

**Biom mineralisation of the
calcitic-shelled, inarticulated
brachiopod, *Neocrania anomala*.**

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Thesis submitted for the Doctor of Philosophy

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Declaration

The material presented in this thesis represents the research carried out over a three year period in the Department of Geology and Applied Geology at the University of Glasgow, under the supervision of Dr. Maggie Cusack and Dr. Gordon Curry. This thesis is a result of my own independent research and any previously published or unpublished work used has been given full acknowledgment in the text.

Karen Brown
January 1998.

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Abbreviations.

APS	Ammonium persulphate
CaCO ₃	Calcium carbonate
CAPS	(3-[Cyclohexylamino]-1-propanesulphonic acid)
CBB R-250	Coomassie Brilliant Blue
DIEA	Diisopropylethylamine
DTT	Dithiothreitol
EDTA	Ethylene diamino tetraacetic acid
g	grams
GuHCl	Guanidine hydrochloride
HCl	Hydrochloric acid
hplc	High performance liquid chromatography
kDa	kilodaltons
mg	milligrams
mmol	millimoles
mM	milliMolar
NaOCl	Sodium hypochlorite (bleach)
ng	nanograms
nm	nanometres
pI	isoelectric point
PITC	phenylisothiocyanate
pmol	picomoles
PVDF	Polyvinylidene fluoride
rRNA	Ribosomal Ribonucleic acid
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
TCA	Trichloroacetic acid
TEM	Transmission electron microscope
TEMED	N, N, N', N'-tetramethylethylene-diamine
Tris	[hydroxymethyl]-aminomethane
μl	microlitres
μm	micrometres
μmol	micromoles
18M Ω water	ultrapure water

Three letter and one letter codes for amino acids.

Amino acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Aspartic acid	Asp	D
Asparagine	Asn	N
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Iso	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Abstract.

The inarticulated calcitic shelled brachiopod, *Neocrania anomala* belongs to the craniids, the sole Recent members of the class Craniata within the subphylum Craniiformea. Articulation is associated with calcitic valves in the Rhynchonelliformea and inarticulation with apatitic valves in the Linguliformea.

Biominerals have organic material, including proteins and glycoproteins, intimately associated with the mineral phase, reducing the nucleation energy required for mineral precipitation and influencing the formation of the biomineral. The characteristics of the structure of shell are therefore a consequence of the protein component of the constituent biomineral. The intracrystalline proteins of *N. anomala* are examined here to gain information relating to the formation of the biomineral and therefore the shell of *N. anomala*.

The intracrystalline protein fraction of the shell of *N. anomala*, is here defined as the protein which is resistant to strong chemical oxidation by incubation of powdered shells in an aqueous solution of sodium hypochlorite (1% v/v) for one hour. Mineral-associated proteins were extracted from the intracrystalline and intercrystalline fractions of the dorsal valve and the intracrystalline fraction of the ventral valve. The proteins were purified to homogeneity using gel electrophoresis (SDS PAGE) and subsequently analysed by partial N-terminal sequencing and amino acid analysis.

A 44 kDa protein is present in both the intracrystalline and total protein extracts of the dorsal valve and also in the intracrystalline fraction of the ventral valve. In addition, the intracrystalline fraction of the dorsal valve contains a 60 kDa protein. The partial N-terminal sequence obtained for the 44 kDa protein does not resemble previously sequenced brachiopod proteins, nor any other protein in the protein databases searched.

The dorsal valve has a higher concentration of protein than the ventral valve. Differences exist between the two valves in terms of the amount of protein present in different locations within the shell. The dorsal valve has twice as much intracrystalline protein per weight of shell than the ventral valve. The higher proportion of protein in the dorsal valve may be related to the presence of semi-nacre in this valve. The 44 kDa protein of the intracrystalline fraction of the dorsal

valve has high levels of aspartic acid/asparagine and glutamic acid/glutamine, as well as glycine and serine. The EDTA-insoluble material contains significantly more protein than the EDTA-soluble protein extract from the intracrystalline fraction of the dorsal valve and differences exist between the two extracts in terms of amino acid composition.

The soluble protein of the dorsal valve can both initiate and inhibit crystal nucleation *in vitro*, depending on the concentration of the protein in the system. Whole extracts were added to the system, so it is not possible to identify which protein(s) influences crystal nucleation. The 44 kDa and the 60 kDa proteins both possess a calcium-binding ability, as determined by reaction with Stains-all and this may be suggestive of the proteins involvement in biomineralisation *in vivo*. The 60 kDa protein is glycosylated, whereas the 44 kDa protein is not.

Chemical etching of the laminar calcite that comprises the semi-nacreous secondary layer of the dorsal valve of *N. anomala* reveals a high order structural arrangement that resembles the ultrastructures observed in nacreous bivalve shells. Nucleation of new crystals occurs uniformly over the surface of the individual tablets, an arrangement which is also observed in the semi-nacre of the cyclostome bryozoans. The non-specific nucleation observed in these groups may be related to the formation of semi-nacreous shell layers. The presence and pattern of the ultrastructures of the *N. anomala* secondary shell suggests that lophophorates possess a more complex system of biomineralisation than the Lophophorate-Mollusc discontinuity concept suggests.

Chapter One

Introduction

- 1.1 The Brachiopoda.**
 - 1.1.1 Classification of the Brachiopoda.
 - 1.1.2 Historical background of the Craniida.
- 1.2 The Study of biominerals.**
 - 1.2.1 The origin of mineralised tissues.
 - 1.2.2 Features of the organic component of shell.
 - 1.2.3 The role of the organic component of the shell in biomineralisation.
 - 1.2.4 Proteins in biominerals.
- 1.3 Ultrastructure of the shell of *N. anomala*.**
 - 1.3.1 Ultrastructure of *N. anomala* shell.
 - 1.3.2 Growth of semi-nacre by screw dislocation.
 - 1.3.3 The protein component of brachiopod shells.
 - 1.3.4 The protein component of the shell of *N. anomala*.
- 1.4 Aims and approach of this study.**

1.1 The Brachiopoda.

Brachiopods, or lampshells, as they are commonly known, are benthic marine organisms, possessing soft internal body parts, enclosed within a mineralised bivalve shell (Figure 1.1) (Williams, 1965). The phylum is interesting in terms of valve calcification, as it not only comprises species with calcium carbonate shells, but also those with phosphatic shells. In general, brachiopods with carbonate shells possess articulated valves, which are joined at the posterior by a hinge mechanism. The phosphatic shelled species, however, are inarticulated, possessing no hinge. The exceptions are the craniid brachiopods, which are inarticulated, yet have a calcitic shell (Figure 1.2).

All brachiopods are filter-feeders, obtaining food by way of the lophophore, a complex food gathering organ. The two valves of the shell, the dorsal and ventral, are usually dissimilar in size and are symmetrical about a median plane extending from one end of the shell to the other (Williams, 1965). The majority of brachiopods are epifaunal, attached by way of the stalk-like pedicle or cemented directly onto the substrate surface by the ventral valve.

An exception to the epifaunal habit is the burrowing mode of life exhibited by members of the genus *Lingula* (James *et al.*, 1992). Brachiopods are fairly cosmopolitan in distribution, but most are found in the warm shallow seas around Japan, Australia and New Zealand. Many species, however, inhabit colder waters and can sometimes be found in depths of up to 5 kilometres. The size and shape of adult shells can vary considerably, as can the external ornamentation. Recent adult brachiopod shells are usually between 1 and 7 centimetres in length. (McLeish, 1991).

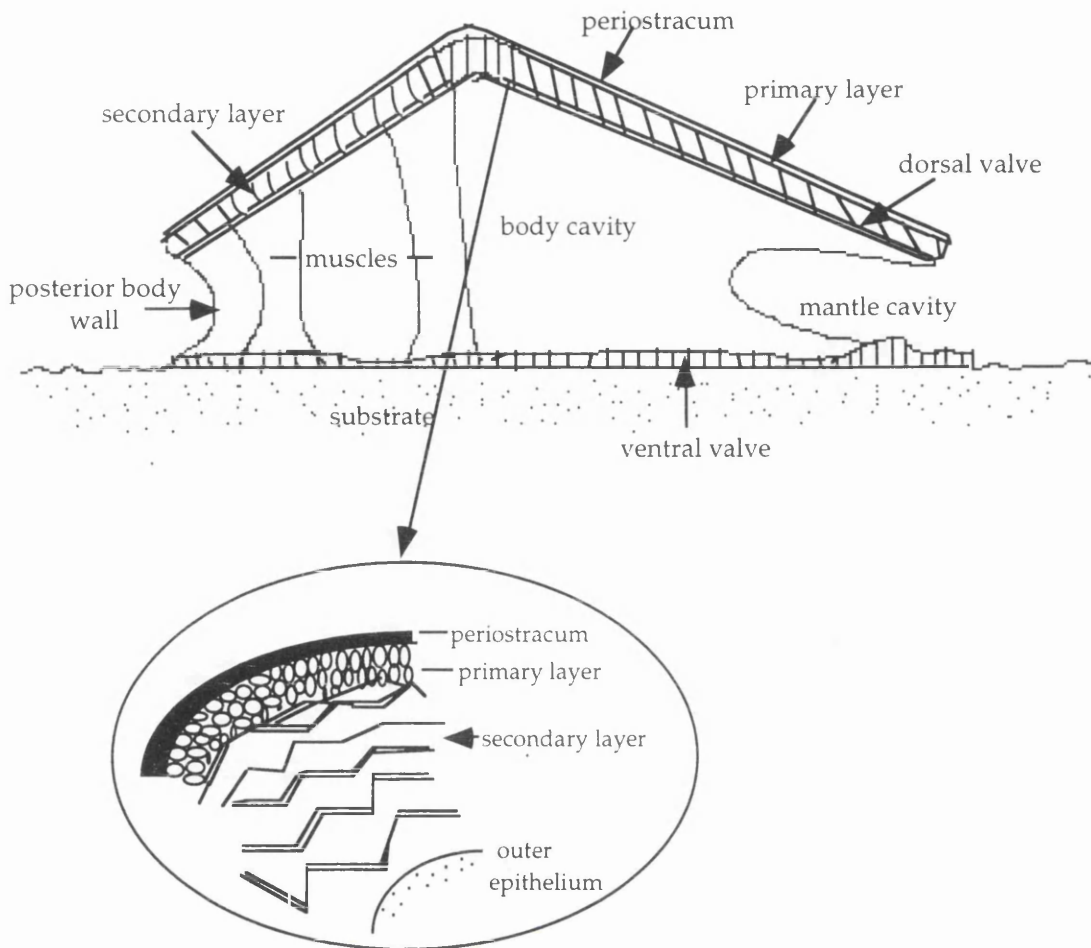


Figure 1.1 Diagram of the interior structure of *Neocrania anomala*, with expanded section highlighting the secondary layer, on which the present work concentrates (after Williams & Wright, 1970).

Brachiopods are one of the few groups that have existed throughout the Phanerozoic era (Rudwick, 1965; H199). The geological record of the phylum extends from the early Cambrian to the present day, a period of around 545 million years. During the Cambro-Ordovician, brachiopods were widespread and profuse members of most marine ecosystems, but today only a fraction of the original diversity remains, having suffered a catastrophic mass extinction at the Permian-Triassic boundary (James *et al.*, 1992). Around one hundred Recent genera exist, but over 95 percent of the genera comprise extinct species (Williams *et al.*, 1996).

"The importance of the study of the Brachiopoda must be obvious to all. They are among the first well known indications of life in this world, and they have

continued to be very extensively represented up to the present time. They are, as Mantell would have termed them, sure medals of creation, the date of their appearance firmly stamped upon them and their distinctive characters so legibly impressed as to defy misinterpretation" (Davidson, 1852-53). The study of brachiopods is still as important to palaeontology and biology as it was when Davidson wrote the above in his monograph almost a century and a half ago.

