone	x	(x)	x	gluconeogenesis
Melanocyte	melanocyte stim. hormone	x	(x)	x	melanin dispersion
Sea urchin egg	*sperm*	x	x	(x)	fertilization
Slime mold	cAMP	x	x	(x)	aggregation
Dividing cells	?	(x)	x	?	cleavage furrow formation spindle shortening

"x" indicates yes; "(x)" probably yes; "—" no; "(—)" probably not.

many systems in which a stimulus elicits a secretory response, Ca^{2+} is a necessary component in the external medium and further that intracellular calcium levels rise following stimulation. Rasmussen (1970) and Rasmussen et al. (1972) documented and explored the concept of calcium serving as a second messenger. As shown diagrammatically in Fig. IV-1.

i. An external stimulus touches the cell surface. Although in most experimental systems the stimulus is chemical, it may well be electrical, mechanical or possibly even thermal.

ii. Subsequently there is an increase in cytoplasmic calcium. This may occur in milliseconds, as in muscle or in the light emitting *Coelenterates*, (see section IV.C.3.a.). In most systems such rapid responses cannot be measured.

iii. Many of these systems also show a parallel increase in cAMP levels. However the relationships between cAMP synthesis and hydrolysis and calcium release and sequestration are not understood.

iv. The target of the calcium is usually an enzyme; however the spasmoneme protein (IV.C.2.e.) is an exception. Calcium may bind to the target enzyme directly, e.g. aequorin, or it may transmit its information via a modulator, as troponin with the myosin ATPase as the ultimate target.

So many cellular responses involve movement of either the entire cell or of organelles such as vesicles, that we have described muscle and microfilaments in some detail. Specific enzymes which might be affected by calcium are mentioned in section IV.C.3. It will become apparent that the membrane is the most important and least understood component of the system. It is considered in section IV.D.

2. Movement

a. Muscle. Both the structure and contractile mechanism of vertebrate skeletal muscle have been thoroughly studied and well reviewed (Fuchs, 1974a, Weber and Murray, 1973 and Huxley, 1973). It is generally assumed that smooth and cardiac muscle as well as cytoplasmic myosin and actin (microfilaments) have a similar but less regular organization (Fig. IV.2.) and have a similar mechanism of contraction.

Structure. The thick filament consists of polymerized myosin. The myosin "hexamer" (MW 460,000) consists of two heavy chains (MW 190,000) and four light chains. Both of the heavy chains have a globular head which contains the ATPase activity and the ability to interact with the actin of the thin filament. They also have α-helical tails which intertwine with one another to form a coiled coil. Myosin can be selectively cleaved with trypsin at the flexible hinge region thereby generating heavy meromyosin (HMM, MW 330,000) and light meromyosin (LMM MW 130,000). LMM consists of most of the coiled coil tail. Further cleavage of HMM produces F-2 (subfragment) the proximal part of the tail, and F-1, the pair of myosin heads. Squire (1972) has proposed a model for the packing of myosin tails along the length of the thick filament and on into the bare zone where tails of opposite polarity overlap.

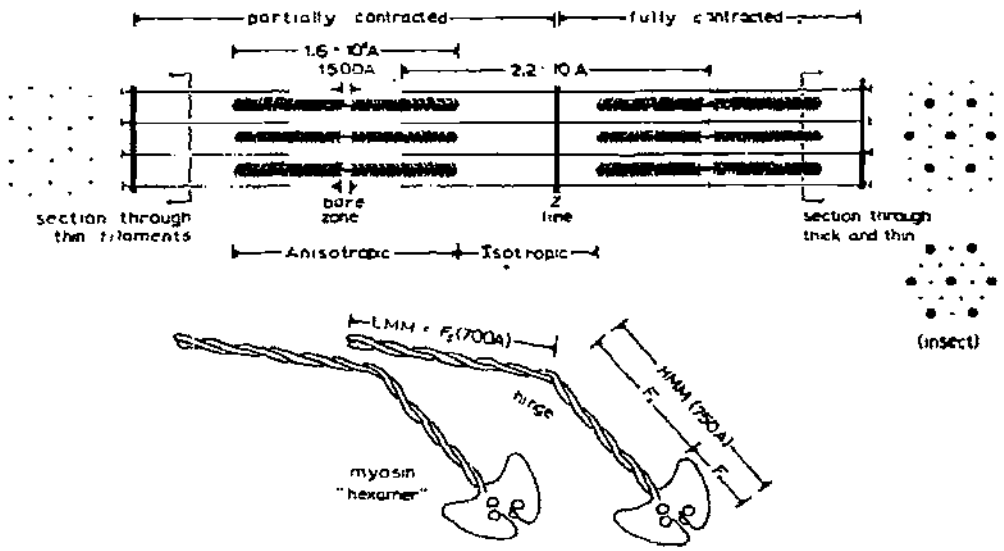


Fig. IV-2. Diagram of muscle structure. The thick filaments are composed of polymerized myosin "hexamers" each consisting of two (near) identical heavy chains and four light chains. Myosin is easily cleaved at its hinge region, thereby producing two fragments light meromyosin and heavy meromyosin. The thin filaments consist of "F" or polyı ized actin plus tropomyosin and troponin.

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Skeletal myosin muscle contains four light chains, two with a free —SH group which is easily reacted with DTNB (DTNBLC, MW 18000 each) and two which can be extracted with 0.1 M alkali (ALC, MW 16500 and 20700). Szent-Györgyi et al. (1973) showed that mollusc myosin contains two —SH light chains and one light chain which can be extracted with EDTA (EDTALC MW 18000).

The thin filament consists primarily of a double helical polymer of globular actin (MW 41800, see Elzinga et al., 1973, for amino acid sequence). The screw operation for a single strand is $Z = 55$ Å, $\phi = 360°/14 = 26°$. Tropomyosin consists of two intertwined α-helices (dimer MW 67000, length 400 Å). One troponin "trimer" (MW 79000) occurs every 385 Å along each groove, apparently near the end of each tropomyosin.

The *Z* line is about 1000 Å thick and has as its main, but not sole, protein α-actinin (MW 95000, Robson and Zeece, 1973). Seen face on it has a square grid pattern with one thin filament inserting at every grid point. It is important to learn its geometric arrangement, because this determines that the thin filaments point out with opposite polarity on the two sides. A protein similar to α-actinin may provide the insertion site of microfilaments into membranes.

Mechanism. We consider first the binding and hydrolysis of ATP by myosin heads, then the movement of the myosin head relative to the body of the thick filament. In the following section we discuss Ca^{2+} control via troponin and/or myosin light chains. According to the model of Burke et al. (1973)

there are two free—SH groups on each myosin head which form a $(-SH)_2$—Mg-ATP inactive complex under physiological conditions of $1-3 \times 10^{-3}$ M Mg^{2+}. Although the —SH groups are not part of the ATP binding site involved in hydrolysis, they are near enough so that the $(-SH)_2$—Mg-ATP complex blocks access. The ATPase can be activated under four circumstances:

i. No Mg^{2+}; ATP can bind in the catalytic site.

ii. $[Ca^{2+}] > [Mg^{2+}]$; CaATP will not complex with the two —SH groups and is bound in the catalytic site.

iii. Actin; during in vivo function, actin "covers" one or both —SH groups, thereby displacing bound MgATP and causing the "initial burst" of ATP hydrolysis. At rest some 90% of the sites are occupied by MgATP.

iv. —SH blocking; if one of the —SH groups is chemically blocked, the MgATP cannot chelate and is obliged to bind at the active site.

In frog sartorius muscle, the object of many physiology experiments, Ca^{2+} in the range pCa 8—4 increases ATP binding to and hydrolysis by myosin (Chaplain and Gergs, 1974). Huxley (1972) obtained X-ray diffraction patterns of toad semitendinosus muscles which had been stretched so far that there was no longer any overlap of thick and thin filaments. Upon addition of physiological amounts of calcium there appeared to be a movement of the myosin head away from the thick filament. He offered as one explanation that there may be "a second activation mechanism present in vertebrate striated muscle" which "holds the cross-bridges away from the actin filaments in a resting muscle and releases them during contraction." The actual force generating step involves the swinging out of the myosin head to bind actin, the actin induced release of ADP and phosphate, and a "rowing motion" which translates the thick filament relative to the thin filament. At rest (nearly) all of the heads lie back against the thick filament; during rigor or ATP depletion all of the heads are extended and bound to actin. Apparently the binding of ATP allows the heads to dissociate from actin and to recover after the rowing stroke.

Calcium regulation. The troponin "trimer" consists of TN-T (MW 37000), TN-I (MW 23000) and TN-C (MW 17846, Collins et al., 1973). Potter and Gergely (1974) have combined their results with the previous models of Parry and Squire (1973), of Hitchcock et al. (1973) and of van Eerd and Kawasaki (1974) and proposed the model of Fig. IV-3.

TN-T binds to tropomyosin either free or complexed with actin. In addition the TN-C, TN-I complex, which is quite stable, binds strongly to actin-tropomyosin in the absence of Ca^{2+}. Consistent with the model, the affinity of troponin for the actin, tropomyosin complex is reduced in the presence of calcium (Hitchcock, 1973). TN-I alone or as the TN-I, TN-T complex has a weak inhibitory effect on actomyosin ATPase (Eisenberg and Kielley, 1974). TN-C binds to neither actin nor tropomyosin. Parry and Squire (1973) have interpreted their X-ray data in terms of tropomyosin shifting deeper into the actin groove following Ca^{2+} binding to troponin.

In the absence of Ca^{2+} tropomyosin is out of the groove in a position to

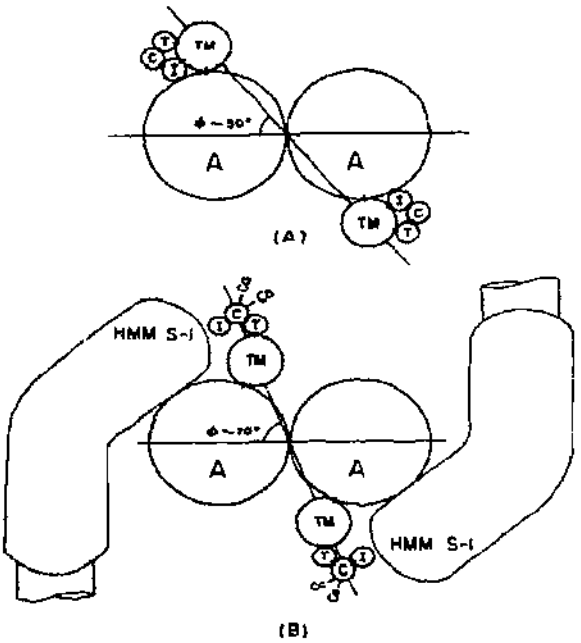

Fig. IV-3. Model of troponin, tropomyosin, actin interaction. In this model from Potter and Gergely (1974) the troponin (I,C,T) complex interacts with actin and with tropomyosin in such a way that tropomyosin blocks the myosin interacting site of actin. Even though the two troponin molecules bind at 385 Å intervals along the thin filament, the tropomyosin blocks the actin site along the entire 385 Å length. Following the binding of calcium by TN-C, the TN-I binding to actin, tropomyosin is weakened and the entire tropomyosin, troponin complex shifts to a position deeper within the groove(s) of the actin helix, thereby allowing interaction between actin and the myosin head (HMM S-1).

block the interaction of actin with the myosin head. The binding of two calcium ions to TN-C would weaken the TN-I interactions with both actin and tropomyosin. Tropomyosin, along its entire 400 Å length, would shift to a binding site deeper within the actin groove, thereby allowing actin, myosin-head interaction (Bremel and Weber, 1972).

In contrast to this calcium control exerted at the thin filament via troponin, Szent-Györgyi et al. (1973) found that in various lower vertebrates Ca^{2+} activates the thick filament directly. Since myosin and particularly actin have evolved very slowly, the thick and thin filaments from very different animals as well as cytoplasmic actin and myosin can cross react. This cross reaction was exploited to classify all muscles as thick filament or thin filament calcium controlled. However, Lehman et al. (1974) found that in the flight muscle of the insect, *Lethocerus cordofanus*, both thick and thin filaments bind Ca^{2+}. The muscle is synergistically controlled. Bremel (1974) reported that chicken gizzard smooth muscle lacks troponin and that calcium regulation is myosin linked.

Removal of the mollusc EDTALC completely desensitizes the myosin. Both native and resensitized myosin bind about 1.4 mole of Ca^{2+} with pK_d 6.8; while desensitized myosin binds 0.8 mole, pK_d 6.5. The residual Ca^{2+} binding and non-integral stoichiometry may well be due to incomplete removal of EDTALC. The important point is that the removed EDTALC does not bind Ca^{2+}. Kendrick-Jones (1974) showed that the rabbit DTNBLC, which does bind Ca^{2+}, can replace the EDTALC, which does not bind Ca^{2+} in its isolated form, in sensitizing molluscan myosin. Rabbit myosin binds two Ca^{2+} with $pK_d = 4.9$; all of this binding capacity is associated with the DTNBLC. Werber et al. (1972) had previously determined for the DTNBLC pK_d values of 5.2 and 4.0, with $n = 2$ for Ca^{2+} binding and $pK_d = 3.0$, $n = 1$ for Mg^{2+}, from the decrease in tryptophan fluorescence caused by the cations. Morimoto and Harrington (1974) determined $pK_d = 4.9$, $n = 1$ for the DTNBLC, Ca^{2+} binding by the Chelex method. Werber and Oplatka (1974) treated myosin with DTNB and found that the actomyosin complex lost Ca^{2+} sensitivity in parallel with the release of DTNB light chains.