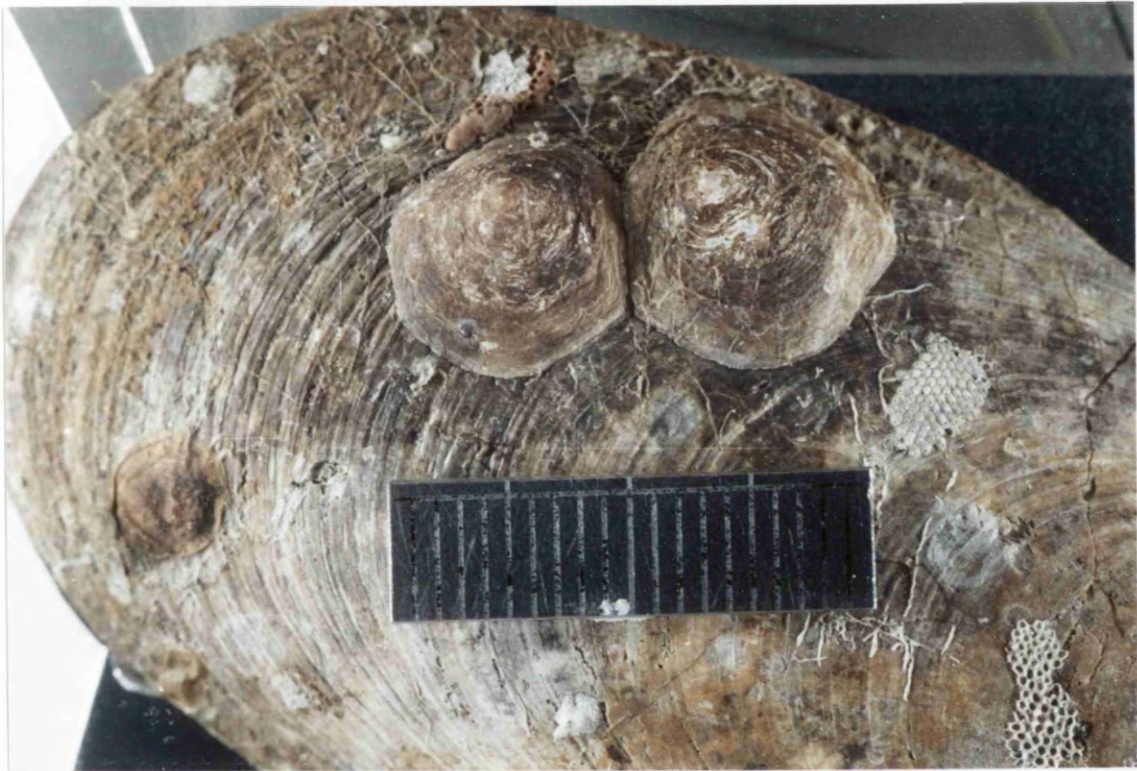


Figure 1.2 Two specimens of *N. anomala*, cemented to a mussel shell.

Only the dorsal valves are visible, the ventral valves are cemented to the shell substrate. The specimens were dredged from the sea bed at a maximum depth of 200 metres in the Firth of Lorne, Scotland. The scale bar is 2 cm.

Although Recent brachiopods form a relatively small part of present day marine communities, their geological record and distribution are almost unsurpassed. The genus *Lingula* has remained virtually unaltered from Silurian to Recent times, a period of around 450 million years and so is an exceptional example of evolutionary stability (Rudwick, 1970).

1.1.1 Classification of the Brachiopoda.

Due to the abundance of the phylum within the fossil record, palaeontologists have been conducive in the development of a classificatory scheme for the group. However, the lack of agreement between this work and that on Recent members of the phylum, has resulted in some discrepancy within the taxonomy of the phylum. One possible solution to the lack of reliable and consistent data is the application of molecular biology and biochemistry in an attempt to define some satisfactory taxonomic order. Advances in the fields of genetics, immunology and biochemistry and the introduction of techniques such as scanning electron microscopy, have enabled workers to obtain more precise data. The development of new and more advanced computerised taxonomic methods has enabled workers to analyse data more efficiently. It is a combination of these techniques that has played such a central role in the development of the present system of classification, as detailed in the revised Treatise (Williams *et al.*, 1997; Volume I). Although anomalies still exist within the system, we are closer than ever before to determining the exact inter-relationships of this ancient group of organisms.

There is, however, a high degree of discordance within the classification of the Brachiopoda. Such problems in the classificatory system are largely due to the abundance of extinct groups within the phylum, leading to a reliance on fossil specimens and the consequential construction of phylogenies based on incomplete character sets. Also, in comparison with other phyla, there is relatively little information on extant forms. The phylum shows many examples of heterochronous homeomorphism, i.e. similar forms or structures that have arisen at different geological dates from different lines of ancestors (Thomson, 1927). This has resulted in numerous mis-interpretations within the classificatory scheme, many of which have yet to be resolved (Williams *et al.*, 1996).

The shell is the most obvious structure of the brachiopod and so there is a reliance on the shell to provide the basis for the taxonomy of the phylum. Brachiopods, however, are very simple in terms of morphology, so any classificatory system will be based on the existing features. There is, however, no agreement over which feature has the most taxonomic significance. As a consequence, every major morphological variation that has appeared during

the evolution of the brachiopod shell must be taken into account when forming any systematic classification (Williams, 1955).

The origin of the Brachiopoda is a contentious issue, with support for diphyletic (Halanych *et al.*, 1995), polyphyletic (Wright, 1979) and monophyletic origins (Rowell, 1981a, b; Holmer *et al.*, 1995; Nielsen, 1991; Carlson, 1991, 1995; Popov, 1992). The current view, supported by detailed 18S rRNA analyses performed by Cohen & Gawthrop (1996), is that all Recent brachiopods are monophyletic. Cohen & Gawthrop (1996) found the traditional two-class system of inarticulated and articulated brachiopods to be valid, but would include the phoronids within the Brachiopoda, possibly as a sister group to the craniids. Nineteenth century naturalists tended to regard brachiopods as molluscs (Lamarck, 1801), or classified them alongside bryozoans and phoronids. The latter view was adopted by Hatschek (1888) in his proposal of the phylum Tentaculata, which was later renamed the Lophophorata by Hyman (1958). However, by the twentieth century, the belief that brachiopods formed a separate phylum was becoming increasingly popular (Hyman, 1940; Cooper, 1944). Percival (1944) suggested the erection of two brachiopod phyla, with the articulated carbonate-shelled species separate from those with inarticulated, phosphatic shells.

The long-established division of the phylum into two classes was based on the hinge mechanism and the mineralogy of the shell, with the Articulata possessing hinged calcitic valves, and the Inarticulata unhinged phosphatic valves. The construction of these two classes was originally proposed by Huxley (1869) and remained in use for over a century. Indeed, the 1965 Treatise on Invertebrate Paleontology, Part H, with the exception of the Kutorginida, classed the eleven orders as either articulated or inarticulated. Williams, (1965, H189) stated that brachiopods with inarticulated valves appear earlier in the geological record than those with articulated valves, and that calcium carbonate shells are likely to have evolved from calcium phosphate shells by a paedomorphic change in the secretory mechanism. Cladistic analysis, does not however, support the two class division (Hennig, 1966) and several taxa exist which possess features of both the articulated and inarticulated classes (Section 1.1.2). One such group are the inarticulated, carbonate-shelled craniids. Gorjansky & Popov (1985a, b) attempted to alleviate the problem of the craniid classification in their proposal of a new class, the Lingulata, consisting of all phosphatic-shelled inarticulates. In this scheme, the class Inarticulata consisted of the carbonate-shelled inarticulated

species, while the Articulata comprised the articulated calcitic-shelled brachiopods. A further revision of this scheme by Popov *et al.*, (1993) retained the Lingulata, but proposed the class Calciata, embracing the calcitic articulated and the calcitic inarticulated species, the latter comprising the subclass Craniiformea. In the revised Treatise, the original two class division is rejected in favour of a classification in which extant and extinct brachiopods are grouped into three subphyla, composed of eight classes. Figure 1.3 illustrates this proposed arrangement, superimposed onto a geological time scale. The phylogenetic position of the craniids however, has yet to be resolved. For a comprehensive discussion of previous cladistic models of brachiopod phylogeny, the reader is referred to Holmer *et al.* (1995).

1.1.2 Historical background of the Craniida.

Since the establishment of the classes Articulata and Inarticulata by Huxley in 1869, craniids have been regarded as members of the Inarticulata, principally on account of the lack of articulation between the valves. However, the structure and composition of the craniid shell does not conform to that of the typical inarticulated brachiopod. Craniids first appeared in the Ordovician and although many species later became extinct, a proportion of species survived until the present day.

The first account of a craniid was that of Linnaeus, in his description of the species *Anomia craniolaris* for his *Systema Naturae* (1758). The genus *Crania* was described more than twenty years later by Retzius (1781), which he based partly on the late Cretaceous species, *Crania brattenburgensis* from Sweden and partly on *Anomia craniolaris*, a species from the Phillipines. The type species of *Crania* is now accepted as *A. craniolaris*, as figured by Chemnitz (1785) in his redescription of Linnaeus' work. However, many uncertainties existed within the classification of *Crania* and so in an attempt to resolve the discrepancies, Lamarck (1819) proposed to rename some of the earlier described members of the genus. Between the years 1818 and 1885, interest in the genus developed and subsequently led to an increase in the number of publications dedicated to the description of new species of Cretaceous to Recent age within the genus *Crania* (Defrance, 1818; Sowerby, 1822; Hoeninghaus, 1828; Davidson, 1852, 1856; Lundgren, 1885). Reeve (1862), however, was the first to publish a comprehensive account of Recent species within *Crania*.

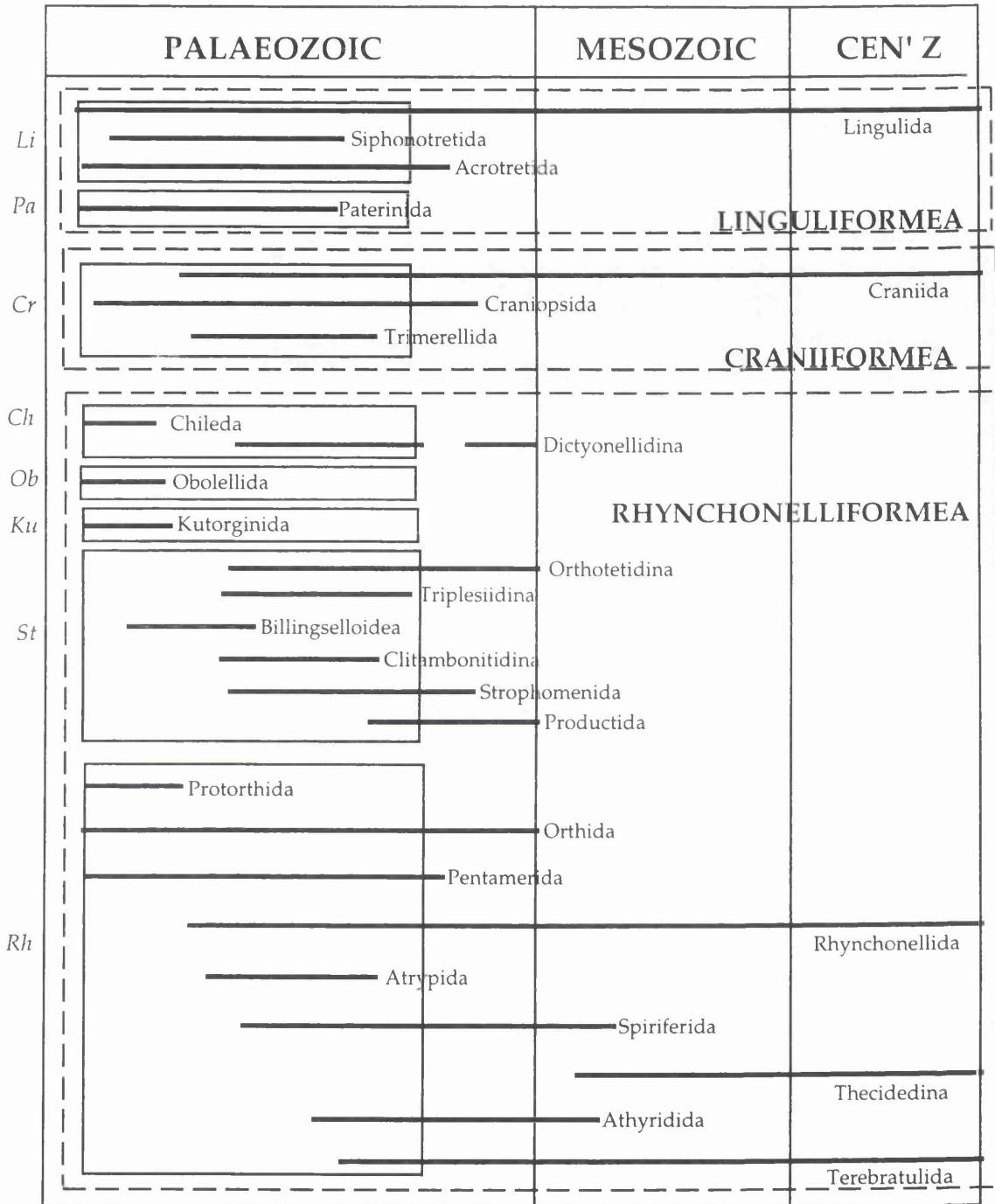


Figure 1.3 The proposed supra-ordinal classification of the Brachiopoda.