In addition there are two ALC's whose amino acid sequences are identical except that the heavier chain has an additional 41 residues at the N-terminus (Frank and Weeds, 1974). Both Weeds and McLachlan (1974) and Tufty and Kretsinger (1975) postulated four regions within ALC which are homologous to the EF-hands of MCBP and of TN-C (see Table II-4). Nonetheless, the isolated ALC does not bind Ca^{2+}; just as the isolated EDTALC of molluscan myosin does not bind Ca^{2+}.

It seems reasonable to predict that ALC, like TN-C, consists of two pairs of EF-hands (see section II.B.1.). Very probably the DTNBLC and the EDTALC will also prove to be homologous. The calcium binding affinities of these light chains will depend not only on the number of carboxylate groups in their respective calcium binding loops, but also on conformational distortions induced by their binding to the heavy chains of myosin.

b. Microfilament related processes The structure(s) and suggested functions of microfilaments have been thoroughly reviewed — Berl et al. (1973), Durham (1974), Huxley (1973), Pollard and Weihing (1974), Wessells et al. (1971), and Hepler and Palevitz (1974). The conclusions of these reviews may be summarized as follows:

i. Prokaryotes do not contain microfilaments. Although there are few examples from plants (*Nitella*, *Physarum* and oat coleoptile), all eukaryotic cells appear to contain microfilaments. This may prove to be one of the more profound generalizations of molecular evolution.

ii. Microfilaments consist of polymerized actin and are so defined. This is demonstrated by "decoration" with heavy meromyosin thereby forming the distinctive arrowhead pattern. When HMM is added to muscle thin filaments the arrowheads always point away from the *Z*-line. Palevitz et al. (1974) "decorated" the microfilaments of the soil alga, *Nitella*, with heavy meromyosin. Recently Lazarides and Weber (1974) succeeded in obtaining anti-

bodies to actin and by immunofluorescence traced the distribution of fibers within various cells (Fig. IV.-4.).

iii. Cytoplasmic actins are, by a variety of criteria, remarkably similar to one another and to muscle actin. They certainly have similar amino acid compositions and are homologous.

iv. Cytoplasmic myosin is defined by Pollard and Weihing as "a class of enzymes with actin-activated ATPase activity that binds reversibly to actin filaments." Their characteristics seem somewhat more variable than do those of actins from different classes. Usually they consist of two myosin heavy chain protomers and about four light chains. The purified and reconstituted myosin of the slime mold, *Dictyostelium discoideum* can be re-aggregated into bipolar filaments 0.5—1.0 μM long and are similar in appearance to those reassembled from vertebrate muscle (1.5 μM). (Clarke and Spudich, 1974).

Acanthamoeba myosin is of molecular weight 170,000 and consists of two light chains and what appears to be a "normal" myosin head but no tail (Pollard and Korn, 1973). If the tail was not lost by some artifact of preparation, this myosin may prove awkward to incorporate into some general theories of microfilament function.

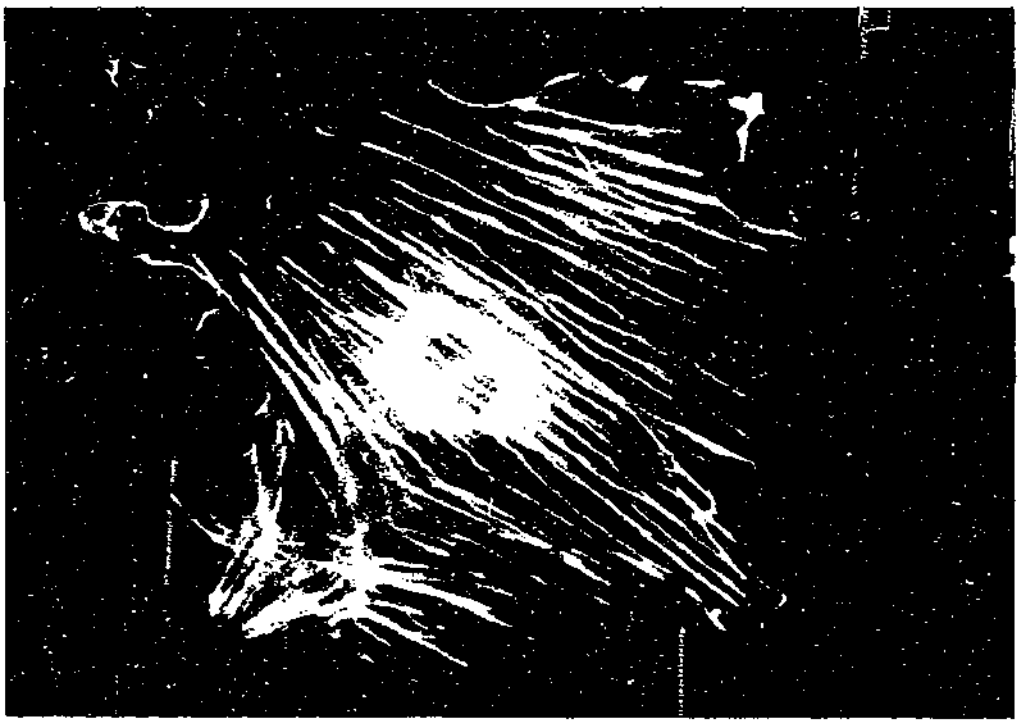

Fig. IV-4. Fluorescent antibody labeling of microfilaments. Indirect immunofluorescence photograph of primary rat embryo fibroblast stained with antibody against actin. Supplied by E. Lazarides (Lazarides and Weber, 1974).

v. Whereas actin may account for a large fraction of the protein of many cells — 10% of total protein in *Acanthamoeba* and 20% of the soluble proteins in chick neurons — cellular myosin is much less concentrated. In vertebrate skeletal muscle the molar ratio of actin monomer to myosin "hexamer" (two heavy and four light chains) is 3.4/1.

vi. Tropomyosin has been extracted from platelets, brain tissue and *Physarum*. The evidence for troponin is solid in platelets and tentative in *Physarum* and in brain (Puskin and Kochwa, 1974).

vii. Although the evidence is inferential, cytoplasmic myosin probably does interact with microfilaments, hydrolyze ATP, and upon release of phosphate, translate relative to the actin polymer.

viii. This contraction is calcium regulated via either a troponin homolog acting on the microfilament or via a myosin light chain. Nachmias and Asch (1974) suggested that the ATPase of cytoplasmic actomyosin of the myxomycete, *Physarum polycephalum* is calcium mediated through the thin filament.

ix. Some microfilaments, e.g. *Dictyostelium* (Spudich, 1974), "insert" into the plasma membrane as though it were the *Z*-line; when they are myosin decorated the arrowheads definitely point away from the membrane. In chick myoblasts, microfilaments span the entire length of the cell and often radiate from "focal points" which appear to be on the cell surface. This has been shown by indirect immunofluorescence with antibodies to actin (Lazarides and Weber, 1974) and to myosin (Weber and Groeschel-Stewart, 1974) (Fig. IV-4).

In spite of this wealth of information, many key questions remain unresolved.

i. Microfilaments have been implicated in many processes — amoeboid movement, cytoplasmic streaming, cleavage furrow formation, cytokinesis, axoplasmic transport, cortical contraction and general maintainence of cell shape. Yet in all of these processes their involvement remains inferential. Their involvement in platelet contraction is fairly well established. Of most relevance to calcium regulation is their possible involvement in vesicle secretion, which will be discussed later.

ii. Microfilaments might exist in equilibrium with monomeric actin. The possibility that actin polymerization might be a parameter in cell movement is reinforced by the experiments of Tilney et al. (1973). When echinoderm sperm touch egg jelly they extend an acrosomal process 90 μM long and 0.1 μM in diameter in less than 30 seconds. It consists of newly polymerized actin which, when decorated, has all arrowheads pointing away from the sperm nucleus.

iii. Equally important are the questions of whether all cytoplasmic myosins can polymerize, if so whether these "heavy chains" are always bi-directional and finally whether they can be attached to membranes.

iv. Microfilaments are often seen near microtubules yet there is little evidence that they interact or that their functions are related. It is tempting to consider that the calcium induced depolymerization of microtubules and

the calcium dependent contraction of microfilaments are coordinated in processes like cell division or pseudopod extension.

v. Again to be discussed in conjunction with exocytosis is the question of whether cAMP has any direct effect on microfilaments. For instance, Adelstein et al. (1973) have found that one of the two light chains of platelet myosin is phosphorylated, with ATP being the phosphate donor. TN-I, TN-T and the DTNBLC of skeletal muscle are phosphorylated. More probable than any direct reaction of cAMP with a microfilament kinase, is the possibility of its regulating calcium release from endogenous sources such as mitochondria or reticulum.

Cytochalasin, Fig. IV-5, is often assumed to interact specifically with microfilaments; unfortunately, it does not seem to be so specific as, for example, colchicine is for microtubules.

Finally we emphasize one of the points in the theory of control of nonmuscle movements presented by Durham (1974). Although much remains to be learned of the mechanism of contraction, the key questions are how, when and where selected regions of the cell membrane release or transmit Ca^{2+}.

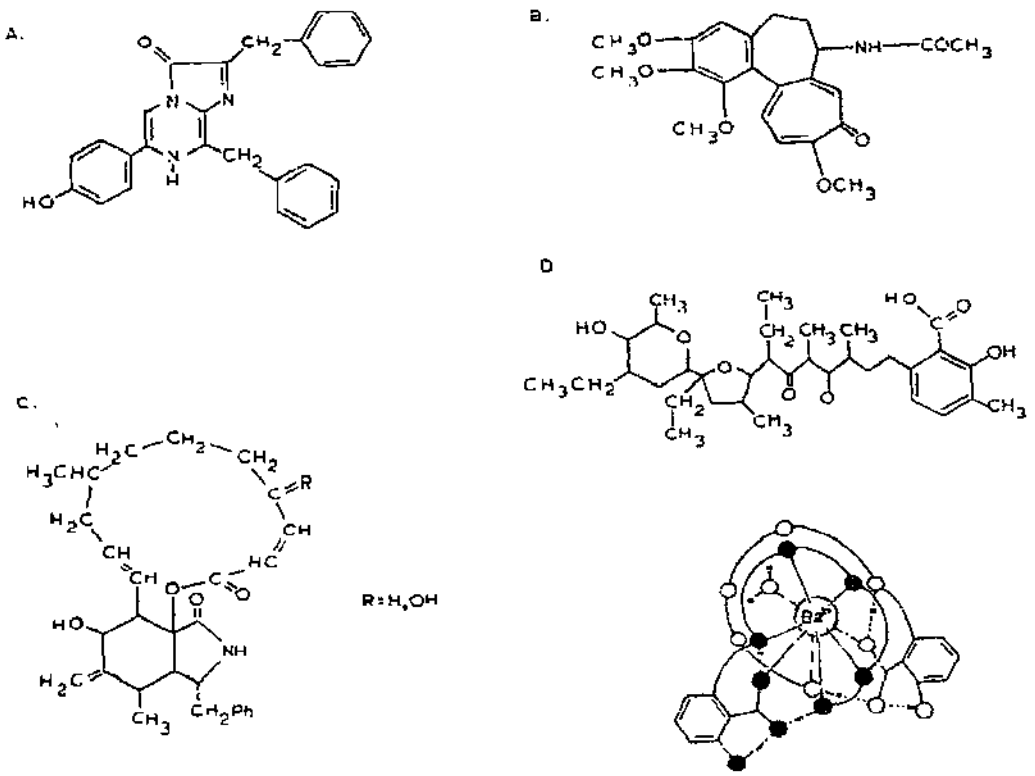

Fig. IV-5. Structural formulae. A. Luciferin B. Colchicine C. Cytochalasin B D. X-537A (The crystal structure, as determined by Johnson et al. (1970), is shown schematically.).

c. Platelets and blood clotting. Blood platelets are the most common source of microfilaments for experimental work. Cohen and DeVries (1973) have formed clots in cylindrical tubes and measured their contractile properties. The clot can be made alternately to contract, then relax by additions of Ca^{2+}, and then of EGTA. The apparent Ca^{2+} pK_d is 6.7. Human platelets contain 5.8×10^{-8}M Ca^{2+}/mg platelet protein (Steiner and Tateishi, 1974). This is over five times more than most other mammalian tissue. 98% of the Ca^{2+} is protein, not lipid bound. Skaer et al. (1974) showed in electron probe (section III.B.8.) analyses of frozen-dried, unstained material that most of the calcium is found in the so-called dense bodies with some calcium at the membrane. The dense bodies also appear to contain most of the platelet phosphorus, supposedly stored in the form of $(Ca^{2+})_2ATP^{4-}$. Upon aggregation some 90% of the platelet Ca^{2+} is released, along with ADP, serotonin, thromboplastin and Factor $XIII_a$. Apparently this calcium is involved in initiating actomyosin (thrombosthenin) contraction, but this is not yet proven. That calcium is crucial to the clotting process is seen at the clinical level by the use of calcium chelators, such as oxalate or citrate, as anticoagulants. There seems to be adequate free Ca^{2+} in the serum for fibrin formation without additional release from the dense bodies of the platelets. That is, even though Ca^{2+} is critical to clot formation, it does not seem to be a controlling factor.

The various enzymatic steps involved in the formation of the fibrin clot are outlined in Fig. IV-6. Prothrombin (Fig. IV-7) is a single polypeptide chain of MW = 72000 (see review by Esmon and Jackson, 1974). It can be cleaved at

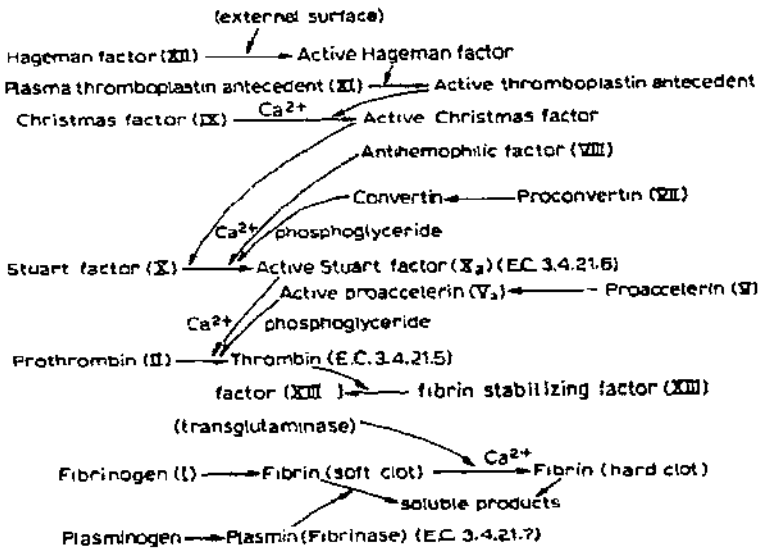

Fig. IV-6. Summary of blood clot formation. Horizontal arrows indicate reaction pathways. Other arrows indicate involvement of the indicated factor in the reaction.

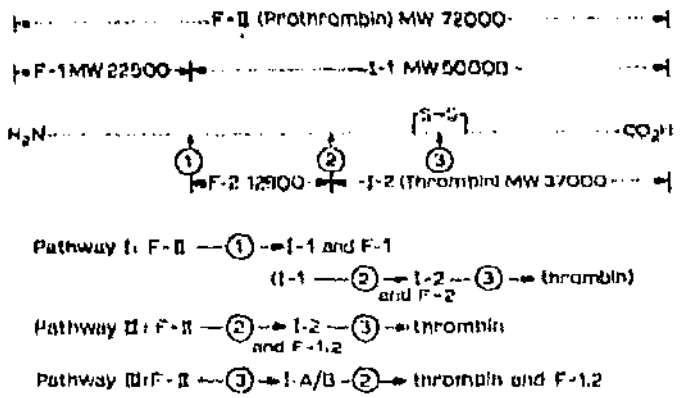

Fig. IV-7. Summary of prothrombin cleavage. Proteolytic cleavage sites and reactions are indicated by circled numbers. The different pathways have different sequences of cleavages.

three sites "1", "2", and "3" in any or all of the sequences:"1", "2", "3" (Pathway I; "2", "3" (Pathway II) or "3", "2" (Pathway III). Factor X_a (without factor V_a) can catalyze both cleavages "2" and "3", primarily via pathway II. However the physiological process actually involves the complex: Factor X_a, Factor V_a, phospholipid and Ca^{2+}. Thrombin cleaves at site "1". Factor V_a, though not obligatory for thrombin activation, greatly enhances the rate relative to that achieved by Factor X_a (plus phospholipid and Ca^{2+}) alone. It interacts with the fragment 2 region of prothrombin. The actual relationship of Ca^{2+} or phospholipid to Factors X_a or V_a is not known.

Not only do Factors X_a and V_a apparently interact directly with calcium, their substrate prothrombin (or Factor II) binds calcium. Benson et al. (1973) found that I-1 does not bind calcium whereas Factor II binds 10 calcium ions while the smaller F-1 binds 12—15 Ca^{2+}, both with $pK_d \simeq 3.2$ as determined both by equilibrium dialysis and gel filtration techniques. Stenflo and Ganrot (1973) reported a similar value; however they found that the first three calcium ions do bind cooperatively.

Nelsestuen et al. (1974) showed that one (and by inference some eight others) glutamic acid of F-1 is modified to γ-carboxy glutamic acid. This modification is vitamin K dependent and is required for calcium binding (supposedly one Ca^{2+} per pair of carboxylate groups) and for phospholipid binding. Several other factors are vitamin K sensitive (F-VIII, IX and X). Factor X may have fourteen extra carboxylate groups.

Fibrin stabilizing factor of plasma has four subunits (a_2b_2, MW = 2 × 81000 + 2 × 81000 = 320,000). Thrombin activation of the zymogen produces Factor ($XIII_a$) (a'_2b_2, MW = 2 × 77000 + 2 × 81000 = 310,000). The b chain, which contains carbohydrate is unchanged. Platelets also contain Factor XIII of structure a_2. Factor $XIII_a$ (as does liver transglutaminase) catalyzes formation of a fibrin dimer

$(\alpha\text{-S-S-}\beta\text{-S-S-}\alpha)_2 \rightarrow \alpha\text{-S-S-}\beta\text{-S-S-}\alpha\text{-}\alpha\text{-S-S-}\beta\text{-S-S-}\alpha$

where the basis reaction is

$$\alpha_1\text{—}\overset{\overset{O}{\|}}{C}\text{—NH(Gln)} + H_2N\text{-}\alpha_2(\text{Lys}) \rightarrow \alpha_1\text{—}\overset{\overset{O}{\|}}{C}\text{—NH—}\alpha_2 + NH_3$$

Cooke (1974) and Curtis et al. (1974) confirmed that Ca^{2+} is required for enzymatic activity of Factor $XIII_a$.

Calcium increases the rigidity of fibrin clots not only by binding to Factor $XIII_a$, thereby enhancing the degree of covalent cross linking, but also by direct interaction with fibrin itself (Endres and Scheraga, 1972). Shen et al. (1974) found that the elastic modulus of fibrin clots in 8×10^{-3} M Ca^{2+}, measured as a function of time, plateaued near 150 dyne/cm^2 while the modulus was 75 dyne/cm^2 for clots in calcium free medium.

d. Microtubule related processes. Microtubules are found in nearly all eukaryotic cells (see reviews by Bryan, 1974, Wilson et al., 1974, Borisy et al., 1974, and Olmsted and Borisy, 1973). In addition to their long recognized presence in cilia and flagella, they are present in the spindles seen at cell division (Inoué et al., 1974, Rebhun et al., 1974). They are strongly implicated in secretory processes — thyroid (Williams and Wolff, 1970) nerve termini (Thon et al., 1972) — and in transcellular movement of procollagen (Ehrlich and Bornstein, 1972).

Induced granulocyte chemotaxis correlates with microtubule assembly and a net flux of calcium from the cytoplasm into the external medium and into the granular (supposedly the mitochondrial) fraction (Gallin and Rosenthal, 1974). Their high concentration in brain tissue remains unexplained though they are assumed to be involved in axonal transport. In a wide variety of cell types particles move over distances and at speeds which cannot be accounted for by Brownian motion. Such saltatory movement (see review by Rebhun, 1972) as well as cytoplasmic transport often appears to involve microtubules. In most of these processes calcium, as well as cAMP, serve as modulators; hence the tubulin—calcium interaction is potentially significant. Whether the microtubules play only a structural role or are directly involved in force generation by either a polymerization depolymerization equilibrium or a dynein-like ATPase is unknown.

Tubulin is a dimer of homologous but non-identical α and β subunits. Polymerized tubulin is the main, in some instances the sole, constituent of microtubules. The α, β dimer molecular weight from a variety of tissues from different animals is 120,000 ± 8%. The subunits can be dissociated only in denaturing agents such as SDS or guanidine-HCl. The α and β subunits can be resolved on the basis of electrophoretic mobility provided they are in a solution of low ionic strength. Lee et al. (1973) determined by several techniques that the molecular weights of the α and β subunit mixtures are 54000 ± 1000.

Both reassembled brain microtubules (Erickson, 1974) and outer doublet microtubules of cilia and flagella (Amos and Klug, 1974) consist of 13 longitu-

dinal filaments whose side to side arrangement is staggered by 9.2 Å. This forms a left handed, 3-start helix; the distance between tubulin dimers along one filament is 40 Å. When flattened, the distance between filaments is 51 Å. Both groups' optical reconstructions of electron-micrographs show the basic 40 by 50 Å subunit, alternately α or β, to be dumbbell shaped, with the handle running from lower right to upper left. Apparently the α and β subunits are very similarly shaped and oriented. The 80 Å α, β dimer repeat is not observed by Erickson and is weak and variable in Amos and Klug's work. They suggest one subunit has a radial extension from 90 to 130 Å while the other is 80 to 120 Å. The ten (or eleven) filament "B" subfiber of the flagellar outer doublet has a structure similar to the complete 13 strand "A" fiber. Amos and Klug suggest it may be attached to the "A" fiber by a different protein.

Microtubules assembled in vitro appear to have the same structure and drug binding capacities as do those isolated from spindles or cell cytoplasm (Borisy et al., 1974). The following observations seem well established for mammalian brain tubulin polymerization: i. Colchicine both inhibits polymerization and induces, with no time lag, depolymerization. ii. Maximal levels of polymerization, reached after twelve minutes, are observed at 37°; at 15° there is no polymerization. iii. the pH optimum is 6.8—6.9. iv. Either GTP or CTP or ATP (10^{-3} M) are required for polymerization. Di- or mononucleotides do not work. v. Ca^{2+} at 10^{-4} M inhibits polymerization. Haga et al. (1974) measured pig brain polymerization by flow birefringence. Free calcium ion at 10^{-5} M both inhibits polymerization and caused depolymerization.

Although the various interactions of microtubules are not understood at the molecular level, it appears that Ca^{2+} will prove to be one of their controlling factors.

e. Spasmoneme. The spasmoneme, an intracellular organelle, is responsible for flexure of the stalk in vorticellids. The addition of 10^{-7} M Ca^{2+} causes a conformational change in the fibrous protein with an actual shortening speed fifteen times faster than the fastest known striated muscle No chemical bond energy is expended during contraction. Apparently all of the work is entropic and is performed by the enclosing membrane in pumping out calcium prior to contraction (Weis-Fogh and Amos, 1972). The spasmonemal proteins of *Zoothamnium geniculatum* are readily dissolved in 1% SDS (Amos, 1975). In acrylamide gel electrophoresis one band, MW $\simeq$ 20000, accounts for 60% of the protein. Its pI is 4.8 as judged by its mobility on electrophoresis and it binds one to two moles of Ca^{2+} with pK_d 6—7. The spasmoneme, isolated free of surrounding membrane, behaves like a rubber; it returns to its rest conformation after repeated stretching to four times its rest length. The phylogenetic distribution of this mechanism of motility has not been determined. Nonetheless this is one of the most intriguing proteins yet characterized.

f. Cilia. Kung and Naitoh (1973) have examined a mutant of *Paramecium aurelia* which cannot swim backwards as can the wildtype. The alteration appears to reside in a membrane protein, possibly a calcium ionophore, since the

reversed ciliary beat can be restored by altering the cell membrane with a detergent and placing the paramecium in a calcium solution over 10^{-6} M (Eckert, 1972).

3. Other intracellular, Ca^{2+} modulated enzymes

a. Aequorin and luciferin binding protein. Cormier et al. (1974) recently published a clarifying review of the field of calcium induced "Bioluminescence in *Coelenterates*". A nervous impulse signals the release of Ca^{2+} from, or through, the cell membrane thereby initiating the events leading to light emission. Hence the situation is similar to that in muscle. One system (from phylum *Cnidaria*, Class *Anthozoa*) requires oxygen; the other (from Phylum *Cnidaria*, Classes *Hydrozoa* (e.g. *Aequorea*) and *Scyphozoa* and from Phylum *Ctenophora*) does not. In simplified form:

Anthozoans

LBP—luciferin + $Ca^{2+} \rightleftharpoons$ LBP—Ca + Luciferin.

Luciferin + O_2 + Luciferase → Luciferase—oxyluciferin (monoanion)* + CO_2.

Luciferase—oxyluciferin (monoanion)* → Luciferase + Oxyluciferin + Light.

Hydrozoans

Aequorin (with oxyluciferin precursor) + $Ca^{2+} \rightleftharpoons$ Apoaequorin—Ca(with oxyluciferin monoanion)*.

Apoaequorin—oxyluciferin (monoanion* → Apoaequorin + Oxyluciferin + Light.

The two systems are closely analogous. One is tempted to speculate that they are homologous. The luciferin binding protein and luciferase genes might have fused to form the aequorin gene.

b. Phosphodiesterase activator protein. Cyclic nucleotide phosphodiesterase (EC 3.1.4.1.) hydrolyzes cAMP and cGMP, thereby preventing the build up of these second messengers. Of the several forms of phosphodiesterase found in heart muscle (see review by Wang et al., 1975) one, called PI, is inactive without a protein activator which in turn requires calcium to be active. Miki and Yoshida (1972) noted the functional similarity of the activator to TN-C; Wang et al. suggest a common evolutionary origin. The heart muscle activator appears to be similar to that initially isolated from rat(Kakiuchi et al., 1973 and Teshima and Kakiuchi, 1974) and bovine (Cheung, 1971) brain. Calcium does not bind directly to phosphodiesterase, but instead binds rather specifically to the activator, thereby producing a conformational change which must precede the activator, enzyme interaction. TN-C will not activate phosphodiesterase, (Wang et al.) even though the amino acid compositions of TN-C and the activator are remarkably similar.