The scheme is superimposed on a geological time scale and shows the ranges of the main taxa within three subphyla (broken line boxes), which consist of eight classes (solid line boxes), labelled: Li (Lingulata); Pa (Paterinata); Cr (Craniata); Ch (Chileata); Ob (Obolellata); Ku (Kutorginata); St (Strophomenata) and Rh (Rhynchonellata) (from Williams et al., 1996).

Several attempts to subdivide the genus have been made. Dall (1871) put forward a subdivision in his formation of the genus *Craniscus* and the subgenus *Craniopsis*. Jaekel (1902) advocated a further subdivision in advancing *Crania egnabergensis* (Retzius) as the type species of the genus *Isocrania*. In 1964, Rosenkrantz proposed *Crania tuberculata* (Nilsson) as the type of a new subgenus *Danocrania*. Such segregation of the group has been largely disregarded by brachiopod workers. For example, although the genera *Ancistrocrania* and *Isocrania* have been present in scientific publications for over sixty years, acceptance of these within the scientific world has been minimal. Carlsson (1958), in his study of Swedish *Crania*, disregarded these two genera and although other workers such as Rosenkrantz (1964) and Kruytzer & Meijer (1969) recognised their existence, they did so only at subgeneric level.

A major revision of extant species of the genus *Crania* was carried out by Lee & Brunton (1986). The authors compared the extant forms to the late Cretaceous type species, *A. craniolaris* and found that several major distinctions were apparent. Lee & Brunton (1986) felt that these differences, which included details of shell morphology, attachment mechanisms, form of growth and musculature, justified the formation of a new genus, *Neocrania*. This new classification, based on *Patella anomala* (Muller), was proposed to include many Recent and some Tertiary species formerly placed within *Crania* (Lee & Brunton, 1986). The genus *Neocrania* ranges from the Eocene to Recent and the Recent species *N. anomala* is the subject of the present study.

Traditionally, morphological and embryological comparisons have provided the means by which phylogenetic inferences have been made and the fossil record has acted as a time scale for evolutionary sequences (Lowenstein, 1986). The last forty years however, has witnessed a dramatic improvement in biochemical and molecular biological techniques, which in turn have provided vast amounts of taxonomic information. Such new data sources have been momentous in studies of brachiopod phylogeny. Up until the publication of the new *Treatise*, the craniids were classed with the inarticulated, phosphatic-shelled brachiopods. Most workers agree on the inaccuracy of this classification, although Carlson (1991) attempted to re-establish confidence in the taxonomic position of the group, following her cladistic analyses of the phylum. The development of non-morphological

taxonomic techniques, has however, been instrumental in the widespread rejection of the inclusion of the craniids within the Inarticulata, with most workers maintaining that the phylogenetic position of the group is between the two classes. The biochemistry of the craniid shell appears to support the intermediate position of the group, since the shell proteins of the group possess characteristics of both classes (Jope, 1967a). Phylogenetic analysis also supported the equivocal position of the craniid group, although Hennig (1966), following his cladistic analyses of the phylum, proposed that the craniids have a closer affinity with the articulated, carbonate-shelled brachiopods than with the inarticulated, phosphatic-shelled species. A later study by Nielsen (1991) on the ontogeny of *Crania* also supported a relationship with the carbonate-shelled brachiopods. A restructuring of the class system was proposed by Gorjansky & Popov (1985a, b) and a later revision of these cladistic studies led Popov *et al.* (1993) to advance the new class Calciata, which embraced both the calcareous articulated and calcareous inarticulated species, with the latter comprising the subclass Craniiformea.

Due to the equivocal position of the craniid group, Williams *et al.* (1996) were prompted to form the subphylum Craniiformea, which consists of a single class, the Craniata (see Figure 1.3). This class then comprises the extinct orders Trimerellida and Craniopsida and the extant Craniida, of which *N. anomala* is a member. This classification is adopted in the revised Treatise, Part H, Brachiopoda (Williams *et al.*, 1997). Detailed cladistic analyses that led to this new supraordinal classification have re-affirmed that the craniids have affinities with both articulated carbonate-shelled and inarticulated phosphatic-shelled brachiopods. An assignment to one or other group would depend on how certain characters are weighted.

1.2 The study of biominerals.

Biomineralisation is the process whereby organisms convert dissolved ions into solid minerals (Simkiss, 1990). This process of mineralisation exists within fifty-five phyla of living organisms and the phenomenon exhibits a great deal of diversity in the minerals themselves and also in the mechanisms by which they are made (Lowenstam, 1981). Organisms may produce crystalline or amorphous minerals and some are able to manufacture both types (Wilbur & Watabe, 1963; Belcher *et al.*, 1996). The main compounds constituting the crystalline biominerals are calcium carbonate, found in shells,

corals and sponges, and calcium phosphate, found in bones and teeth. Of the approximately sixty types of biogenic mineral known, around half of these possess calcium as the major cation (Lowenstam & Weiner, 1989).

The history of biomineral studies began in the last century with the discovery and subsequent development of the systematic nomenclature of many new species. With the hard mineralised parts of the organisms often being the most conspicuous, these were frequently used in species descriptions by the scientists of the time. The light microscope was widely used in these early investigations. Boggild (1930) used light microscopy to study the ultrastructure of mollusc shells. The introduction of X-ray diffraction and improvements in microscopic and histological methods later that decade, enabled further advancements in the field (Lowenstam & Weiner, 1989). With this new knowledge came the realisation within the medical field of the importance of biomineralisation studies, in terms of mineral-related diseases in bones and teeth. The later introduction of the transmission electron microscope (TEM) and the scanning electron microscope (SEM) led to an increase in the number of mineralisation studies during the 1960s and 1970s. Biochemical investigations into the mechanisms of membrane transport systems and the associations between organic material and the skeletal framework also contributed to the increasing interest in the process of biomineralisation (Simkiss & Wilbur, 1989).

In addition to having applications within molecular biology, biochemistry and medicine, biomineralisation has great importance for the fields of geology and ecology, due to the scale of related processes occurring within the Earth's structure. Mineral formation by organisms contributes significantly to the formation of many sedimentary rocks and influences the cycling of minerals throughout the Earth's outer layers (Westbroek *et al.*, 1983). Another consequence of these large scale processes is the influence on the chemical composition of fresh and sea water and on ocean sediments. For example, silicification by diatoms directly affects the silica cycle in lakes. The effects of biomineralisation processes are however, much better documented in the oceans than on land (Lowenstam & Weiner, 1989).

1.2.1 The origin of mineralised tissues.

One of the most important changes to come with the beginning of the Phanerozoic was the sudden abundance and rapid spread of calcareous hard tissues in many living organisms. Most of the major kinds of skeletal materials appeared relatively abruptly, within forty to fifty million years of the Cambrian Period. Since then, only the corals, some algae, the cheilostome bryozoans and the vertebrates have developed new skeletons (Simkiss & Wilbur, 1989; Taylor, pers comm.). The oldest fossils to exhibit biomineral production are of blue-green algae (cyanophyte) colonies, known as stromatolites, which have been detected in rocks dated at three billion years old (Weidensaul, 1994). However, the fossil record of skeletal hard parts extends back to around late Vendian times (around 550 Ma). Up until this time, fossils were all of soft-bodied organisms and are thus very rare. The advent of mineralisation dramatically increased the likelihood of preservation, providing tissues with the strength and resistance to survive relatively intact until fossilisation had occurred.

Studies of late Vendian and Early Cambrian fossils illustrate that the widespread adoption of biomineralisation was neither instantaneous, nor simultaneous in all taxa, but there was a relatively rapid increase in eukaryotes with hard parts in the Early Cambrian (Stanley, 1976). Attempts to explain this sudden increase have included theories of significant environmental alterations, destruction of any previous fossil record by geological processes, the evolution of new morphological structures and changes in the ecosystem (Lowenstam & Margulis, 1980).

Degens *et al.* (1985) suggested that the development of biomineralisation was an attempt by organisms at detoxification in a sea where calcium concentrations were increasing. Kretsinger (1983) suggested that the increase in calcium mineralisation was related to metabolism. In order to combat the increasing calcium levels within cells caused by the environment, calcium was secreted from the body. The resulting low concentrations of calcium ions within the cells allowed the subsequent evolution of many calcium-binding proteins. An alternative suggestion is that environmental changes were responsible. Towe (1970) put forward the theory that biomineralisation is oxygen-dependent and was therefore constrained until photosynthetic processes increased the atmospheric oxygen concentration to a level sufficient

for biomineralisation to occur. However, despite detailed research into Precambrian environments, there seems to be no evidence to link the actual onset of biomineralisation with any changes in the biosphere. Ocean salinity, atmospheric composition, global climate, sedimentary regimes, the Earth's orbit, nor any other environmental factor examined has given evidence to suggest any significant change at the Precambrian-Cambrian boundary (Fischer, 1965). Thus, the relatively sudden onset of biomineralisation 550 million years ago remains an enigma.

In the Late Precambrian and Early Cambrian, calcium carbonate (calcite), calcium phosphate (apatite) and silica, typically in the form of opal, were the minerals most widely utilised in eukaryotic hard parts. The fossil record indicates that apatite mineralisation was much more abundant during the Late Precambrian and Early Cambrian than later in the Phanerozoic, when carbonate mineral secretion increased. Omori (1990) suggested that the higher levels of phosphate, over carbonate in the late Precambrian may have been due to upwellings of deep water in the oceans around this time, causing phosphate saturation of shallow waters (Omori, 1990). The level of phosphate in surface waters is controlled organically and so the utilisation of either carbonates or phosphates in mineral secretion may have been a result of the organic activity of other organisms. Lowenstam & Margulis (1980) however, suggested that the switch was initiated by the metabolic advantage of utilising carbonates, which require a lower energy input in their utilisation by the body than the phosphates. The decrease in phosphate mineralisation and the simultaneous increase in the use of carbonates later in the Cambrian may have been due to a global environmental change, where the phosphate concentrations of the early Palaeozoic sea decreased, necessitating adaptation to carbonate utilisation. Organisms unable to make this switch may have become extinct (Lowenstam & Margulis, 1980).

1.2.2 Features of the organic component of shell.

The organic fraction is composed of a variety of constituents, each of which may fulfil a different role within the biomineral. Despite extensive studies of a number of mineralised tissues, a complete list of organic constituents does not exist, but for the majority of mineralising organisms, the organic fraction appears to consist mostly of proteins and polysaccharides (Weiner *et al.*, 1983). Small amounts of lipids (Curry *et al.*, 1991b); alcohols and carbohydrates

(Collins *et al.*, 1991b) and nucleic acids (Cohen *et al.*, 1991) are also present within the organic fraction. The amount of organic material present in biominerals varies between and within phyla. e.g. 0.01 to 10 percent by weight for molluscs (Hare & Abelson, 1965) and 0.5 to 25 percent for brachiopods (Jope, 1967a). (See Section 1.2.5). The organic constituents of the shell show varying degrees of solubility in organic solvents.

The organic component of biominerals may be classified in terms of the position within the shell structure. The intercrystalline organic material is located between the mineral crystals and is therefore destroyed by chemical oxidation, whereas the intracrystalline organic component is found within the mineral itself and is therefore protected from oxidation. Proteins from both locales play important roles in the control of mineral formation (Weiner & Addadi, 1991). Although there is much variation between organisms in the amounts and proportions of compounds in the organic component of a biomineral, proteins and polysaccharides constitute the bulk of the material (Weiner *et al.*, 1983).