Wolff and Brostrom (1974) have isolated from pig brain, as well as from bovine adrenal medulla and testis, a calcium binding protein ($pK_m \simeq 5.4$) which activates phosphodiesterase. This "calcium dependent activator" appears to be identical to CaBP-II mentioned in section IV.D.2.

c. Phosphorylase b kinase. Phosphorylase b, a dimer, is rather inefficient in catalyzing the conversion of glycogen to glucose-1-phosphate. The phosphorylated form of phosphorylase is called phosphorylase a (see review by Walsh and Krebs, 1973). The phosphorylase kinase [EC 2.7.1.38] is activated by Ca^{2+} in blow fly flight-muscle (Sacktor et al., 1974) and in rabbit muscle (Brostrom et al., 1971). Phosphorylase kinase has twelve subunits (A_4,B_4,C_4). Drummond and Duncan (1968) concluded that "Kinase-activating factor is a calcium-activated proteolytic enzyme, that kinase-inhibitory factor is a proteolytic inhibitor and that activation of phosphorylase b kinase by Ca^{2+} involves proteolysis". However, they were reluctant to suggest a physiological function because of the apparent need to resynthesize a peptide bond in order to inactivate the phosphorylase kinase. It is possible that direct calcium interaction with phosphorylase kinase provides short term modulation, while proteolytic activation is long term and irreversible.

d. Adenylate and guanylate cyclases. The formation of 3′: 5′-cyclic AMP + pyrophosphate from ATP is catalyzed by adenylate cyclase [EC 4.6.1.1]. Since cAMP and Ca^{2+} are so often observed functioning together as second messengers (Rasmussen et al. 1972), it is very important to characterize the calcium—adenylate cyclase interaction. However, since the enzyme is apparently associated with membranes, it is not yet possible to be certain that the calcium is even interacting directly with the enzyme. As an example, the adenylate cyclase of mouse parotid was solubilized in 1% Lubrol PX. The activity of this preparation was inhibited some 50% by 5×10^{-4} EGTA; and was almost fully active in 5×10^{-4} M EGTA, 5×10^{-4} M $CaCl_2$ (Franks et al. 1974).

A guanylate cyclase [EC 4.6.1.2] activity has been demonstrated in various tissues, including bovine retinas (Pannbacker, 1973) and rat smooth muscle (Schultz et al. 1973). Again, as with adenylate cyclase, cGMP sometimes functions as a second messenger in conjunction with Ca^{2+}; so it is particularly important to understand the Ca^{2+}, enzyme interaction.

e. Cytoplasmic enzymes, not Ca^{2+} modulated. Several other enzymes (Table IV.3.) from the cytosol of eukaryotes have been reported to bind Ca^{2+}. In general their Ca^{2+} affinities are so low that it is doubtful that this calcium binding has any physiological significance (see review by Kretsinger, 1976, for details).

f. Extracellular enzymes, Ca^{2+} activated. Many extracellular enzymes (Table IV.4.) require Ca^{2+} for either maximal activity, thermostability or protection against proteolysis. None of them appear to be modulated by Ca^{2+}. It is unlikely that any of these enzymes or those of the cytoplasm which are not Ca^{2+} modulated are EF hand homologs.

4. Cell proliferation

Cell division is one of the most complex, as well as significant, of all cell

TABLE IV-3

Summary of intracellular enzymes not calcium modulated

Protein	E.C.	$pK_d(Ca^{2+})$	$pK_m(Ca^{2+})$
a. Phosphofructokinase (ATP:D-Fructose-6-phosphate 1-phosphotransferase)	2.7.1.11		~3 (inhib)
b. Fructose diphosphatase (D-Fructose-1,6-bisphosphate 1-phosphohydrolase	3.1.3.11		~4 (inhib)
c. Pyruvate kinase (ATP:Pyruvate phospho-transferase)	2.7.1.40	3.7 (inhib)	
d. Creatine kinase	2.7.3.2		~3
e. α-Glycerophosphate dehydrogenase (sn-Glycerol-3-phosphate:NAD^+ 2-oxidoreductase	1.1.1.8		(7.2) (mitochondria)
f. Alkaline phosphatase (Orthophosphoric-monoester phosphohydrolase)	3.1.3.1		~4 ($\overline{c}$CaATPase)
g. Pyrophosphatase (Pyrophosphate phosphohydrolase)	3.6.1.1	3.8 (Gd^{3+} 7.1)	(yeast)
h. Nicotinamide nucleotide transhydrogenase			3.7 (bacteria)
i. Kynurenine aminotransferase (L-Kynurenine:2-oxoglutarate aminotransferase)	2.6.1.17		~3 (mitochondria)
j. 25-Hydroxycholecalciferol-1-hydroxylase			3.5 (mitochondria)
k. Phosphatidylinositol hydrolase			6.8
l. Prostaglandin dehydrogenase			5.8 (inhib)
m. Protein kinase	2.7.1.37		4.7 (inhib)
n. Endonuclease (Deoxyribonucleate oligonucleotidohydrolase)	3.1.4.5.		~3

processes. The overall response of cell division is the result of many component processes — enzyme activation, DNA synthesis, spindle formation and shortening, cleavage furrow formation, etc. — all of which Ca^{2+} might modulate. At this stage most of the research is phenomenological. Some specific ef-

TABLE IV-4

Summary of extracellular enzymes

Enzyme	E.C.	$pK_d(Ca^{2+})$	n	MW	Sub-units	Source
a. Thermolysin	3.4.24.4	4.7 >6	2 2 } 4	34600	1	B. stearothermophilus
b. Clostridiopeptidase B	3.4.22.8	3.3	1	~60000	1	C. histolyticum
c. Proteinase (or Pronase)	3.4.99.?	low	?	~30000	1	S. griseus
d. Collagenase	3.4.24.3	low	?	vary	vary	bact. → vert.
e. Trypsinogen	3.4.21.4	3.2 1.5	1 1 } 2	23990	1	mammal pancreas
f. Prothrombin (F-11) fragment 1	3.4.21.5	3.2 3.2	10 13	72000 22500	1 1	human plasma
g. Transglutaminase (Factor $XIII_a$)		~3.0 3.8	1 2	81000 310000	1 4	mammal liver human plasma
h. Phospholipase A_2 (Phosphatide 2-acyl-hydrolase)	3.1.1.4	~3 (pH 7) ~4 (pH 8)	1 2	14600 29800	1 2	pig pancreas Crotalus venom
i. Phospholipase C (Phosphatidylcholine cholinephospho-hydrolase)	3.1.4.3	low (inhib)		?	?	C. perfringens
j. Nuclease (Nucleate 3'-oligonucleotidehydrolase)	3.1.4.7	~3 (pK_m)	1	16500	1	S. aureus
k. DNase A (Deoxyribonucleate oligonucleotido-hydrolase)	3.1.4.5	4.8 3.7	2 3 } 5	31000	1	mammal pancreas
l. Lysozyme (Mucopeptide N-acetylmuramoyl-hydrolase)	3.2.1.17	low	1?	14400	1	vertebrate
m. α-Amylase 1,4-α-D-Glucan glucanohydrolase)	3.2.1.1.	~6 >6 low	1? 2 2 } 4	45000 48000	1 2	hog pancreas B. subtilis

fects are discussed in other sections — microfilaments and cleavage furrow formation, microtubules and spindle shortening, etc.

Whitfield et al. (1973) concede that it is too early to suggest a detailed model of cell proliferation; however they review the observations which must ultimately be incorporated. The calcium activity of the extracellular fluid "directly determines the extent of cell proliferation in the bone marrow and thymus gland". "Calcium seems to control the multiplication of avian fibroblasts" as well as of rat thymic lymphoblasts "by promoting the initiation of DNA synthesis". The fact that chicken fibroblasts, infected with Rous sarcoma virus, multiply rapidly in a low calcium medium, in which normal fibroblasts cannot proliferate, is of great interest in cancer research (Balk, 1971). Mazia et al. (1972) identified a CaATPase activity in the mitotic apparatus of sea urchin eggs. They speculate that it is associated with either the microtubules of the spindles or with small vesicles dispersed between the spindles and near the poles.

The second consideration involves several cAMP effects which are somewhat contradictory. As discussed by Rasmussen et al. (1972), one does not yet understand all of their interactions nor whether the second messenger effects of cAMP and of Ca^{2+} operate in series and/or parallel.

Using the divalent specific ionophore, A23187, Maino et al. (1974) induced the transformation of lymphocytes. The sequence of events — enhanced uptake of ions and metabolites, increased cAMP levels and phospholipid metabolism and finally DNA synthesis and mitosis — induced by A23187 plus 10^{-3} M external Ca^{2+} are the same as those produced by the natural mitogen, phytohaemagglutin. They "suggest that lymphocyte transformation is the result of a direct effect of the mitogen on the permeability of the plasma membrane to Ca^{2+}".

Intracellular Ca^{2+} is essential for yeast cell division. A23187 treatment in a medium deficient in Ca^{2+} and Mg^{2+} inhibited cell division with an ion efflux, lowering the total cell calcium by a factor of 7 and the total magnesium to half its normal value (Duffus and Patterson, 1974).

Steinhardt et al. (1974) treated a variety of unfertilized eggs — from sea urchins, mollusca, bat-star, toad and hamster — with 5×10^{-6} M A23187 and observed the usual signs of normal fertilization — elevation of the fertilization membrane, change in plasma membrane conductance and increase in protein and DNA synthesis. In contrast to lymphocyte transformation, ionophore activation does not depend on exogenous calcium; ^{45}Ca prelabeled eggs show increased efflux. They "propose that release of intracellular Ca^{2+} may be the universal factor promoting activation of egg metabolism at fertilization." This proposed release of internally stored Ca^{2+} is consistent with the finding that externally applied A23187 releases Ca^{2+} from the sarcoplasmic reticulum of single barnacle muscle fibers (Hainaut and Desmedt, 1974). Further support comes from Nakamura and Yasumasu (1974), who found that for the complete homogenate of the sea urchin egg the Ca^{2+} pK_d for the unfertilized egg was 4; this rose to 3 within 10 min after fertilization.

D. Calcium and membranes

1. Membrane structure

a. General model. In section IV. C. we considered Ca^{2+} as a second messenger focusing on its targets and their modes of interaction. Yet at a functional level the conceptual key concerns the membrane. How is a Ca^{2+} gradient established? How does a signal cause the release or allow the passage of Ca^{2+}?

In the generally accepted fluid mosaic model of Singer and Nicolson (1972 the phospholipids and cholesterol form a lipid bilayer some 40 Å thick from polar group to polar group, (Fig. IV-8). The porosity and fluidity of the lipid bilayer vary as a function of composition, temperature and ionic environment. Intrinsic, amphipathic proteins are imbedded in the bilayer with only a small fraction of their surface exposed, on either or both sides, to the aqueous environment. The protein, lipid interactions may be rather strong, specific and critical to protein function. Various extrinsic proteins may associate with the membrane surface. They are functionally defined as being easily extracted without using protein denaturing or lipid disrupting solvents. The membrane may be thought of as a two dimensional liquid in which both the lipids and the proteins diffuse in both rotational and translational senses. The more ordered arrangement, with the lipid in the myelin sheath or the nicotinic receptor protein lattice (Cartaud et al., 1973), would appear to be the exception.

A more recent finding, which is certainly consistent with the Singer model, is that many membranes show a sidedness or markedly asymmetric distribution of both their lipid and their protein components (see review by Bretscher, 1973). This asymmetry is of potential use not only for transport mechanisms but also as a site of insertion for structural and contractile elements inside the cell. Many intrinsic proteins have covalently bound sugar groups, which seem to point exclusively to the outer, aqueous side. Finally, it is certainly relevant to cation sequestration that most plasma membranes have an external glycan, glycoprotein layer which is poorly understood. In plants and bacteria this is thickened and cross-linked to form a cell wall.

b. Phospholipid, Ca^{2+} interactions. In addition to the control of Ca^{2+} fluxes by membranes, calcium itself may significantly alter the properties of membranes. The effects of calcium on synthetic bilayers of phosphatidylserine (PS) and "spin-labelled" phosphatidylcholine (PC*) were studied by ESR spectroscopy. Ohnishi and Ito (1974) found that Ca^{2+}, in concentrations as low as 10^{-3} M, greatly reduced the fluidity of the membrane, supposedly by forming O—Ca—O cross bridges between the PS carboxylate or phosphate groups. Sr^{2+} and Ba^{2+}, but not Mg^{2+}, also induce PS "patch" formation. Ca^{2+} appears to facilitate the fusion of PC and PS vesicles (Maeda and Ohnishi, 1974). From calorimetric measurements Verkleij et al. (1974) showed that Ca^{2+} can condense and raise the so-called phase transition, or characteristic temperature of phosphatidylglycerol suspensions even at molar ratios as low as 1 : 100. Montal (1973) added Ca^{2+} to either side of an asymmetric lipid bilayer and

observed no reaction with the neutral PE side but a binding to the anionic PS side. This binding reduced the electrical resistance and the mechanical stability of the membrane. Both Seimiya and Ohki (1973) and Hammermeister and Barnett (1974) have also observed a general condensing effect of Ca^{2+} on synthetic membranes with apparent preference for the carboxylate groups of PS.

Seimiya and Ohki found that the various binding constants were critically dependent on the area of the phospholipid in the membrane. Selected values of pK_d (assumed $K_d = [A^-]\,[Ca^{2+}]/[CaA^+] = [A^-]\,[CaA^+]/[CaA_2]$):

	Group	pK_d
Stearic acid	$-CO_2^-$	2.8
PS 70 A^2/Molecule	$-CO_2^-$	3.2
	$-OPO_2^-O-$	2.9
PE 150 A^2/Molecule	$-OPO_2^-O-$	2.9

c. Ionophores. Ionophores are compounds which carry ions. They may be proteins, as CaATPase normally found in the sarcoplasmic reticulum membrane. Usually the term refers to small molecules, which are often antibiotics. Some are mobile carriers, while others are channel formers. Generally they are used either in synthetic systems as a means of studying membranes themselves, or in cellular systems as a means of "inserting" cations such as Ca^{2+} into the cytoplasm with minimal damage to the cell membrane.

During the past year many systems have been investigated using the Ca^{2+} ionophores — X-537A and A23187. Examples considered in other sections include: muscle (Levy et al., 1973, Hainaut and Desmedt, 1974); neuromuscular junction (Kita and van der Kloot, 1974); parotid (Selinger et al., 1974); salivary gland (Rossignol et al., 1974); mitochondria (Wong et al., 1973); leucocytes (Schell-Frederick, 1974, Cochrane and Douglas, 1974, Maino et al., 1974); and unfertilized eggs (Steinhardt et al., 1974).