Demineralisation of the shell by the calcium chelator, ethylene diamine tetraacetic acid (EDTA) results in an EDTA-soluble and an EDTA-insoluble organic fraction (Meenakshi *et al.*, 1971; Jope, 1979; Weiner, 1979). The soluble and insoluble fractions differ in composition, but both consist primarily of protein and carbohydrate (Crenshaw, 1972; Weiner & Hood, 1975). However, the EDTA-soluble fraction consists of similar types of macromolecules, irrespective of the organism being studied, whereas the composition of the insoluble fraction varies greatly between taxa. Proteins from the insoluble fraction of molluscs are generally hydrophobic, consisting primarily of alanine and glycine residues, whereas the soluble protein fraction is predominantly acidic. Differences exist between different phyla in terms of the proportions of EDTA-soluble and EDTA-insoluble material in the intracrystalline fraction. The intracrystalline fraction of bivalves is mostly EDTA-insoluble (Addadi *et al.*, 1991), whereas the intracrystalline component of sea-urchin tests (Addadi *et al.*, 1991) and gastropod shells (Watabe, 1965; Crenshaw, 1972; Weiner, 1979) are almost entirely soluble in EDTA. Intracrystalline material extracted from the shells of brachiopods is composed of both EDTA-soluble and EDTA-insoluble fractions, although the proportions differ greatly within the phylum (Cusack, pers comm.).

1.2.3 The role of the organic component of the shell in biomineralisation.

Lowenstam (1981) proposed a distinction between different means of biomineral formation based on the degrees of control exerted by the organic material. He thus proposed the term "biologically induced mineralisation", for processes resulting in mineral formation in a non-specific manner. Biologically induced mineralisation is the main process used by the prokaryotes, fungi and protists. Such induction of mineral precipitation can occur in instances where, for example, metabolic end products are introduced, where the cell releases cations, or where charged cell surfaces are formed (Lowenstam & Weiner, 1989). In many situations, however, biomineralisation is controlled by structures and processes specifically employed for that function, a process which Lowenstam (1981) termed "organic matrix-mediated mineralisation". Mann (1983) later renamed this process more appropriately, "biologically controlled mineralisation".

The majority of minerals formed by either of these biological processes can also be produced by inorganic processes. However, biogenic minerals are formed at ambient temperatures and pressures, conditions under which many inorganically produced minerals would not normally form. The precise means by which living organisms produce complex biominerals, often single crystals, at ambient temperatures and pressures, is still not fully understood (Cusack *et al.*, 1992). Biomineralisation produces a vast array of crystal shapes and sizes that are able to fulfil their intended biological function, due to the involvement of the organic material within the biomineral itself (Mann, 1983). This organic matter also appears to be the main regulatory factor in determining the form of the biomineral. Polymorphs of calcium carbonate exist as calcite, aragonite or vaterite, although calcite and aragonite are the most commonly formed calcium carbonate biominerals (Weiner, 1984). Soluble shell proteins can determine which calcium carbonate polymorph will be formed *in vitro*. (Falini *et al.*, 1996). For example, the presence of soluble mollusc shell proteins is sufficient to control the switch between the formation of aragonite or calcite (Belcher *et al.*, 1996).

The degree of control exerted over the mineralisation process can vary from very little to being highly regulated (Weiner, 1986). The roles of the mineral-associated proteins are extremely diverse and although some of the general functions have been determined, the precise mechanisms by which mineralisation is controlled are largely unknown (Weiner & Hood, 1975;

Termine *et al.*, 1980; Wheeler *et al.*, 1981; Berman *et al.*, 1988, 1990; Addadi *et al.*, 1991, 1994; Aizenberg *et al.*, 1994, 1997). Some of the functions of mineral-associated proteins include: (1) Induction of mineral nucleation (Simkiss & Wilbur, 1989); (2) Inhibition of mineral deposition by protein binding onto the crystal structure (Wheeler *et al.*, 1981). (3) Influence on crystal strength by the organic material becoming incorporated into the crystal structure (Silyn-Roberts & Sharp, 1986); (4) Influence on the flexibility of the mineral by the presence of intracrystalline protein molecules (Weiner, 1986) and (5) Formation of organic chambers in which crystals grow until restricted by the surrounding protein "envelope" (Watabe, 1981; Simkiss & Wilbur, 1989). Crystal size and shape can therefore be controlled. Wheeler & Sikes (1984) preferred to label the mineral-associated organic material as "regulatory", since it was considered that the organic fraction possesses many functions yet to be determined. For a comprehensive review of the properties and proposed functions of mineral-associated organic material, and of proteins in particular, the reader is referred to Lowenstam & Weiner (1989).

1.2.4 Proteins in biominerals.

Mineral associated proteins perform a multitude of different and varied functions (see Section 1.2.3) and some of these roles are governed by their amino acid composition (Weiner & Hood, 1975). Studies of a wide variety of mineralised tissues have indicated that the associated EDTA-soluble intracrystalline proteins tend to be glycosylated and are acidic, being rich in aspartic and glutamic acid (Weiner & Hood, 1975; Meenakshi *et al.*, 1971; Cariolou & Morse, 1988). Carboxyl groups of aspartic and glutamic acid residues on acidic macromolecules bind calcium ions and this induces localised ion supersaturation required for nucleation (Wilbur, 1976). A common mode of structural mineral formation is the growth of crystals within preformed intercrystalline "envelopes" of protein (Lowenstam, 1981). The size and shape of the growing crystals is thus controlled (Simkiss, 1986). Crystals grown in such a manner tend to be symmetrical and are often in crystallographic alignment with each other. A highly organised structure results, consisting of crystals surrounded by protein sheaths (Berman *et al.*, 1993).

Many *in vitro* studies of the effects of the mineral-associated proteins have been performed. A common method of analysis is the examination of crystal growth in the presence and absence of mineral proteins (Termine *et al.*, 1980;

Wheeler *et al.*, 1981; Aizenberg *et al.*, 1995). Studies such as that of Addadi & Weiner (1985), Addadi *et al.* (1991) and Aizenberg *et al.* (1994) have shown that proteins stereoselectively interact with specific planes of the growing crystal, resulting in inhibition of growth of the interacting crystal face in relation to unaffected faces. The actual shape of the crystals is dependent partly on the pattern of defects caused by incorporation of the protein into the ordered crystal lattice (Aizenberg *et al.*, 1995). Shell proteins also play a role in determining the polymorph of a particular mineral deposited in the shell (Falini *et al.*, 1996; Belcher *et al.*, 1996). Many organisms, such as molluscs, are able to deposit two polymorphic forms of calcium carbonate, calcite and aragonite, in different areas of their shell structure (Watabe & Wilbur, 1960). Specific soluble polyanionic proteins regulate the switch in morphology between the two mineral polymorphs (Falini *et al.*, 1996). By controlling the expression of these proteins, the organism is able to exert a high degree of control over the growth of the biomineral and thus can rapidly select the polymorph to be deposited at a particular site within the shell (Belcher *et al.*, 1996). Such studies demonstrate the unprecedented ability of the mineral associated proteins to exquisitely control the formation of the minerals with which they are so closely linked.

1.3 Ultrastructure of the shell of *N. anomala*.

1.3.1 Ultrastructure of *N. anomala* shell.

The structure of the craniid shell is known principally through the classic studies of Carpenter (1853) and Blochmann (1892) and more recently at the ultrastructural level, using electron microscopy (Williams & Wright, 1970). The dorsal valve of *N. anomala* is punctate (Williams & Rowell, 1965; H64) (Figure 1.4). These endopunctae are unusual, in that they branch at one end. The dorsal valve consists of both a primary and a secondary layer, with the former lying directly beneath the periostracum (Williams, 1970). The two layers differ in appearance. The primary layer is composed of acicular calcite (Figure 1.5), with a small amount of protein interspersed among the crystallites. The abrupt transition to secondary calcite is illustrated in Figure 1.6. The mineral of the secondary layer is in the form of tablets, or laminae, approximately 320 nm thick and up to 6 μm long (Williams & Wright, 1970). The layers of laminae resemble a series of steps. Between the laminae are layers of intercrystalline protein, which form an "envelope" for each lamina

(Williams, 1970). Further away from the primary layer, the lamina succession is "corrugated", as rounded ridges between punctae (Williams & Wright, 1970). (Figure 1.7).

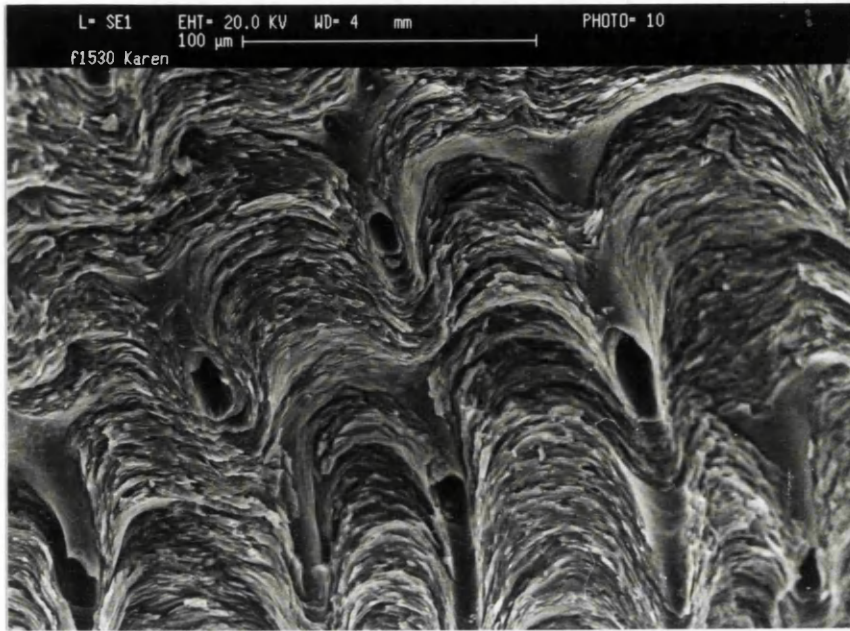


Figure 1.4 Scanning electron micrograph of a cross-section of the secondary layer of the dorsal valve of *N. anomala*.

The scale bar is 100 μm.

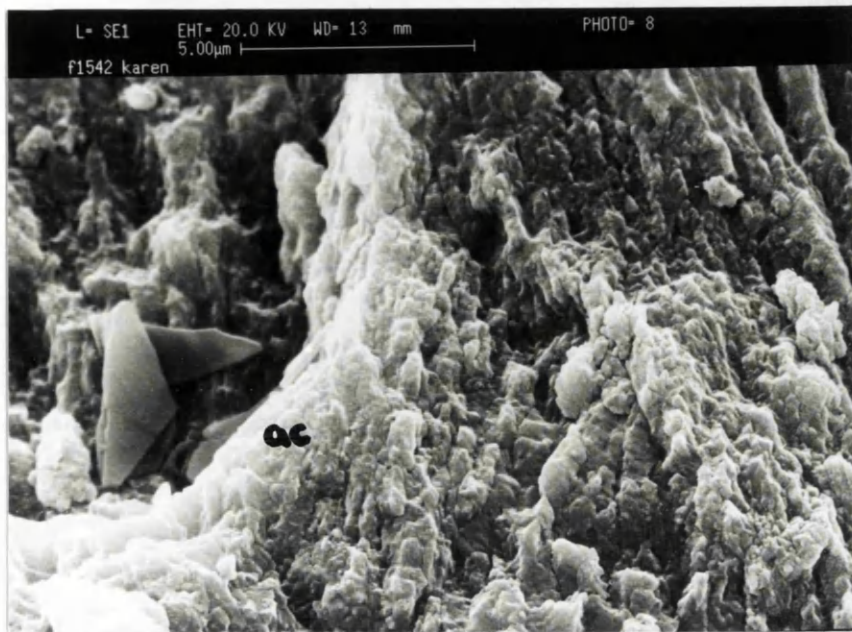


Figure 1.5 Scanning electron micrograph of the primary layer of the dorsal valve of *N. anomala*, showing the acicular calcite (ac)

The scale bar is 5 μm.

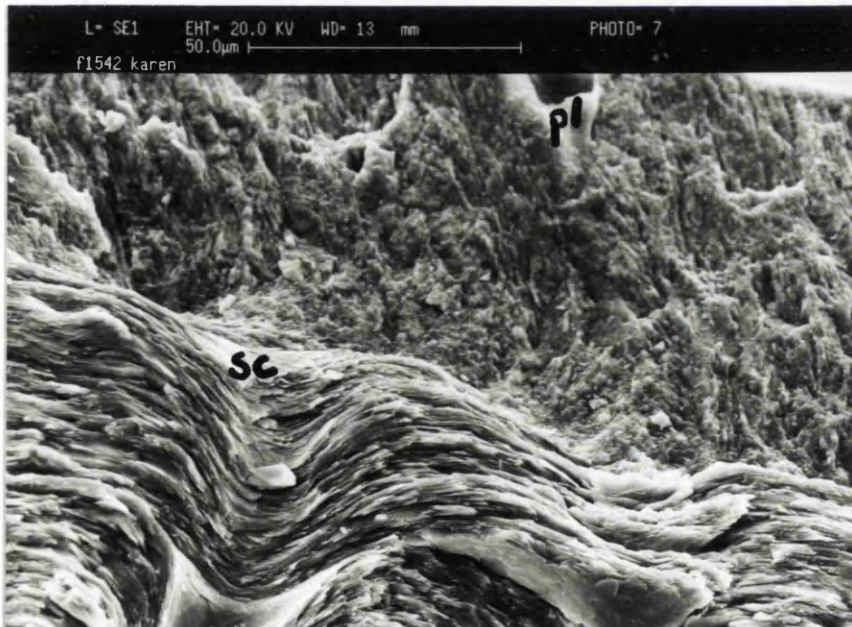


Figure 1.6 Scanning electron micrograph of the transition between the primary (pl) (top) and secondary (sc) (base) layers in the dorsal valve.

The scale bar is 50 μm.

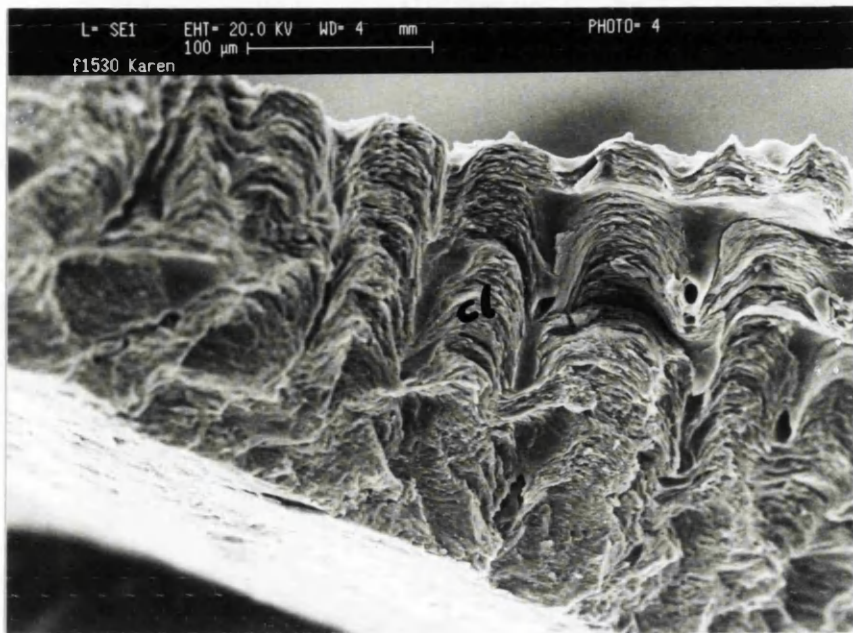


Figure 1.7 Scanning electron micrograph of the dorsal valve of *N. anomala* illustrating the corrugation of the laminae (cl) comprising the secondary layer (top of photograph).

The scale bar is 100 μm .

In contrast to the dorsal valve, the ventral valve consists only of a primary layer of acicular calcite and a periostracum (Figure 1.8). The inorganic component of the primary layer of the ventral valve is identical in composition to that of the dorsal valve, but morphological differences exist, resulting from the attachment and close conformity of the ventral valve to the underlying substrate (Williams & Wright, 1970). The calcitic layer of the ventral valve is very thin, consisting of a concentric ridge of mineral and a thin transparent layer in the centre (Figure 1.9).

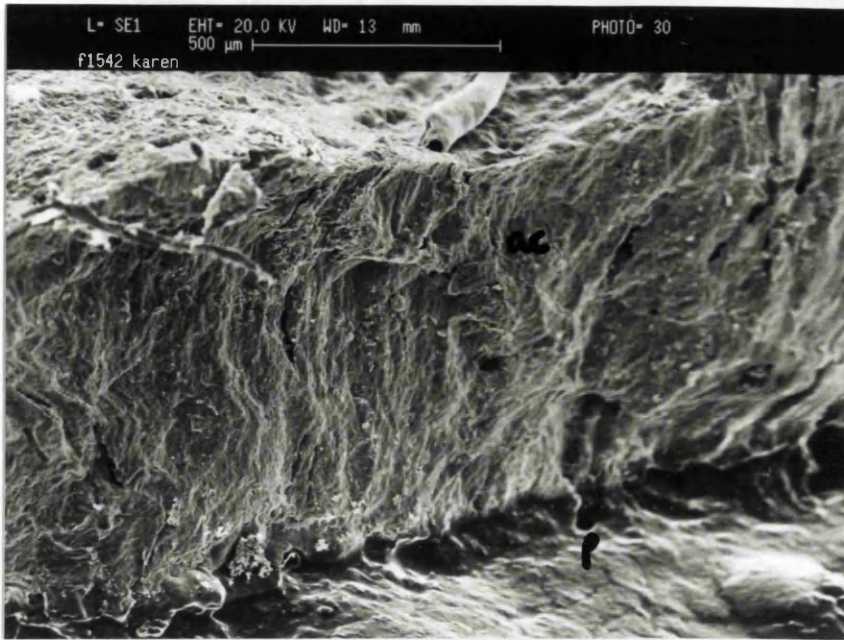


Figure 1.8 Scanning electron micrograph of the ventral valve, consisting of acicular calcite (ac) and a periostracum (p) (base of photograph).

Scale bar is 500 μm .



Figure 1.9 Ventral valves of *N. anomala*, adhering to the mussel shell substrate.

Each valve is 1 cm in diameter.

1.3.2 Growth of semi-nacre by screw dislocation.

The high resolution provided by the scanning electron microscope has led to the discovery of several microscopic structures within shells, some of which are common to several phyla. One such ultrastructure is nacre, or mother-of-pearl, which is composed of aragonitic laminae, separated by layers of organic material. Nacre is restricted to molluscs (Wise, 1970; Carter, 1979). Semi-nacre however, is similar in structure to nacre, but may be calcitic or aragonitic and exists in organisms other than molluscs. Semi-nacre may also be distinguished from nacre in having more screw dislocations (Weedon & Taylor, 1995). Semi-nacre has been identified in the secondary layer of the dorsal valve of *N. anomala* (Williams, 1970; Williams & Wright, 1970) (Figure 1.10) and also in the cyclostome bryozoans (Weedon & Taylor, 1995).

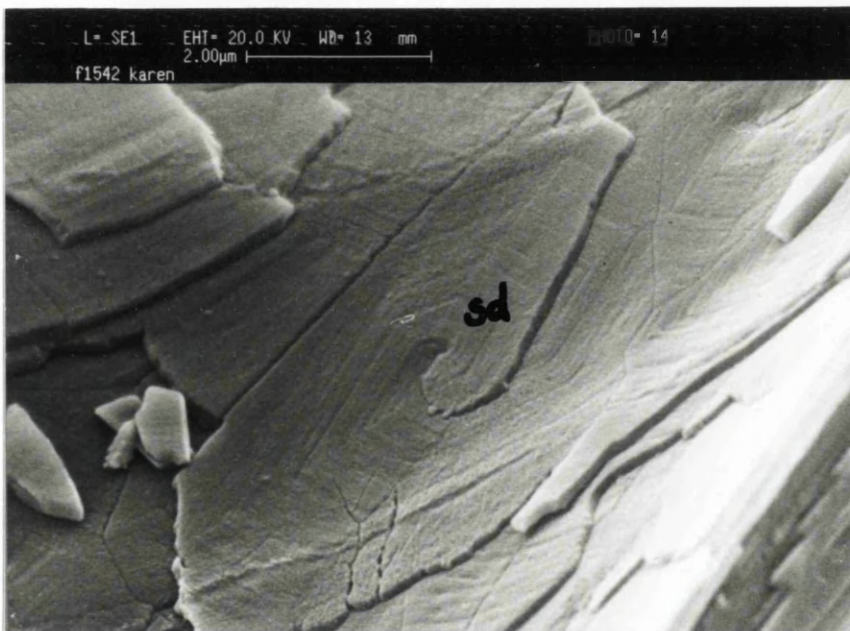


Figure 1.10 Scanning electron micrograph of the laminae of the secondary layer of the dorsal valve of *N. anomala*, showing screw dislocations (sd).

Etching of the mineral with NaOCl (20% v/v) for 24 hours enhances the screw dislocations. The scale bar is 2 µm.

Screw dislocations on a biomineral surface are characterised by a spiral growth pattern (Figures 1.10 and 1.11). The spiral shape results from the accumulation of foreign ions or mismatching between constituent ions, in the top layer of the lattice. This causes steps to form, which extend spirally as growth proceeds (Nielsen & Christoffersen, 1982). Screw dislocations may occur as single or double spirals and a crystal may possess several spirals (Williams & Wright, 1970). The edges of adjacent laminae are indicated by fine cracks (Figure 1.12), but nuclei may coalesce, especially if crystallographically aligned, to form composite laminae. Protein sheets cover the surface of growing laminae, resulting in alternating organic and inorganic layers (Williams, 1970).



Figure 1.11 Scanning electron micrograph of the interior of a puncta in the dorsal valve of *N. anomala*, showing numerous left and right handed screw dislocations (sd).

The scale bar is 5 µm.

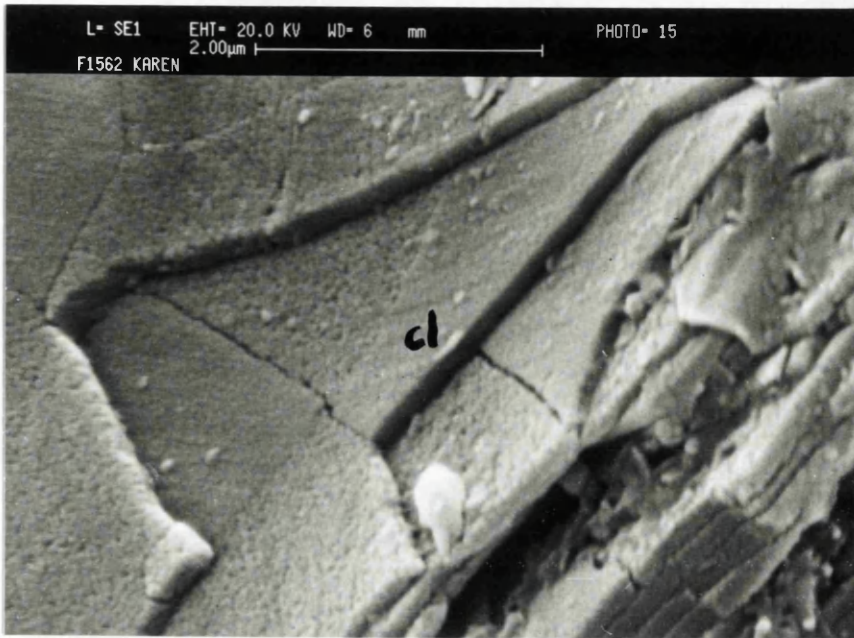


Figure 1.12 Scanning electron micrograph of several laminae (cl) in the dorsal valve of *N. anomala*.

The borders between adjacent laminae, marked by fine cracks in the mineral surface, are enhanced by incubation in NaOCl (20% v/v) for 48 hours. The scale bar is 2 μm.

1.3.3 The protein component of brachiopod shells.

The brachiopod shell resembles the exoskeleton of arthropods and molluscs in that it comprises protein layers, alternating with layers of mineral (Jope, 1965; H156). Protein is the main organic component of all brachiopod shells, but the amount, form and position varies between taxa. The amount of protein present within the shell appears to be governed by the type of mineral constituting the shell structure. The protein content of phosphatic shells tends to be high (i.e. *Lingula*, 25% of shell weight; *Discina*, 15%), whilst carbonate shells in comparison, contain little protein (0.5 to 2% of shell weight) (Jope, 1967a). In endopunctate species, such as *N. anomala*, organic material may also originate from the punctae (Williams, pers comm.).