The mechanism of ion transport by ionophores can be represented by four steps (XH is the protonated ionophore, M^+ is the cation):

(i) outside: $XH + M^+ \rightleftharpoons XM + H^+$ (or $2\,XH + M^{2+} \rightleftharpoons X_2M + 2H^+$)
(ii) $XM_{out} \rightleftharpoons XM_{in}$ (translocation)
(iii) inside: $XM + H^+ \rightleftharpoons XH + M^+$
(iv) $XH_{in} \rightleftharpoons XH_{out}$ (translocation)

Net $M^+_{out} + H^+_{in} \rightleftharpoons M^+_{in} + H^+_{out}$

Although there are many variations on this basic scheme, it does focus attention on the various dissociation constants and solubility products involved, as well as the desirability of knowing the structure(s) of "X". Further it emphasizes the fact that the process is driven by a preexisting cation gradient, with charge balance maintained by countertransport of protons (or other cations).

X-537A is a monocarboxylic antibiotic. In the crystal (Johnson et al., 1970) of its 2 : 1 complex with Ba^{2+}, the cation is nine coordinate with oxygen, with

one carboxylate and one hydroxyl group of one X-537A molecule, one carboxylate, two hydroxyl, two ring ether and one carbonyl of a second, plus a water molecule (see Fig. IV-5). The 2 : 1 Ca^{2+} complex is probably similar.

Degani and Friedman (1974) have completed a thorough study of the circular dichroic and fluorescence spectra and binding constants of X-537A with a series of mono and divalent cations. They were particularly interested in the molecular species involved in the translocation steps (ii) and (iv) and chose methanol and hexane as solvents; the former being more, and the latter less, polar than the interior of a phospholipid bilayer. They identified species $(M^+X^-)^0$, $(M^{2+}X^-)^+$ and $(M^+H^+X^-)^+$ in methanol and $(M^+X^-)^0$, $(M^{2+}X_2^-)^0$ and $(M^+H^+X_2^-)^0$ in hexane. Haynes and Pressman (1974) also used fluorescence spectroscopy and found that the quantum yield increases with decreasing solvent polarity.

Concentration dependence studies indicate the permeant species -- HX_2^- and $CaHX_2^+$ (Celis et al., 1974). From biionic potential measurements they confirm the selectivity sequences

$H^+ >> Cs^+ > Rb^+, K^+ > Na^+ > Li^+$ and $Ba^{2+} > Ca^{2+} > Mn^{2+} > Sr^{2+} >> Mg^{2+}$

7.6	3.43	3.56	3.58	2.57	1.68	6.46	4.57	4.40	5.47	3.83

Dissociation constants, pK_d in methanol at 25°C (Degani and Friedman, 1974) are listed for comparison. The molecular mechanisms involved in the ionophoric action of X-537A remain to be elucidated.

Beauvericin is a neutral cyclic hexadepsipeptide with an alternating sequence of three D-α-hydroxyisovaleryl and three N-methyl-L-phenylalanyl residues. The cation selectivity appears to be $Rb^+ > Ba^{2+} > K^+ > Na^+ >> Ca^{2+} >>> Li^+$ (Roeske et al., 1974) in the presence of picrate, but $Ca^{2+} > K^+ > Cs^+ > Li^+ > Na^+$ in various other solutions (Prince et al., 1974).

A23187 is more specific for divalent cations than is X-537A (Reed and Lardy, 1972); however, less work has been done on its characterization.

d. Exocytosis and Secretion. The various phenomena related to secretion will be discussed in terms of questions which are suggested by the model, illustrated in Fig. IV-1.

An external stimulus — electrical, mechanical or chemical — reaches the cell surface.

i. What is the stimulus?

ii. What is its site of interaction on the cell surface?

There is a subsequent rise in the intracellular Ca^{2+} concentration.

iii. What is the source of the Ca^{2+}?

iv. Is there a rise in cAMP?

v. If so, what is the Ca^{2+}, cAMP relationship?

The contents of a vesicle are secreted.

vi. Is a vesicle actually involved?

vii. If so, did it fuse with the cell membrane?

viii. Is actin or myosin associated with the vesicle?

ix. Are microtubules involved?

x. Are charges on the membrane surfaces neutralized?

As implied by the last three questions, three basic mechanisms have been suggested, any combination of which may obtain in a specific cell. Actin is attached to the cell membrane and either actin or myosin to the vesicle, and contraction draws the vesicle to the cell surface; and/or microtubule fenders prevent vesicles from touching the cell membrane, and depolymerization allows contact which leads to fusion; and/or vesicles randomly diffuse to the membrane but only in the presence of Ca^{2+} can they fuse.

i. Stimuli. The various stimuli which elicit secretion are reviewed by Rasmussen et al. (1972) and by Douglas (1974). The electrical stimulus can be self generated, e.g. the nervous impulse travelling down the nerve axon to its own terminus. Although nervous transmission between cells normally involves cholinergic or adrenergic chemotransmitters, some cells have direct electrical coupling. Mechanoreceptors appear to operate Na^+ and/or Ca^{2+} gates; however, as discussed for *Paramecium* in section IV. C.2.f., it is not known whether the Ca^{2+} influx is the primary or secondary response to touch.

Most excretion experiments use systems which are stimulated by chemicals, usually hormones or more recently various ionophores.

ii. Site. Specific receptor proteins have been isolated for various hormones. The hormone, receptor interactions do not require Ca^{2+}. The possible interaction of such receptors with either a Ca^{2+} gate or adenylate cyclase remains to be proven. A mechanoreceptor ionophore interaction remains to be demonstrated.

iii. Source. That Ca^{2+} is usually, if not always, involved in "stimulus, secretion" is well established. In addition to the examples previously cited, Phillis (1974) has reviewed the evidence that serotonin and adrenalin "produce their post-synaptic effects on neuronal activity in the central nervous system, through a calcium dependent mechanism". The secretory granules of individual mast cells of the rat mesentery were seen in the light microscope to be extruded following microinjection of Ca^{2+}, but not Mg^{2+} (Kanno et al., 1973). Cochrane and Douglas (1974) and Kagayama and Douglas (1974) observed the same response using the ionophores A23187 and X-537A. Miledi (1973) microinjected Ca^{2+} into the terminus of the squid giant axon and recorded transmitter release. Neither Mg^{2+} nor Mn^{2+} worked. Even in the presence of external Mn^{2+}, which is normally inhibitory, injected Ca^{2+} causes release of transmitter.

Admittedly, to elicit some response by injecting Ca^{2+} with either a micropipette or an ionophore does not prove that extracellular Ca^{2+} is the physiological second messenger. However in many ^{45}Ca studies it has been shown that stimulated cells incorporate more calcium.

What remains to be shown is whether Ca^{2+} on the inner aspect of the membrane is displaced by the stimulus. Nor is it known whether Ca^{2+} might be released from endoplasmic reticulum or mitochondrion by another second mes-

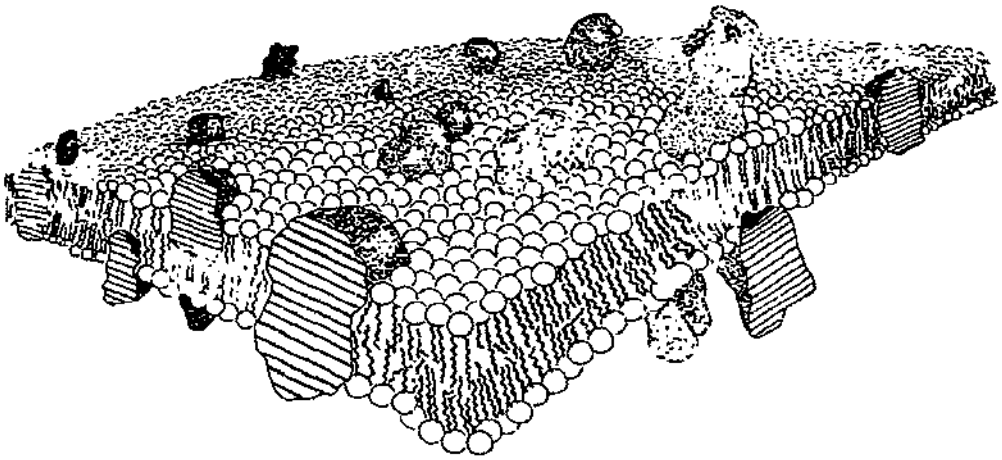

Fig. IV-8. Model of membrane structure. The "lipid, globular protein mosaic model" is drawn after Singer and Nicolson (1972). The phospholipid molecules form a two dimensional liquid. The intrinsic proteins are embedded in the hydrophobic region of the lipid bilayer; some extend through the membrane. The extrinsic proteins can be extracted without disrupting the membrane. The sugar groups, frequently found on the proteins on the outer surface of the membrane, are indicated by "X's".

senger, such as cAMP, thereby making Ca^{2+} a third messenger.

iv. cAMP. The question begs the answer. Sutherland (1972) as well as Rasmussen et al. (1972) have thoroughly discussed and documented the systems in which there is a rise in cAMP following stimulation. Recently Ueda et al. (1973) found that synaptic membrane fractions from rat cerebrum contain two intrinsic membrane proteins (I, 86000 MW and II, 48000 MW) which can be phosphorylated by a protein kinase which is cAMP dependent. Protein I is specific to neural tissues; Ueda et al. suggest that it may be involved in neurosecretion.

v. cAMP and Ca^{2+}. The systems in which there is an increase in both cAMP and Ca^{2+} are listed in Table IV-2. As discussed in section IV. C. 1., the question of series versus parallel response is, for most systems, not resolved. The possible effects of Ca^{2+} on adenylate cyclase, various kinases and phosphatases is considered in section IV.C.3.

Many proteins — troponin, DTNBLC, tubulin, vitamin D CBP etc — are phosphorylated. In several instances the responsible kinase is cAMP sensitive.

vi. Nonvesicular secretion. A few systems involve secretion without the involvement of vesicles. The fly salivary gland secretes fluid at the luminal border following stimulation by either the hormone, 5-hydroxytryptamine, or by A23187. Prince et al (1973) suggested that an increase in cytoplasmic Ca^{2+} is adequate to increase the permeability of the terminal membrane to Cl^-, with a resulting K^+, Cl^- secretion. The source of Ca^{2+} may be either mitochondrial or extracellular. Although an increase in cytoplasmic cAMP normally follows

stimulation, an increase in cytoplasmic cAMP without Ca^{2+} increase does not stimulate secretion.

In rat parotid gland slices, A23187 simulates the effect of adrenalin on the α-adrenergic receptor and stimulates massive K^+ release. Ca^{2+} is necessary in the external medium and cannot be replaced by Sr^{2+} or Mg^{2+} (Selinger et al., 1974).

vii. Fusion. There is little evidence that vesicle membranes derive from, or permanently fuse with, the plasma membrane. As will be discussed, in several instances the vesicular membranes can be separated from the plasma membrane. They have different lipid and protein compositions.

Nonetheless the secretory vesicles do seem to fuse temporarily with the plasma membrane so that the interior of the vesicle is topologically continuous with the extracellular space. For instance, Werning (1972) has analyzed acetylcholine release from crayfish neuromuscular junction and confirmed that the observed response is accounted for by the release of the contents of one or a few vesicles.

viii. Vesicle interaction with actin and/or myosin. Studies using the electron microscope suggest some sort of "fuzz" or basket-like structure surrounding some synaptic vesicles. Berl et al. (1973) separated the vesicle fraction from the synaptic membrane fraction of rat brain synaptosomes. "Neurin" and "Stenin" (40% and 60% by weight) accounted for 10% of the total synaptosomal protein. Over 70% of the myosin was associated with the vesicles; over 70% of the actin with the synaptic membranes. Subsequently Puskin and Kochwa (1974) isolated an "actin-relaxing factor complex" from synaptosomal membranes which resembles muscle thin filaments.

Blitz and Fine (1974) have characterized the protein in synaptosomes by SDS acrylamide gel electrophoresis and peptide composition.

Fraction	Actin	Tr'myosin	Myosin	Tubulin	X(50000 MW)
Synaptosome	12%	7%	4%	27%	2%
Synaptosome membrane	13%	8%	2%	23%	—
Vesicle	5%	3%	4%	19%	38%
Soluble	17%	—	4%	17%	—

ix. Microtubules. Although the evidence for microtubule involvement in exocytosis is somewhat weaker than is the evidence for the involvement of microfilaments or calcium modulated membrane fusion, it is still an important consideration. As noted in section IV. C.2.d., brain is a particularly rich source of tubulin. Microtubules are very abundant in axons and are assumed to play a key role in axonal transport. These microtubules extend to the synaptic region but their role in neurosecretion is still uncertain. As previously tabulated, Blitz and Fine (1974) find a tubulin like protein as 27% of the total synaptosome protein.

Both Burns and Pollard (1974) and Gaskin et al. (1974) have preliminary

evidence for a dynein-like protein in brain which co-purifies with tubulin. It is conceivable that axonal and perhaps synaptic microtubles possess an inherent contractibility.

Thoa et al. (1972) reported that both colchicine and vinblastine (as well as cytochalasin B) inhibited the release of noradrenalin and dopamine-β-hydroxylase from hypogastric nerve termini. They suggested that microtubules (as well as microfilaments) are involved in exocytosis.

Tubulin has been implicated in other secretory processes. Secretion of thyroid hormone involves thyroid stimulating hormone binding increase in cellular cAMP, endocytosis of thyroglobulin from the follicular lumen, fusion of the thyroglobulin vesicle with lysosomes, hydrolysis of thyroglobulin to individual iodinated amino acids and finally exocytosis. Colchicine blocks thyroid secretion apparently by interfering with microtubules involved with formation of the vesicles containing thyroglobulin. Ehrlich and Bornstein (1972) suggested, from colchicine and vinblastine inhibition, that microtubules are involved in the transcellular movement and secretion of procollagen. Similarly, microtubules may be involved in glucose-induced insulin secretion, a process which is known to involve a Ca^{2+} influx.

x. Charge neutralization. Miledi (1973) showed that micro-injection of some 10^5—10^6 calcium ions into the squid presynaptic nerve terminus caused the release of acetylcholine from vesicles. Muller and Finkelstein (1974) have considered the model of Ca^{2+}, Mg^{2+} competition for sites on the presynaptic plasma membrane which might potentially bind acetylcholine-containing vesicles. Aside from any considerations of the hypothetical sites, they maintain that the Mg^{2+}, as well as the K^+, inhibition of vesicle release need not even involve Mg^{2+} binding to the sites but might be due to the "decrease in surface potential on the outer surface of the presynaptic terminal".

Secretory granules containing adrenalin, noradrenalin and ATP are stored in the chromaffin cells of the adrenal medulla. Isolated chromaffin granules take up calcium by an undefined mechanism. As suggested in the Katz and Miledi "calcium hypothesis" (see Miledi, 1973, for recent results and discussion), the entry of Ca^{2+} following a stimulus should neutralize the anionic charge of the granules allowing fusion with the plasma membrane. Dean and Matthews (1974) determined that isolated granules, of average diameter 2985 Å, have 10800 anionic sites and adsorb 4200 calcium ions in total, and 1300 at $K_d \approx 10^{-2.8}$ M. Neuraminidase treatment removes about 20% of the calcium binding sites. In addition about for times as much calcium was actually taken up by the granules.

Brooks and Siegel (1973) suggested that the calcium binding protein, (section IV:D.2.d) which they isolated from beef adrenal medulla and other adrenergic tissue, might correspond to the "mobile receptor" mentioned in the Katz and Miledi "calcium hypothesis of stimulus-secretion coupling".

In contrast, Madeira and Antunes-Madeira (1973) isolated the synaptosomal fraction from sheep brain and found a divalent cation binding site for which Ca^{2+} and Mg^{2+} competed. Poste and Allison (1973) considered membrane

fusion in both cell fusion and exocytosis. They do not suggest any involvement of microfilaments but suggest a four step model: contact, induction, fusion and stabilization. During "induction" the Ca^{2+} and ATP^{4-} normally bound to the membrane are displaced by the neurotransmitter or hormone which stimulated the secretory cell. This implies that the calcium observed following a stimulus is not a second messenger at all but a displaced blocking factor.

2. Calcium gradients

a. Overview. Probably all eukaryotic cells, and possibly prokaryotic cells as well, have mechanisms for actively extruding Ca^{2+} (as well as Na^{+}). Most eukaryotes also contain mitochondria and an endoplasmic reticular (ER) system. In muscle cells this is highly specialized and called the sarcoplasmic reticulum (SR). Other cells have specialized granules or vesicles which appear not to be derived from the ER. In any case, these organelles, in particular the SR and the mitochondria, can sequester Ca^{2+}. One assumes that the usual mechanism involves a CaATPase; however, the designation, [EC 3.6.1.3] certainly does not imply homology or even functional similarity between the enzymes of different organisms. In some instances a CaATPase may be identified in the plasma membrane fraction, and the membrane may be involved in calcium transport. Yet that particular ATPase may be involved in the transport of some metabolite and not the transport of calcium. Various CaATPase containing systems will be outlined. The SR, about which most is known, will be discussed in detail (section IV.D.3.a.).

In other systems there is not a specific CaATPase; the pumping of Ca^{2+} is dependent upon the establishment of a Na^{+} gradient as in some nerves and muscle, or of an anion (or H^{+}) gradient as in the mitochondrion.

We have used the term "membrane", as illustrated in Fig. IV-8, referring to a molecular structure some 80 Å thick. However, when thinking in terms of entire organ systems, a "membrane" is an entire cell thick. In, for instance, the gut or kidney there is not only the problem of transporting Ca^{2+} across a molecular membrane, but also of transcellular transport of calcium.

Finally, there are a variety of calcium, membrane effects in plants which might not even involve CaATPase but which deserve far more research than they have received.

b. Ca^{2+}, (Mg^{2+})—ATPase systems

i. Bacteria. The Ca or Mg—ATPase of *E. coli* consists of four subunits of molecular weights 60000, 56000, 35000 and 13000 (Hanson and Kennedy, 1973). Full enzymatic activity is retained by the two larger subunits (Nelson et al., 1974). The membrane bound as well as the reconstituted enzymes show cooperativity with regard to Ca—ATP as well as to the inhibitor, Na^{+}. The membrane free enzyme is active but shows no cooperativity (Moreno et al., 1974). It is apparently involved in coupling oxidation to phosphorylation and in various active transport processes. Plate et al. (1974) have used mutants defective in Ca, Mg—ATPase activity to analyse the effects of colicin K. They suggest that colicin K de-energizes various energy dependent transport systems

or the cell membrane. There is no suggestion that it is involved in calcium transport; nor is the free Ca^{2+} concentration in bacterial cytoplasm known.

ii. Fungi. The filamentous coenocytic fungus *Achlya* requires calcium for growth and takes up calcium by facilitated diffusion in the presence of metabolic inhibitors. A low molecular weight glycopeptide of the cell wall and a component from the membrane bind Ca^{2+} and are involved (LéJohn et al., 1974).

iii. Molds. *Physarum* has calcium sequestering vacuoles possibly similar to endoplasmic reticulum (Braatz and Komnick, 1970). The accumulation is ATP dependent and supposedly related to the regulation of microfilaments involved in cytoplasmic streaming. Aldridge et al. (1973) reported that the oscillations in glycolysis normally seen in yeast can be specifically and reversibly altered in media 1.5×10^{-4} M in Ca^{2+}.

iv. Erythrocytes. The reason why erythrocytes maintain a low intercellular calcium ion concentration is unknown. It may relate to regulation of internal enzymes, or to the regulation of a putative contactile system, or to maintaining the inner membrane in a deformable state (see review, Juliano, 1973). Various indirect considerations have shown rather conclusively that the Ca^{2+} stimulated, Mg—ATP dependent ATPase is located on the inner surface of the membrane and is responsible for the active extrusion of Ca^{2+}. This Ca, Mg—ATPase is distinct from and independent of the Na and K, Mg—ATPase (Knauf et al., 1974 and Schatzmann, 1974 (references to previous work)). Knauf et al. suggest as the obvious candidate for the enzyme a membrane protein, MW 150,000, which is phosphorylated by ATPase only in the presence of Ca^{2+}. Schatzmann determined $K_d = 10^{-6.0}$M for Ca^{2+} binding to the membrane ATPase. This value seems more realistic than $K_d = 10^{-4.0}$ M determined by Buckley (1974).

In erythrocytes, as in liver cells and in cultured L-cells, the Ca^{2+} and the Na^{+} fluxes do not appear to be coupled as they are in squid axon. Olson and Cazort (1974) could find no amino acid to accompany the transported Ca^{2+}. Porzig (1973) has studied Ca^{2+}, Sr^{2+} and $^{40}Ca^{2+}$, $^{45}Ca^{2+}$ exchange in energy depleted ghosts and suggested that in addition to the CaATPase system of active transport, there is "a specific saturable system mediating a one-for-one exchange diffusion". Using p-nitrophenyl phosphate as a substrate, Rega et al. (1973) have found a calcium dependent phosphatase activity in human erythrocyte membranes. Both ATP and Mg^{2+} are required; the activity is located on the inner surface and appears similar to the Ca, Mg—ATPase.

The CaATPase binding appears to involve a tyrosine as suggested by Tb^{3+} fluorescence (section III.C.5.) measurements (Mikkelsen and Wallach, 1974).

v. Liver. Even though liver plasma membranes have high affinity Ca^{2+} binding sites ($K_d = 10^{-5.5}$ M, $4.5\ 10^{-5}$ M Ca^{2+}/g. membrane protein), Chambaut et al. (1974) could find no Ca—ATPase activity nor any membrane phosphorylation following Ca—ATP incubation. They suggest that the "calcium extruding activity is mediated by a mechanism involving no direct ATP hydrolysis at the membrane level."

vi Kidney. In the proximal tubule of the kidney cortex, Ca^{2+}, as well as Mg^{2+}, Na^+ and K^+, are reabsorbed from the primary urine to the plasma against a concentration gradient. The involved CaATPase does not require Mg^{2+}, K^+ or Na^+, but it can be activated by Mg^{2+} and Sr^{2+} (Parkinson and Radde, 1971). Kinne-Saffran and Kinne (1974) used preparative free flow electrophoresis to separate apical (luminal) from basal—lateral plasma membrane fragments and showed that the CaATPase (as well as the Na,K—ATPase) is located only in the basal—lateral membrane. The apparent K_d is 10^{-3} M; the enzyme is not inhibited by ruthenium red, as is erythrocyte CaATPase, or by ouabain, as are most Na,K ATPase's. The mechanism(s) of apical membrane transport and of transcellular transport remain unknown; however the finding of a vitamin D induced calcium binding protein (section IV.D.2.d.) in the kidney cortex is certainly suggestive.

vii. Intestine. Melancon and DeLuca (1970) found that the CaATPase of chick intestinal brush border increased several fold a day after vitamin D administration to rachitic chicks. How or whether the enzyme itself is modified is unknown.

viii. Placenta. Both guinea pig (Shami and Radde, 1971) and human (Miller and Berndt, 1973) placenta contain a CaATPase activity which is assumed to be involved in mother-to-fetus calcium transport. It is distinct from the (Na + K), Mg—ATPase, is ouabain insensitive and has a pH optimum over 8.0.

ix. Gill. Ma et al. (1974) report that the plasma membrane of gills of trout, *Salmo gairdneri*, contain a Ca (or Mg) ATPase with apparent $pK_d = 3.5$. If, as Ma et al. suggest, this CaATPase is involved in regulating calcium levels within the body, then the gill cells as well as those of placenta, kidney and gut epithelium, must have transcellular calcium transport mechanisms in addition to the CaATPase.

x. Brain. Ca^{2+} and/or Mg^{2+} activated ATPase have been isolated from a variety of nervous tissues. Stefanovic et al. (1974) suggest that the Ca^{2+} activated ATPase of cultured mouse neuroblastoma cells is found on the external surface of the cell membrane. Roufogalis (1973) reported a ouabain-insensitive (Ca^{2+} + Mg^{2+})ATPase in the microsomal fraction of rat brain. Its calcium pK_d is 6.5. Tetracaine inhibits Ca^{2+} stimulation. Although the physiological significance is not understood, Ca^{2+} competitively inhibits the formation of the phosphorylated ATPase intermediate which is involved in Na^+ transport (Tobin et al., (1973). This pattern of competition is seen not only in rat brain but also in kidney, liver and probably erythrocytes.

xi. Retina. Hagins (1972) in his review presented a specific model involving Ca^{2+} fluxes in visual reception. The rod cell of the vertebrate retina consists of a cell body and a constricted neck connecting to a cylindrical rod outer segment (ROS). The membrane of the ROS is deeply invaginated then pinched off forming a "disc" whose "inside" is topologically "outside" the cell. The entire ROS is filled with up to a thousand discs. In each of these disc membranes, imbedded rhodopsin accounts for 80% of the total protein; they also contain a great deal of (probably) bound calcium. With no illumination there is

a so-called "dark current" of Na^+ passing extracellularly from the cell body to the ROS. Upon illumination, 11-cis-retinaldehyde absorbs a photon and changes its conformation to all trans. The rhodopsin to which it is bound changes its conformation and situation within the membrane. Bound calcium is released from the disc to the "interior" of the ROS. Ca^{2+} blocks the inward flow of Na^+ across the ROS plasma membrane; the Na^+ dark current is thus interrupted; and the cell body initiates a nerve impulse.

Hendriks et al. (1974) measured 11 moles of calcium per mole of rhodopsin in isolated frog ROS. Illumination does not alter the total calcium content but shifts its distribution from a disc sequestered fraction to a soluble fraction. There is 1.4 times as much Mg^{2+} present in ROS as there is Ca^{2+}; however, illumination causes no change in Mg^{2+} distribution. Mason et al. (1974) also demonstrated Ca^{2+} release following pigment bleaching. Further they demonstrated that disc membranes can accumulate Ca^{2+}, apparently utilizing a Mg—ATP system. Brown and Blinks (1974) used aequorin to measure a rise in intracellular Ca^{2+} following illumination of the ventral eye of *Limulus*.

xii. Secretory vesicles. As is discussed in section IV. D.1.d., many secretory vesicles contain Ca, Mg—ATPase activity. This is assumed somehow to be related to the process of membrane fusion (Poste and Allison, 1973) which accompanies most exocytotic processes. For instance, the chromaffin granules of the chromaffin cells in the bovine adrenal medulla have a CaATPase and can actively accumulate Ca^{2+} from the cytosol (Serck-Hanssen and Christiansen, 1973). A similar situation exists in the granules of blood platelets.

xiii Plants. Kylin and Kahr (1973) found a CaATPase in the microsomal fraction from wheat with apparent $K_a = 10^{-4}$ M. It is not known whether it is involved in calcium transport. A wide variety of phenomena are ascribed to Ca^{2+}, membrane interactions although the mechanism(s) are unknown. Shay and Hale (1973) found that sugar exudation in peanut plants is inhibited when the growth medium contains over 3×10^{-4} M Ca^{2+}. Poovaiah and Leopold (1973) showed that 10^{-3}—10^{-2} M Ca^{2+} deferred the senescence of leaf discs, supposedly by contributing to membrane stability. Dela Fuente and Leopold (1973) suppressed the basipetal transport of the growth hormone auxin, indoleacetic acid, in sunflower stem sections by EDTA treatment. Addition of 10^{-4}—10^{-3} M Ca^{2+} restored transport; Mg^{2+} and La^{3+} are about ten times less effective. They cite similar experiments with anion, cation and metabolite transport.

c. Na^+ Driven, Ca^{2+} Transport. The following summary hardly does justice to the excellent reviews of Blaustein (1974) and of Baker (1972). The free ion concentrations for Na^+ and Ca^{2+} are 1.45×10^{-1} M and 1.2×10^{-3} M in human blood plasma and 4.37×10^{-1} M and 4.0×10^{-3} M in squid hemolymph. In both fluids the ratio of Na^+ to Ca^{2+} activities is about 310 : 1. This is the ratio found for most animal plasmas. The true Ca^{2+} concentration within the axoplasm is difficult to determine but most measurements give values of $10^{-5.8}$—10^{-7} M. The ratio of Na^+ activities, $a_{Na(out)}/a_{Na(in)}$, is about 10.