The structure of the brachiopod shell is complex and varies considerably, not only between subphyla, but also between classes, families and even orders. The main shell protein of the articulated calcitic-shelled species occurs between and within the calcite fibres of the secondary layer (Jope, 1977). Most of the shell protein of the inarticulated carbonate shelled craniids is associated with the calcitic laminae of the secondary layer, with only a small amount of protein interspersed with the acicular calcite of the primary layer (Williams &

Wright, 1970). In the shell of the inarticulated *Lingula* and *Discina*, the protein is located within and around the granules of calcium phosphate that make up the shell structure (Jope, 1977).

Shell proteins are secreted by the mantle epithelial cells, which deposit protein particular to each cell layer as they mature and grow away from the generative zone. As the protein in the mantle is profoundly different from that in each of the shell layers secreted by the mantle, it appears that just one set of mantle cells is able to produce all of the different protein types required in each layer to fulfil the different requirements of the whole shell structure (Jope, 1965; H156).

The amino acid composition of the protein from the brachiopod shell not only differs between the subphyla, but also within them; i.e. between taxa of ordinal, familial and even generic level. Although the general composition includes fairly high glycine and alanine levels, there are significant differences which support the sub-phyletic division proposed by Williams *et al.* (1996) (Figure 1.3). The inarticulated phosphatic species possess low glycine but high alanine and hydroxyproline, whilst the articulated carbonate species have high glycine and low alanine, but lack hydroxyproline (Jope, 1967a). Proteins extracted from *N. anomala*, are rich in glycine, possess moderate levels of alanine, but lack hydroxyproline (Jope, 1967a).

Since the pioneering work of Margaret Jope in the 1960's and 1970's, few studies have attempted to characterise the proteins of brachiopod shells. However, Cusack *et al.*, (1992) succeeded in isolating and partially characterising an intracrystalline chromoprotein responsible for the red colouration of some terebratulid brachiopod shells. Tanaka *et al.* (1988) isolated a calcium-binding protein called apsilin from the shell of *Lingula*, although the location of the protein within the shell has yet to be determined. The present study succeeded in obtaining an N-terminal sequence for the main dorsal valve protein and amino acid composition for the shell protein extracts of *N. anomala* (Sections 3.3.2 and 3.3.3.2). Such proteins may have a function in the process of biomineralisation, but as yet, the precise mechanisms of shell formation are unknown (Cusack *et al.*, 1992). Much more research is required before the precise functions of brachiopod shell proteins can be determined.

1.3.4 The protein component of the shell of *N. anomala*.

The shell protein of *N. anomala* occurs both within the mineral crystals and also surrounding each calcite lamina. The protein aids the process of shell formation by interacting with the growing crystals, which perpetuate the screw dislocations that make up the shell (Williams, 1970). This method of shell growth is utilised by some molluscs and the bryozoans, but within the phylum Brachiopoda, is unique to the genus *Neocrania*. The laminae grow in a spiral fashion, with each forming on top of a sheet of protein, the newly formed tablet thus becoming the base for the growth of another sheet. The protein layers finally come together, resulting in a shell structure of alternating protein and mineral layers (Williams, 1970). The growth of the shell by screw dislocation is discussed in more detail in Section 1.3.2.

The amino acid composition of shell proteins expresses the partial genetic make-up of the organism, allowing a phylogenetic reconstruction based on the proteins extracted from brachiopod shells (Jope, 1977). The amino acid data of Jope (1967a, 1969a, 1973) and of the present study (Section 3.3.2), support the theory that the craniids are of anomalous taxonomic affinity, with a phylogenetic position intermediate between the calcitic-shelled articulated and phosphatic-shelled inarticulated brachiopods.

1.4 Aims and approach of this study.

Traditional methods of taxonomical analysis, such as morphological comparison, have largely failed to determine the phylogenetic position of the craniid brachiopods. The difficulty is that the craniids are taxonomically in an intermediate position, having affinities with both articulated carbonate-shelled and inarticulated phosphatic-shelled brachiopods. Differential weighting of different synapomorphies assign the craniids to one or other group.

This study examines the proteins which are intimately associated with the calcite of *N. anomala* valves, in particular, the secondary layer of the dorsal valve. The main aim of this study is to use the knowledge of these proteins to shed light on the mechanisms of biomineralisation occurring in this particular system. The information may also have taxonomic potential, in helping to resolve the phylogenetic position of the craniid brachiopods.

Chapter Two

Materials and Methods

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- 2.2 Methods. Collection and preparation of *N. anomala*.**
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 - 2.2.2 Extraction of insoluble intracrystalline proteins.
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- 2.11 Determination of the N-terminal residues.**
 - 2.11.1 Preparation of samples for sequencing.

2.1 Materials (Appendix)

2.2 Methods.

Collection and Preparation of *N. anomala*.

An abundant population of *N. anomala* exists in a depression in the Firth of Lorne, mid-way between the island of Kerrera and the Island of Mull off the west coast of Scotland (Curry, 1982). Specimens were obtained by dredging the sea bed at a maximum depth of 200m. The dredging was carried out by staff of the Calanus research vessel, belonging to the Dunstaffnage Marine Laboratory in Oban. The dredged material was stored overnight in tanks of sea water at Dunstaffnage Marine Laboratory. Brachiopods were transported to the University of Glasgow, either on the same day, or one day after dredging. Specimens were transported in sea water in a portable tank fitted with a battery powered aerator.

Neocrania anomala is characterised by being attached by cementation of the ventral valve to the substrate, which was in this case, the shell of the horse mussel *Modiolus modiolus* (Linnaeus) (Figure 1.2). Specimens of the articulated brachiopod, *Terebratulina retusa*, also attached to mussel valves, were removed by cutting the pedicle and used by a colleague. The dorsal valves of *N. anomala* were detached from the mussel shell substrate by prising off using dental tools. The ventral valves are strongly cemented onto the mussel shell substrate by way of a mucopolysaccharide, which is first secreted from the mantle epithelium during larval life (Williams, 1970). The strength of attachment makes it extremely difficult to remove the ventral valves. Removal was achieved by sawing at the base of the attached valve with a sharp scalpel blade. Body tissue and the organic periostracum were then removed by abrasion with a small toothbrush and fine sandpaper to ensure complete removal of the organic covering.

2.2.1 Extraction of soluble intracrystalline proteins.

Valves were crushed to a fine powder with a mortar and pestle and the intercrystalline organic material destroyed by incubation in an aqueous solution of sodium hypochlorite (1% v/v) at 22°C for one hour under constant agitation. Sodium hypochlorite was removed by repeated washing of the sample with 18M Ω water and centrifugation. Washes were repeated, typically ten times, until no bleach could be detected by smell. *N. anomala*

shell powders were dissolved by incubating with constant agitation for 2 to 3 days at 22°C with the calcium chelator, ethylene diamino tetra acetic acid, disodium salt (EDTA) (20% w/v, pH 8) at a ratio of 23 ml/g shell powder. Insoluble material in the form of a residue, was removed by centrifugation at 4,000g for 30 minutes and stored at -20 °C.

The supernatant was then concentrated and EDTA removed by ultrafiltration using the Minitan™ tangential flow ultrafiltration system. This device is efficient in the removal of EDTA (Cusack *et al.*, 1992). The sample was concentrated 20 fold and EDTA removed by filtration with sodium phosphate buffer (10 mM, pH 7.2) using 10 kDa molecular weight cut-off filters. Incomplete removal of EDTA tends to distort SDS PAGE gels and interferes with PITC amino acid analysis, by co-eluting with the amino acids Glu, Gly, Ser and Pro and therefore altering the apparent amino acid composition (Walton, 1992). It is essential therefore, to remove EDTA before further analysis. Figure 2.1 shows a summary of the methods used in the extraction of the intracrystalline protein.

The Minitan™ retentate was further concentrated using Centriprep™ concentrators, by centrifugation at 3000g for 40, 10 and 5 minutes in a Suprafuge centrifuge. Finally, Microcon™ concentrators were used in the Microcentaur bench top centrifuge to further concentrate the extract. Filters of 10 kDa molecular weight cut-off were employed throughout. The final volumes were typically 300 µl, from a starting shell weight of 7 g for the dorsal valves and 1 g for ventral valves.

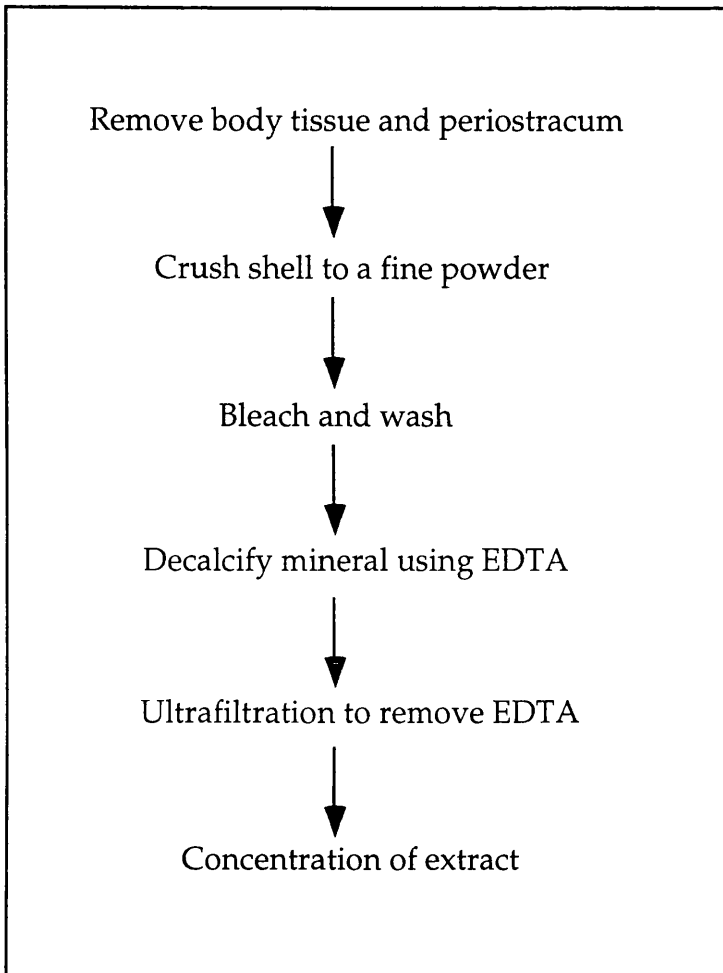


Figure 2.1 Protocol for extraction of intracrystalline proteins from *N. anomala*.

2.2.2 Extraction of insoluble intracrystalline material.

The insoluble material resulting from demineralisation of the dorsal valve by EDTA (see Section 2.2.1) was repeatedly washed in 18M Ω water (typically 20 times), in an attempt to remove EDTA from the sample. A 0.01mg aliquot of the insoluble material was then dissolved in 2N HCl (11 μ l/mg). Samples were analysed on the 420A Amino Acid Analyser to determine the amino acid levels in the EDTA-insoluble intracrystalline material.

2.2.3 Extraction of intercrystalline material.

Two methods were used in the extraction of the soluble intercrystalline material. The first involved simply omitting the bleaching step and following the extraction and concentration steps as shown in Figure 2.1. This was the method most commonly used. Intercrystalline material was also extracted by

incubating the powdered shell in 4M guanidine hydrochloride (GuHCl), buffered to pH 7.4 with hydrochloric acid. GuHCl was added at a ratio of 100 ml to approximately 5 g shell and incubated with constant agitation for 24 hours at 4 °C. GuHCl is a strong hydrogen bonder and thus destroys the ordered water structure of the protein, promoting the unfolding and dissociation of the protein molecules (Matthews & van Holde, 1990). The intercrystalline material is therefore separated from the mineral.

Centrifugation at 4,000g for 10 minutes separated the soluble from the insoluble fractions. The supernatant was stored at -20°C and the denaturing process repeated with fresh GuHCl solution for the insoluble fraction. Both supernatants containing the soluble intercrystalline material were pooled. The intracrystalline material was extracted from the insoluble fraction remaining after GuHCl treatment, as detailed in Section 2.2.1.

Removal of GuHCl and the first concentration step was achieved by filtration through the Minitan™ tangential flow ultrafiltration system and the sample concentrated further using Centriprep™ and Microcon™ concentrators. Any remaining salts were removed by filtration through a Sephadex PD-10 column, having pre-equilibrated with 25 ml 18M Ω water. Twenty 1 ml fractions were collected and the absorbance of each sample at 280 nm was determined using a Digital Ultraviolet Spectrophotometer. Fractions containing protein were pooled and concentrated by centrifugation under vacuum using a Gyrovap. The final volume was around 300 µl, from a typical shell weight of 7 g.

2.3 Purification of soluble intracrystalline material.

2.3.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE).

SDS PAGE was employed to fractionate the protein mixture into the constituent proteins. Separation of individual proteins is based on differences in the molecular weight of the proteins and the fractionation is facilitated by an electric current. The compositions of resolving and stacking SDS gels are presented in Table 2.1. Protein samples were denatured by heating to 100°C for 4 minutes with a sample buffer [Tris buffer (50mM, pH6.8); SDS (4% w/v); Glycerol (12% w/v); β-Mercaptoethanol (2% w/v); Coomassie Blue 250

(0.01% w/v)]. β -Mercaptoethanol cleaves the disulphide bonds and SDS binds to the protein in a constant weight ratio (1.4 g SDS per gram polypeptide). The charge on the polypeptide is thus overcome by the negative charges of the bound SDS detergent, with the result that the proteins have identical charge to mass ratios and are fractionated in the polyacrylamide gel according to molecular weight (Dunn, 1993).

Polyacrylamide gels (10% w/v), with 4% stacking gels were prepared, following the method of Schägger & von Jagow (1987), where glycine is replaced by tricine. Acrylamide and N, N, N',N'-methylene bisacrylamide were polymerised together, initiated by the addition of ammonium persulphate and catalysed by N,N,N',N'-tetramethylethylenediamine (TEMED).

Reagent	Resolving gel	Stacking gel
Acrylamide	10% (w/v)	4% (w/v)
Bis-acrylamide	0.03% (w/v)	0.12% (w/v)
Sodium dodecyl sulphate	0.1% (w/v)	0.07% (w/v)
Ammonium persulphate	0.65% (v/v)	1.57% (v/v)
TEMED	0.075 (v/v)	0.18% (v/v)
Glycerol	13% (v/v)	n/a

Table 2.1. Composition of resolving and stacking SDS gels.

The protein and buffer mixture was loaded into the wells of the stacking gel and a constant 100 volts applied to the gel for one hour. Cathode buffer [0.1 M Tris, 0.1 M Tricine, SDS (0.1% w/v), pH 8.25] and Anode buffer (0.2 M Tris, pH 8.9) facilitated the electric current. The polyacrylamide gel acts as a "molecular sieve", with smaller molecules being less restricted by their size than larger molecules and thus migrating further within the gel. Prestained molecular weight standards were included in every SDS gel. Standards included myosin (H-chain) (214.2 kDa); Phosphorylase B (111.4 kDa); Bovine Serum Albumin (74.25 kDa); Ovalbumin (45.5 kDa); Carbonic Anhydrase

(29.5 kDa); Beta-Lactoglobulin (18.3 kDa) and Lysozyme (15.4 kDa). Molecular weight values presented are apparent molecular weight values for prestained proteins. A standard curve of log molecular weight as a function of the electrophoretic mobility (Figure 2.2), allows the molecular weight of unknown proteins to be determined.

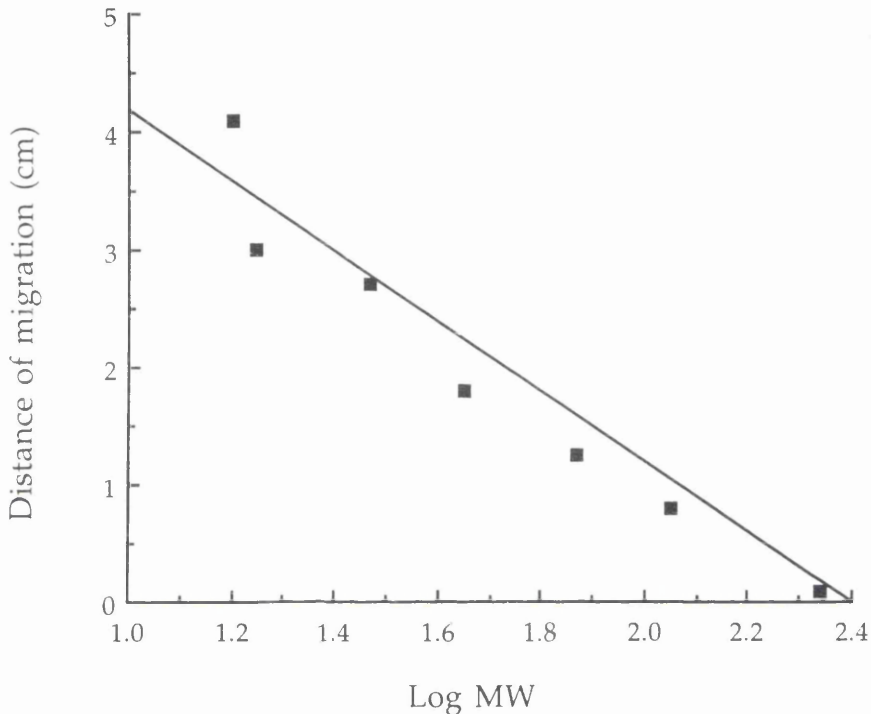


Figure 2.2 Calibration curve of the electrophoretic mobility of the SDS standards as a function of the molecular weight (\log_{10}) of the proteins.

Proteins were electrophoresed in 10 percent polyacrylamide gels. Data was analysed by linear regression and the correlation coefficient was 0.95.

2.3.2 Staining of SDS PAGE gels.

2.3.2.1 Coomassie Blue Staining.

Following electrophoresis, proteins were both fixed and visualised in the gel using one of two stains. Incubation of the gel in a solution of Coomassie™ Brilliant Blue (CBB) R-250 (0.25% w/v) in methanol (45% v/v) and acetic acid (50% v/v), fixes and reveals all the proteins present. Background staining is removed by destaining [methanol (75% v/v), acetic acid (25% v/v)]. Usually

0.2-0.5 μg of any protein can be detected in a single band using CBB-250 (Hames & Rickwood, 1990).

2.3.2.2 Silver staining.

This method is up to one hundred times more sensitive than CBB staining, being able to detect around 1 ng of protein (Hames & Rickwood, 1990). This staining technique involves the reduction of silver nitrate to metallic silver, leading to the deposition of silver grains and thus visualisation of protein. Proteins were fixed in the gel by incubating with methanol (50% v/v); TCA (12% w/v). The gel was then reduced by incubation in dithiothrietol (DTT) solution (0.0005% w/v), exposed to silver nitrate solution [AgNO_3 , (0.2% w/v)] and developed in developer solution [$\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$ (0.8% w/v), formaldehyde (0.05% v/v)]. Staining was terminated by immersion in an aqueous solution of acetic acid (1% v/v).

2.3.3 Electrophoretic transfer of protein to PVDF membrane.

The electrophoretic transfer of protein from a polyacrylamide gel to an inert membrane allows an exact replica of the original protein separation to be made, yet leaves the transferred proteins accessible to further study. Polyvinylidene fluoride (PVDF), a Teflon-type polymer, is widely used in laboratories for this purpose, due to its ability to withstand harsh chemical conditions. A constant voltage induces the transverse movement of the protein molecules from the gel onto the membrane, where the protein then binds to the PVDF membrane through dipolar and hydrophobic interactions (Gooderham, 1984). Problott™ PVDF membrane (Perkin Elmer-Applied Biosystems) was used in this study.

Problott™ membrane was wetted with methanol and placed in electroblotting solution [methanol (10% v/v); CAPS buffer (10mM, pH 11)]. The electroblotting procedure is carried out using a BioRad MiniTransblot Electrophoretic Transfer Cell. A constant 55 volts was then applied for 45 minutes to transfer the proteins out of the SDS gel and onto the Problott™ membrane, to which they adhere.

2.3.3.1 Staining of Electroblobs.

Coomassie Blue. Protein bands were visualised by incubating the membrane in Coomassie™ Brilliant Blue R-250 (CBB-250) (0.1% w/v) for one minute. Destaining in an aqueous solution of methanol (50% v/v), reduced background staining and the blot was then rinsed in 18M Ω water and air dried.

Sulphorhodamine dye. [Sulphorhodamine B (0.005% w/v); methanol (30% v/v); acetic acid (0.2% v/v)] may also be used to stain protein bands and is reported to be more sensitive than CBB-250. This dye is used primarily in protein sequencing applications, when CBB-250 interferes with the Edman chemistry. Incubation of a dry PVDF membrane in the dye solution for a few seconds produces pink protein bands against a white background.

2.4 Carbohydrate detection.

Glycoproteins, in which one or more carbohydrate units have been covalently attached to the protein, are the most common products of post-translational modification (Creighton, 1983). Two protocols were used in the analysis of the intracrystalline fraction, in order to assess whether or not the proteins are glycosylated. The reagents used in the Streptavidin/Biotin protocol are listed in Table 2.2 and those used in the Concanavalin A protocol are listed in Table 2.3.

Phosphate buffered saline (PBS), pH 7.2	Na_2HPO_4 (12% w/w) NaH_2PO_4 (0.02% w/v) NaCl (0.16% w/v)
Sodium acetate/EDTA, pH 5.5	$\text{NaC}_2\text{H}_3\text{O}_2$ (50% v/v) EDTA (0.2% w/v)
Tris buffered saline (TBS), pH 7.2	Tris (0.6% w/v) NaCl (0.16% w/v)

The Streptavidin Biotin protocol for carbohydrate detection exploits the extremely high binding affinity between streptavidin, a tetrameric protein, and the vitamin biotin (Weber *et al.*, 1989). The Glycotrack™ kit (Oxford Glycosystems) is based on this binding reaction. PVDF membrane, with immobilised protein, was washed for 10 minutes in phosphate buffered saline (PBS). Any carbohydrate groups present were then oxidised by incubating the membrane for 30 minutes in the dark in 10 mM sodium periodate in sodium acetate/EDTA solution. The membrane was then washed three times in PBS for 10 minutes. Any aldehyde groups generated by the periodate oxidation were then reacted with biotin hydrazide (0.02% w/v) in sodium acetate/EDTA by incubation in this solution for one hour. Following washing in tris buffered saline (TBS), additional binding sites were blocked by immersion in a proprietary blocking reagent (Oxford Glycosystems), (0.5% w/v) dissolved in TBS for 30 minutes. After washing in TBS, the biotinylated compound was detected by incubation for one hour in the buffer containing the streptavidin conjugate (0.5% w/v) in TBS. Following washing in TBS, the conjugate was detected by addition of the staining solution [Nitroblue tetrazolium (NBT) (0.5% v/v), 5-bromo, 4-chloro, 3-indolyl phosphate (BCIP) (0.4% v/v) in dimethylformamide]. Glycosylated proteins appeared as blue-brown bands on the membrane and staining was terminated by immersion in 18M Ω water.

Table 2.3 Reagents for Concanavalin A protocol.

Fixing solution	Acetic acid (10% v/v) Isopropanol (25% v/v)
Tris buffered saline (TBS), pH 7.4	20mM Tris/HCl 0.5M Na Cl
TBS/cations buffer	1mM CaCl ₂ 1mM MnCl ₂ 4H ₂ O in TBS
TTBS/cations buffer	Tween 20 (0.1% w/v) in TBS/cations buffer

The Concanavalin A method of carbohydrate detection exploits the saccharide-binding property of the lectin concanavalin A. This method has been referred to as affinodetection. Following electroblotting onto PVDF membrane (Problott™), the membrane was rinsed in water and the protein fixed by incubation in fixing solution for 15 minutes. The membrane was then rinsed in water and then in TBS. Remaining protein binding sites on the membrane were blocked by incubation in bovine serum albumin (BSA) (3% w/v) in TBS for one hour at room temperature and the lectin incorporated by incubation in TBS/cations buffer, containing BSA (1% w/v) and 25 mg/ml concanavalin A. After briefly washing twice with TBS, the membrane was washed four times for 15 minutes in TTBS cations buffer. TBS/cations solution containing BSA (1% w/v) and 50 µg/ml horseradish peroxidase was then added and agitated for one hour, after which the blot was washed briefly twice with TBS and four times, for 15 minutes each in TTBS/cations buffer.