The Na^+ gradient is maintained by a ouabain (a cardiac glycoside) sensitive, NaATPase pump. No evidence for a CaATPase pump can be found in various "excitable" tissues, for example axons. Ca^{2+} extrusion can occur in the absence of ATP hydrolysis and appears to be coupled to the total influx of three and the net influx of two sodium ions. The key idea is that the energy stored in the Na^+ gradient drives the Ca^{2+} efflux. In Blaustein's tentative model a carrier, R, has one alkali metal cation (M^+) site and a second site which can bind either Ca^{2+} or two M^+ ions. Although there is no structural precedent for such a (Ca^{2+} or two M^+) site, the general idea is still valid.

(i)	Outside:	$2\ Na^+ + RM^{-2}$	$\rightleftharpoons Na_2RM$
(ii)		Na_2RM_{out}	$\rightleftharpoons Na_2RM_{in}$ (translocation)
(iii)	Inside:	$2\ Ca^{2+} + M'^{+} + Na_2RM$	$\rightleftharpoons CaRM'_2 + 2\ Na^+ + M^+$
(iv)		$CaRM'_{in}$	$\rightleftharpoons CaRM'_{out}$ translocation)
Net		$2\ Na^+_{out} + Ca^{2+}_{in} + M^+_{out} + M'^{+}_{in} \rightleftharpoons 2\ Na^+_{in} + Ca^{2+}_{out} + M^+_{in} + M'^{+}_{out}$	

The M^+ which passes outward, in the CaRM' complex, might be K^+, for instance as occurs following Ca^{2+} injection into *Helix aspersa* neurones (Meech, 1974). In contrast, Begenisich and Lynch (1974) injected Ca^{2+}, from 10^{-3} to 10^{-2} M, into voltage-clamped squid axons and found no effect on K^+ efflux. Obviously the basic idea

$$(Na_2R_{out} + Ca^{2+}_{in} \rightleftharpoons CaR_{out} + 2\ Na^+_{in})$$

has been elaborated to accommodate the total, as opposed to net, inflow of three sodium ions for one Ca^{2+} passing outward.

The actual function of this calcium gradient remains unknown. As Montal (1973) has shown, adding Ca^{2+} to one side of a lipid bilayer can lower its electrical resistance. The asymmetric distribution of calcium may affect the functional characteristics of the membrane. Triggle (1972) has reviewed in detail calcium membrane interactions and their possible effects on hydration and permeability. Triggle also explored the idea that certain local anesthetics, e.g. procaine and butacaine, interact at Ca^{2+} binding sites and function as membrane stabilizers.

Alternately, Ca^{2+} carries part of the net current inflow at the time of nervous impulse. In some nerves this Ca^{2+} pulse can be temporarily resolved from the much larger Na^+ influx or sometimes, as in *Helix* nerves, carry the entire current. As noted in section IV.D.2.b., calcium flux from the intradisc space to the cytoplasm appears to be the initial ion flux event in visual reception. It probably corresponds to the early receptor potential. In invertebrate muscle and in vertebrate smooth and cardiac muscle, tissues with sparse endoplasmic reticulum (section IV.D.3.a.), Ca^{2+} carries a considerable fraction of the inward current. In the ROS disc and in the sparse SR type muscle, the identification of a CaATPase and/or a Ca^{2+}, Na^+ exchange mechanism is still tentative.

As evidence of the controversial nature of the subject, we cite the recent "Molecular Model of Action Potentials" of Dubois and Schoffeniels (1974) in

which they invoke a calcium binding, receptor protein and a CaATPase both of whose calcium binding characteristics are dependent upon the electrical potential.

d. Transcellular transport. The problem of Ca^{2+} transport by cells such as the kidney proximal tubule or the intestinal brush border differs from that of, say, a muscle cell. As noted in section IV.D.2.b., in the kidney the CaATPase is located only in the basal (non-luminal) membrane; hence one would assume that the cytoplasmic concentration of Ca^{2+} is the same as that in the primary urine. Although the mitochondria may have some buffering function, there still exists the basic problem of transporting the calcium across the cells by means other than simple diffusion. There exist several closely related calcium binding proteins whose synthesis is induced by vitamin D. In the gut and kidney they account for some 2% of the soluble proteins. In spite of their water solubility, fluorescent antibodies to chick protein are located almost exclusively on the absorptive surface of the epithelial cells (Taylor and Wasserman, 1970). These proteins are acidic; all of them must be heated to over 65—70°C to be denatured. Those from mammals have MW 10000—13000 (Dorrington et al., 1974, Hitchman et al., 1973, and Fullmer and Wasserman, 1973). The Vit D CaBP from chicken gut has MW 28000 (Bredderman and Wasserman, 1974).

There are also a group of acidic, thermostable calcium binding proteins (S-100, 30.3, L-1, L-2, CaBP-I and CaBP-II) apparently specific to nervous tissue (reviewed by Kretsinger, 1976). They are not enzymes. It appears that they are involved either in transcellular movement of calcium or are modulated by Ca^{2+} and interact with a target protein.

3 Intracellular Ca^{2+} buffers

a. Sarcoplasmic reticulum. The sarcoplasmic reticulum (SR) is an internal membrane system of muscle cells. It corresponds to the agranular endoplasmic reticulum of other cells. In some cells, usually of skeletal white muscle, it is dense and somewhat convoluted; in others, such as smooth muscle it is sparse. The SR forms a set of sleeves or clefts surrounding the cytoplasm, which contains the thick and thin filaments (Frazini-Armstrong and Porter, 1964). When muscle tissue is homogenized, the fragmented SR membranes fuse, forming vesicles which can be isolated. The vesicles represent a valid experimental system in that the SR has a sidedness and can sequester Ca^{2+} "inside", away from the cytoplasm.

The muscle cell membrane, the sarcolemma, has invaginations, the T (transverse) tubules, whose content is continuous with the extracellular environment. In vertebrate skeletal muscle the T tubules occur at the I band. Although the contents of the SR and the T tubules are not continuous, at the "triad" region the terminal sac of the SR touches the T tubule. The nervous impulse depolarizes the sarcolemma, is propagated inward along the T system and is

somehow transmitted to the SR. The SR releases its stored Ca^{2+}, momentarily raising the cytoplasmic calcium concentration to about $10^{-4.5}$ M, thereby causing contraction. The mechanism of this release is unknown.

The second function of the SR is to sequester Ca^{2+} in the resting muscle, lowering the free calcium ion concentration below $10^{-7.0}$ M (Hellam and Podolsky, 1969, Ashley and Ridgway, 1970). Of course the mitochondria and cell membrane can also remove Ca^{2+} from the cytoplasm and their roles are relatively more important in slower muscles which lack the great surface area of rich SR. However the mitochondria have no electrical connectivity with the sarcolemma.

Of most relevance to this review is the mechanism of "Calcium Transport in Sarcoplasmic Reticulum" which has been thoroughly and authoritatively reviewed by MacLennan and Holland (1975). The generally accepted model of Kanazawa and Boyer (1973) is summarized as follows:

(i)	outside:	$MgATP^{2-} + 2Ca^{2+} + E^{3-} \rightleftharpoons Ca_2 \cdot E \cdot MgATP^{-1}$	
(ii)		$Ca_2 \cdot E \cdot MgATP^{-}_{out} \rightleftharpoons Mg^{2+}_{out} + ADP^{3-}_{out} + Ca_2 \cdot EP_{in}$	translocation, phosphorylation
(iii)	inside:	$Ca_2 \cdot EP \rightleftharpoons Ca^{2+} + EP^{4-}$	
(iv)	inside:	$EP^{4-} \rightleftharpoons E^*P^{4-}$	conformational change of CaATPase
(v)	inside:	$E^*P^{4-} + Mg^{2+} \rightleftharpoons MgE^*P^{2-}$	
(vi)		$MgE^*P^{2-}_{in} \rightleftharpoons MgE^* \cdot P_i^{2-}{}_{out}$	translocation,
(vii)	outside:	$MgE^* \cdot P_i^{2-} \rightleftharpoons E^{*3-} + Mg^{2+} + P_i^-$	dephosphorylation
(viii)	outside:	$E^{*3-} \rightleftharpoons E^{3-}$	back to original conformation
Net		$ATP^{4-}_{out} + 2\,Ca^{2+}_{out} + Mg^{2+}_{in} = ADP^{3-}_{out} + P^-_{i\,out} + 2\,Ca^{2+}_{in} + Mg^{2+}_{out}$	

Upon release of calcium, ATP can be regenerated with reasonable efficiency. Note that electrical neutrality is (partially) maintained by exchanging at least one Mg^{2+} for two Ca^{2+}. K^+ may also be transported out.

For the dephosphorylated enzyme Ca^{2+} is bound 3×10^4 more strongly than is Mg^{2+}; while the phosphorylated enzyme, which functions on the inside, binds Mg^{2+} almost as strongly as it does Ca^{2+}. The actual translation or rotation of the membrane bound enzyme has not been demonstrated.

Although the detailed structure and interrelationships of the SR membrane are unknown, enough has been learned in the past few years to make this one of the best understood membrane systems. The Ca, MgATPase as isolated has bound phospholipid up to 50% of the protein weight. Although the enzyme is unstable without the various bound phospholipids, they can all be replaced by PC, with full enzymatic activity retained. In synthetic membrane systems the Ca, MgATPase can function as an ionophore, or as an ion carrier. Even tryptic digest products of a few thousand molecular weight are Ca^{2+} ionophores. Limited tryptic digestion yields two fragments. One, 45000 MW, is hydrophob-

ic and is quite certainly buried in the membrane. The 55000 MW fragment is less hydrophobic, appears to be partially accessible to solvent, can be phosphorylated with ATP and retains full MgATPase activity (Shamoo and MacLennan, 1974).

In addition to the ATPase, which accounts for some 68% of the SR protein, there are calsequestrin (19%) and high affinity calcium binding protein (HACBP) (12%). Calsequestrin (MW 46000) is an extrinsic, or loosely bound membrane protein, apparently located on the inner surface of the SR. Its function is to bind or to store Ca^{2+} or possibly cations in general (Ostwald et al., 1974). It is a bit surprising that calsequestrin with a net negative charge of −98 electrons (131 acidic and 33 basic groups) should bind some 45 calcium ions with a fairly high specificity, $pK_d = 4.3$ (cf. $pK_d(Mg^{2+}) = 2.5$).

MacLennan and Holland (1975) suggested that HACBP (MW 55000) might transfer Ca^{2+} from the ATPase, which is involved in calcium transport, to calsequestrin, the site of calcium storage. This is consistent with the fact that its pK_d(=5.5) is between that of calsequestrin and that of ATPase (pK_d=6.5).

Platelets (section IV.C.2.c.) contain a relaxing factor which appears to be similar to SR. Various unicellular organisms, e.g. the protozoan *Spirostomum ambiguum* (Ettienne, 1970) as well as the alga, *Nitella* (Nagai and Rebhun, 1966) have internal vesicular systems which seem to have a function analagous to SR.

b. Mitochondrion. The mitochondrion has been thoroughly reviewed (Lehninger, 1971, 1972, Lehninger et al., 1967 and Borle, 1973, 1974). Mitochondria are found in all eukaryotic cells. Depending on the cell's metabolic activity it will contain from 10^2 to nearly 10^6 mitochondria. Although their exact morphology and distribution varies with cell type, they are generally 0.5 by 3.0 μm and shaped like prolate ellipsoids. They consist of a smooth outer membrane, an intermembrane space, a highly convoluted inner membrane and an internal matrix. The convolutions of the inner membrane are called cristae; their inner surface is studded with "elementary bodies". Within these four regions are located many enzymes, particularly those involved in oxidative phosphorylation, and the electron transport system.

Less widely appreciated than the mitochondrion's role in intermediary metabolism, but of corresponding importance, is its ability to accumulate Ca^{2+}. When the mitochondrion is respiring, about 4 calcium ions are accumulated per oxygen atom reduced; if the process is driven by ATP hydrolysis the P/Ca ratio is about 1 : 3. If PO_4^{3-} is the external anion, it is transported with Ca^{2+} and $Ca_3(PO_4)_2$ is actually precipitated in the matrix. If other anions are present, H^+ is exchanged for Ca^{2+} to maintain electrical neutrality. Borle (1973), among others, has demonstrated and discussed the Ca^{2+} buffering capacity of the mitochondria. Its membrane as well as the plasma membrane, can establish gradients, $[Ca^{2+}]$—mitochondria/$[Ca^{2+}]$—cytoplasm, up to 3×10^3. Yet the total surface area of all the mitochondria is some 30 times greater than that of the plasma membrane in a "typical" cell. For example, in a cell involved in

calcium transport, as in the gut with a mucosal and a serosal end, the cytoplasmic calcium concentration could be described by the following parameters.

$[Ca^{2+}]_{cyt} = [Ca^{2+}]_{gut} \cdot (K_{gut \rightarrow cyt}/K_{cyt \rightarrow gut})$ and

$[Ca^{2+}]_{cyt} = [Ca^{2+}]_{blood} \cdot (K_{bld \rightarrow cyt}/K_{cyt \rightarrow bld})$ but also

$[Ca^{2+}]_{cyt} = [Ca^{2+}]_{mit} \cdot (K_{mit \rightarrow cyt}/K_{cyt \rightarrow mit})$

The real question posed by this formulation is: how do the various stimuli to the cell surface, which ultimately effect $[Ca^{2+}]_{cyt}$, communicate with the mitochondria? For instance, the stimulus to muscle contraction causes release of Ca^{2+} from the SR. In the muscle the mitochondria might help soak up released calcium between contractions and only during rest would the calcium slowly seep back from the mitochondria, an intermediate sink, to the SR.

Borle lists several regulatory effects on mitochondria. Phosphate increases cellular uptake of Ca^{2+}, not, apparently, by affecting $[Ca^{2+}]_{cyt}$ or $(K_{ext \rightarrow cyt}/K_{cyt \rightarrow ext})$ but by increasing mitochondrial accumulation of Ca^{2+} (and of PO_4^{3-}) by raising $[Ca^{2+}]_{cyt}$ and/or the ratio, $K_{cyt \rightarrow mit}/K_{mit \rightarrow cyt}$. The net result would be deposition of $Ca_3(PO_4)_3$ crystallites within the mitochondrial matrix as observed.

Borle (1974) has shown that in isolated dog kidney, liver and heart mitochondria, cAMP (10^{-7} to optimum 3×10^{-6} M) causes an instantaneous release of calcium. The concentration of calcium in the external medium rises from 10^{-6} to 10^{-4} M within 3 seconds. Pursuing the ideas of Rasmussen (1970) and Rasmussen et al. (1972) in the context of his own model, he suggests that various cell stimuli cause a release of cAMP and that the observed rise in cytoplasmic calcium is due primarily to an increase in the rate of calcium efflux from the mitochondria ($K_{mit \rightarrow cyt}$). This does seem reasonable but it does not explain the necessity of many such systems for calcium in the external medium, nor the influx of external calcium following the stimulus.

Lehninger (1974) has found that the anions of weak acids (H_3PO_4, CH_3CO_2H, $CH_3(CH_2)_2CO_2H$, CO_2, $HAsO_3$) facilitate Ca^{2+} uptake by respiring mitochondria while the anions of strong acids (HNO_3, HSCN, $HClO_3$, $HClO_4$, etc.) do not. In his model,

i. The mitochondrial transport of a pair of electrons, at each of the three energy conserving sites of the respiratory chain, somehow pumps two protons from the mitochondrial matrix to the cytoplasm.

ii. The "weak" acid is drawn into the matrix and its associated protons neutralizes the OH^- generated in the first step by pumping out H^+. The exact site of the $H^+ + OH^- \rightarrow H_2O$ can not be defined.

iii. Finally the negative inside phosphate potential is the driving force for the subsequent entry of Ca^{2+}. Lehninger further suggests that the phosphate inside gradient is also the driving force for the generation of ATP from ADP during oxidative phosphorylation.

It is well established that 1.0 molecule of phosphate and about 1.7 calcium ions are accumulated for each pair of electrons traversing each energy conserv-

ing site. Interestingly, in hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, the Ca/PO_4 ratio is 1 : 67.

The details of mitochondrial function are complex and still not understood. A few recent papers relate to the involvement of calcium. Reynafarje and Lehninger (1974) have explained the "superstoichiometry" of respiration-dependent uptake of Ca^{2+} and ejection of H^+, i.e. $Ca^{2+}/2e^- > 2.0$, as being due to an initial discharge of protons of an energized state of the mitochondrion. In vitro it occurs in the absence of phosphate.

Lehninger (1970) postulated that mitochondria may be the site at which normal and abnormal biological calcification are initiated. Mitochondria from the hepatopancreas of the blue crab *Callinectes sapidue* show up to twelve fold stimulation of respiration on addition of Ca^{2+}. They can accumulate 5.5 μg-atoms of Ca^{2+} per mg protein (Chen, et al., 1974). Calcium phosphate crystallites are formed within the mitochondria. This appears to be the major storage form of calcium phosphate during the intermolt period. It is possible that the crystals are somehow transported out of the cell during shell formation (Becker et al., 1974).

Regardless of the detailed kinetics or stoichiometry of calcium uptake, there is an apparent requirement for a calcium transport protein. In mitochondria which do accumulate calcium there are high and low affinity calcium binding sites ($K_d = 10^{-7} \rightarrow 10^{-6}$ with $1 \rightarrow 10 \times 10^{-9}$ moles/mg protein and $K_d = 10^{-4}$ with 5×10^{-8} moles/mg protein) (Carafoli and Gazzotti, 1973, and Rossi et al., 1974). They bind Sr^{2+} but not Mg^{2+}, K^+ or Na^+. It may prove significant that Reynafarje and Lehninger (1973) found that the apparent affinity of mouse leukemia cell mitochondria for Ca^{2+} during stimulation of oxygen uptake is about three fold greater than in normal liver mitochondria. "Intact rat liver mitochondria contain respiration-independent high affinity Ca^{2+} binding sites whose properties suggest they may be concerned in respiration-dependent Ca^{2+} transport." These sites account for 0.5 to 1.0% of the protein of mitochondria, and contain 27% phospholipid and 8% hexosamine (Gomez-Puyou et al., 1972). Sottocasa and coworkers have described two high calcium affinity glycoproteins from vertebrate mitochondria. The first (Sottocasa et al., 1972) has a molecular weight 42000 of which 30% is phospholipid and 5% carbohydrate. It is water soluble whereas the rat lipo-glycoprotein is not. The second (Sottocasa et al. (1974) has a molecular weight 30000 of which 15% is carbohydrate.

Chloroplasts, the site of photosynthesis in plants, are in many respects similar to mitochondria. Even though they contain half of the calcium of most plants, the function(s) of Ca^{2+} is not known. Gross and Hess (1974) found two classes of binding sites for Ca^{2+}, Mn^{2+} and Mg^{2+} on isolated chloroplasts (Ca^{2+} site I $pK_d = 5.1$, 6.5×10^{-5} moles/mg chlorophyll; site II $pK_d = 4.3$, 5×10^{-5} moles/mg chlorophyll). Bakker-Grunwald (1974) showed that Ca^{2+}, as well as Mg^{2+}, stimulated the incorporation of phosphate into ATP.

Several mitochondrial enzymes have been reported to be activated by Ca^{2+} — glycerophosphate dehydrogenase (Hansford and Chappell, 1967), kynure-

nine aminotransferase (Mason, 1974) and 25-hydroxycholecalciferol-1-hydroxylase (Suda et al., 1973). The proteins are associated with the mitochondrial membrane. It is not known whether they are modulated by Ca^{2+}.

4. Cell adhesion

The regulatory aspects of cell contact appear to be fundamental to normal morphogenesis as well as to tumor metastasis. There is a strong intuitive feeling that the specificity of cell interactions must reside in highly specific glycoproteins or glycolipids. However without invoking those yet uncharacterized templates, one can review some more general effects of Ca^{2+} and of Mg^{2+} (Okada et al., 1974). In the general range of 10^{-4} M, Mg^{2+} appears to be marginally more effective than Ca^{2+} in promoting the adhesion of cells (most often from chick embryos) to various synthetic substrates. There appears to be no requirement for divalent cations if proteins with a net negative charge are omitted from the culture medium. Cell to cell adhesion occurs more rapidly in the presence of Mg^{2+} than in the presence of Ca^{2+}.

From a thorough study of the aggregation of suspended HeLa cells Deman et al. (1974) concluded that "Trypsin-releasable material confers adhesiveness upon the cells". "The adhesive property of this material" which appears to be a glycoprotein "is counteracted by the presence of cell surface sialic acids", which can be removed by neuraminidase treatment. Ca^{2+} at 3×10^{-3} M, but not Mg^{2+}, exerts its aggregating "effect by attenuating the adverse effect of sialic acids".

In contrast, Schudt et al. (1973) found that raising Ca^{2+} concentration from 10^{-4} to 2×10^{-3} M produces an increase from 10% to 90% fusion of cultured chick embryo myoblasts. Mg^{2+} (2×10^{-3} M) markedly inhibits fusion.

Humphreys (1970) showed that dissociated sponge cells from *Microciona prolifera* and *Haliclona occulata* aggregate in a species-specific manner if Ca^{2+} is present in the sea water. The sponge aggregation factor from *Microciona parthena* (Cauldwell et al., 1973 and Henkart et al., 1973) is a "sunburst" shaped proteoglycan of 2.1×10^7 MW comprised of an inner circle, 5×10^6 MW, and some fourteen radiating glycoprotein arms about 1000 Å long. It consists of 49% glycan, of which 21% is uronic acid, and 47% protein of whose residues 28% are aspartic and glutamic acid. There are 1150 calcium binding sites per 2.1×10^7 a.m.u. with a pK_d of roughly 3.5. If the Ca^{2+} concentration is lowered, the arms irreversibly dissociate into subunits of 2×10^6 MW; if it is raised the factor aggregates into a gel. It is not known how the factor attaches to the cell surface, nor how it confers its specificity.

In addition to this glycoprotein from *Microciona* and those of the mitochondria (section IV.D.3.b.), several other Ca^{2+} binding glycoproteins have been described — vitellogenin of the egg yolk of *Xenopus laevis* (Ansari et al., 1971); phosvitin, a phosphoprotein of chicken egg yolk (Grizzuti and Perlmann, 1973); salivary glycoprotein (Betteiheim, 1971); cartilage glycoprotein (Vittur et al., 1972); and goblet cell mucin (Forstner et al., 1974). Neither the structure nor the precise functions of these glycoproteins are known but

their apparent involvement in Ca^{2+} storage and transport would justify much more research.

5. Virus assembly

The spherical bacteriophage PM2 has a protein shell, of net positive charge, extending from radius 300 to 240 Å, and a lipid bilayer, 65% of which is anionic phosphatidylglycerol, from 240 to 200 Å (Schäfer et al., 1974 and references therein). The host, *Pseudomonas*, grows and supports PM2 infection in 3×10^{-5} M Ca^{2+}; however, the final stages of viral assembly require 3×10^{-4} M Ca^{2+} in the growth medium. The released, fully assembled PM2 particle does not contain appreciable Ca^{2+}. Snipes et al. (1974) suggest that Ca^{2+} is required to neutralize the negative charge during phosphatidylglycerol membrane assembly and is subsequently displaced during protein coat formation. Schafer, et al. (1974) have found that coat protein II, which forms the outer shell, has an isoelectric point of 12.3 as isolated from the phage and 9.0 after removal of Ca^{2+}.

Durham and Butler (1975) have speculated that the Pb^{2+} binding site of tobacco mosaic virus coat protein functions to bind Ca^{2+}, thereby stabilizing the virus particle. Following entry into the plant cell, which is assumed to have low [Ca^{2+}], the ion would be released and the coat fall apart freeing the virus RNA.

Various viruses and phages, e.g. MS2, appear to require Ca^{2+} either for attachment, assembly or release. However since growth media usually contain Ca^{2+} as a contaminant, the requirement is unobserved or not investigated.

CONCLUSION.

So much accomplished, so much left to be done.

We need a reliable pCa meter for the entire range 2 to 8 as well as a full range of Ca^{2+} buffers. Total calcium determinations are tedious and consume much material. We anticipate the development of flame absorption spectrophotometers which require microliter quantities and which can monitor enough spectral lines to allow one to correct for 10^4 excess of Na^+ and K^+ and 10^2 excess of Mg^{2+}.

As the interpretations of various spectra — CD, ORD, NMR and fluorescence — become more refined, we should be able to interpret more fully the changes induced in proteins and lipids by Ca^{2+}. More crystal structure determinations of compounds whose calcium dissociation constants are known should provide a better understanding of the relationship of coordination geometry to Ca^{2+} affinity and selectivity.

While the spectrally active lanthanides are the "best available" replacement ions for the study of protein calcium binding sites, caution is advised. In all of these studies, protein tertiary structural perturbations, however subtle they may appear, have been recorded.

Perhaps the ideal approach, and the most challenging, is to develop proce-

dures that will monitor, directly, Ca^{2+} induced events in protein and membraneous structures.

At the physiological level it is very important to localize the various intracellular proteins such as actin, myosin and tubulin. Perhaps even more challenging is the measurement of intracellular Ca^{2+} concentrations. Even though the aequorin technique has provided a great deal of information, it is difficult to insert microelectrodes into most cells. Electron microprobe analyses should be refined both in terms of focal spot size and sensitivity.

Nearly all cell membranes actively maintain a Ca^{2+} gradient. Equally important, following a stimulus the membrane must allow the passage of a pulse of calcium ions. Neither of these processes is now understood.

Finally one might ask why calcium was chosen to play these key informational roles? In many instances organic molecules, such as cAMP, acetylcholine and various hormones, serve to transmit information within or between cells. Perhaps an inorganic ion requires less energy to "synthesize" or to sequester and was first used evolutionarily. Cations seem to be bound with greater affinity and specificity than are the halides or even the simple polyatomic anions. Although Si, Al and Fe are more abundant in the earth's crust, only Na^+ (0.45 M), Mg^{2+} (0.05 M), K^+ (0.01 M) and Ca^{2+} (0.01 M total concentrations) are adequately abundant and soluble in the ocean, from which we evolved, to serve a general messenger function. Although Nature has evolved Na^+ and possibly K^+ specific membrane transport proteins, again we suggest that the ranges of specificities and affinities for Na^+ and for K^+ are significantly smaller than are those for Ca^{2+}. Finally Mg^{2+} quite definitely is not used as a second messenger as is Ca^{2+}. This can hardly be a historical accident; however, the answer is by no means clear.

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