Glycoproteins were visualised by incubation in staining solution [diaminobenzidine (0.03% w/v), hydrogen peroxide (30% v/v) in TBS] and appear as purple/ brown bands within five minutes.

2.5 Determination of saccharide content.

To determine the total amount of carbohydrate in the protein sample, a total sugar assay, developed by Svennerholm (1956) using orcinol, was employed. The assay was performed on a concentrated liquid sample and the products quantitated colourimetrically. A range of glucose standards and protein controls were prepared. Ovalbumin was used as the glycosylated (positive) protein control, while BSA and ribonuclease A were used as the unglycosylated (negative) controls. All standards, controls and a range of sample volumes were prepared to a final volume of 200 μ l and cooled to 4°C before commencing.

Samples were reacted with ice cold orcinol dissolved in concentrated sulphuric acid. The acid hydrolyses any glycosidic linkages present in the sample and dehydrates the resulting monosaccharides. The products react with orcinol to give coloured substances. The solutions were incubated at 80°C for 15 minutes and cooled rapidly to 22 °C. The absorbance readings at 420 nm of each 1ml reaction were then determined using a Digital Ultraviolet Spectrophotometer (Cecil) with a tungsten lamp. Figure 2.3 shows the relationship between glucose concentration and absorbance at 420 nm in this system.

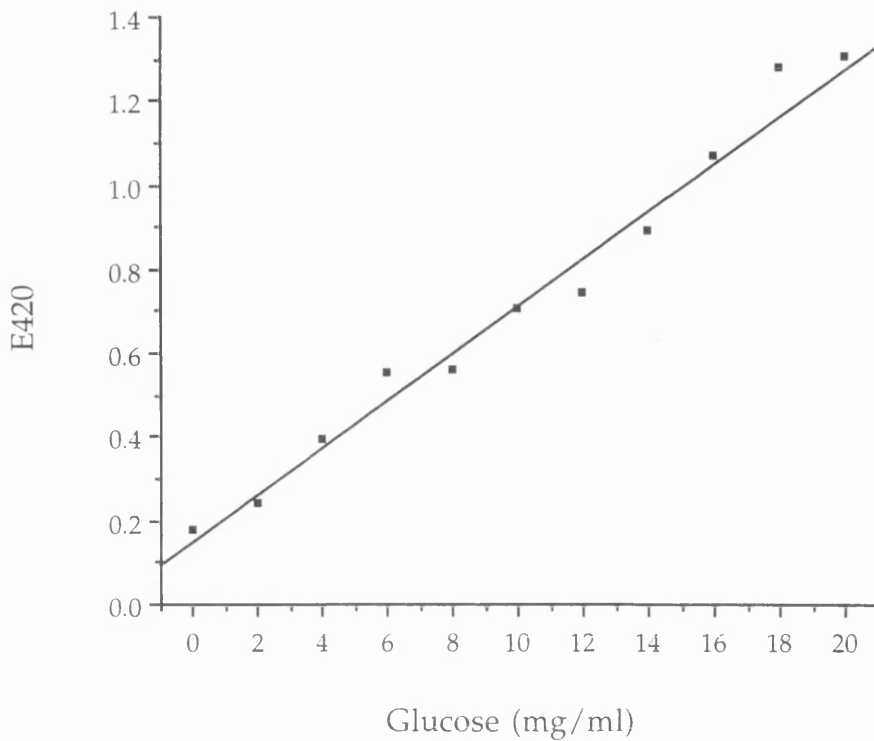


Figure 2.3 Relationship between glucose concentration and absorbance at 420nm after treatment with sulphuric acid and orcinol.

Glucose (0 to 20 mg) was reacted with orcinol, dissolved in concentrated sulphuric acid (0.2% w/v) and mixed by agitation (Section 2.5). Following incubation, samples were cooled rapidly to 22 °C. The absorbance at 420 nm was then determined for each sample. The data were analysed by linear regression and the correlation coefficient is 0.98.

2.6 Determination of calcium binding potential using Stains-all.

The Cationic carbocyanine dye, "Stains-all" (1-ethyl-2-[3-(1-ethylnaptho [1,2d]thiazolin-2-ylidene)-2-methyl propenyl] naptho [1,2d] thiazolium bromide) was used in order to determine whether the extracted proteins were calcium-binding or not. The protocol was carried out according to a modification of the method of Campbell *et al.*, (1983). Proteins in an SDS PAGE gel were fixed by incubating for one hour in an aqueous solution of trichloroacetic acid (12.5% w/v), containing ethanol (50% v/v) and the SDS removed by washing in an aqueous solution of acetic acid (10% v/v), containing methanol (30% v/v) for 2 to 3 days. Further washing was carried out twice for one hour with isopropanol (5% v/v) and cations removed by incubation in EDTA (0.5% w/v), isopropanol (25% v/v). After washing for 20

minutes with isopropanol (5% v/v) and then tris buffer (10 mM, pH 8.8) for a further 20 minutes, the gel was finally stained with Stains-all solution in tris buffer (10% v/v), prepared from a stock solution of 0.1% (w/v) in formamide, for 1 to 3 hours in the dark. Destaining was performed with 18M Ω water in the dark. Acidic and calcium-binding proteins stain bright blue against a pink background.

2.7 *In vitro* calcium carbonate mineralisation.

2.7.1 Slow crystal growth by carbonate diffusion.

Growth of synthetic calcite crystals *in vitro*, in the presence of proteins extracted from biominerals, is a commonly used method in attempting to determine the effect of individual, or mixtures of proteins (Berman *et al.*, 1990, 1993; Albeck *et al.*, 1993). The majority of these studies examine the growth of crystals in the presence of soluble protein in solution, which is believed to have effects on crystal growth distinct from the effects shown in experiments in which crystals are grown in the presence of protein adsorbed onto a surface (Addadi & Weiner, 1985). By understanding the different mechanisms by which protein can affect the growth of crystals *in vivo*, it may be possible to gain information regarding the formation of biominerals *in vivo*.

Calcite crystals were grown according to the method of Addadi & Weiner (1985). Coverslips of 1.3 cm diameter were thoroughly cleaned with an aqueous solution of chromic acid (1% v/v) for one hour, rinsed in 18 M Ω water and dried at 165 °C for one hour. One coverslip was placed in each well of a Sterilin™ 25 well plate and overlaid with 1 ml calcium chloride solution (7.5 mM, pH 8.7). The plate was then covered with aluminium foil which was pierced once above each well. Crystals were grown by slow diffusion of ammonium carbonate (1g) in a desiccator (Figure 2.4) Crystals were identified as calcite by X-ray diffraction using a Philips PW 1050/35 X-Ray Diffractometer with vertical goniometer (Figure 4.2).

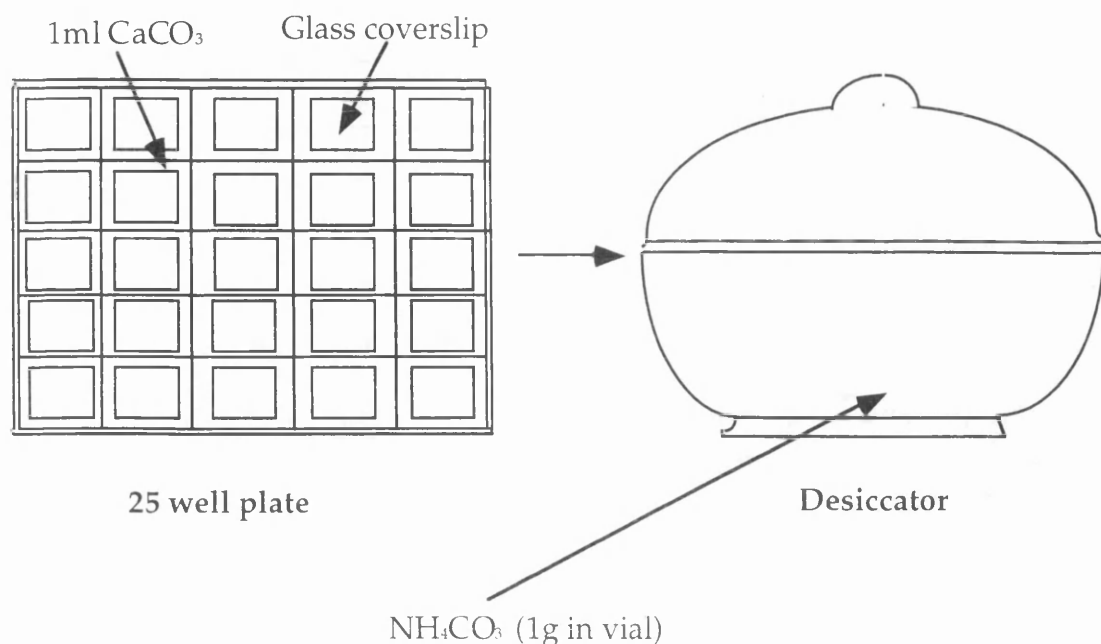


Figure 2.4 Method used in the growth of synthetic calcite crystals.

Glass coverslips were inserted into each well of a 25 well plate and overlain with 1 ml calcium chloride solution. Protein, as a solution, was added to each well in the desired concentration, at the start, or during crystal growth. Water was used as a control. The covered plate was placed in a desiccator alongside an open vial of 1 g ammonium carbonate and incubated for 48 hours at 21 °C.

Sodium phosphate buffer was removed from mineral-associated EDTA soluble proteins by desalting in a Sephadex PD 10 column, equilibrated with 25 ml 18M Ω water. The resulting fractions were concentrated by centrifugation under vacuum in a Gyrovap. The effect of sodium phosphate buffer on crystal growth and morphology was checked in case any buffer remained in the sample. A range of volumes of NaH₂PO₄, (10 mM, pH 7.2) was added to inorganically grown crystals at both the start of the experiment and separately, after 24 hours of crystal growth. Control crystals were grown with 20 μ l 18 M Ω water added instead of buffer, at both the start of the experiment and after 24 hours of crystal growth.

To determine the timecourse of crystal formation, crystals were grown by the above method and coverslips removed from the system between 0 and 168 hours at 24 hour intervals. Coverslips were then rinsed in 18M Ω water briefly six times and air dried. In order to examine the crystals, the coverslips were glued to stubs, sputter coated with gold/palladium and observed using a Cambridge 360 Scanning Electron Microscope.

Synthetic calcite crystals were also grown in the presence of a range of concentrations of total *N. anomala* intracrystalline shell proteins from both dorsal and ventral valves and intercrystalline protein from the dorsal valves. The final concentration of additives ranged from 0 to 2.4 μ g/ml. Controls were included in which crystals were grown with 18M Ω water instead of protein extract. One set of experiments involved addition of protein at the start of the experiment and another involved addition after crystal growth had proceeded for 24 hours. Experiments were terminated after a total of two days of crystal growth.

The effect of the EDTA-soluble protein extract on the abundance of crystals was also determined, by counting the number of crystals in ten 1 millimetre squares drawn at random on SEM photographs and then calculating the mean value for each stage. The photographs were taken at a standard $\times 10.5$ magnification and counting was facilitated using a $\times 10$ hand lens.

2.7.2 Fast crystal growth to measure the rate of precipitation.

To examine the effect of intracrystalline macromolecules of *N. anomala* on the rate of precipitation of calcium carbonate, the *in vitro* mineralisation method of Wheeler *et al.*, (1981), with modifications was followed.

Glass universal tubes were thoroughly cleaned in HCl (6N) and rinsed in 18M Ω water. To two tubes, 5 ml of NaHCO₃ (20 M, pH 8.7) was added and 10 μ l of water, protein extract or NaH₂PO₄ (10mM, pH 7.2) introduced. Five millilitres of calcium chloride (20 mM, pH 8.7) was added to start the reaction. One tube was sealed with Parafilm™ and the pH value of the reaction in the other tube was monitored using a Corning 220 pH meter, with the tube sealed with Parafilm™ around the pH probe. Room temperature was monitored throughout the experiment. The reaction was terminated by removing the solution, washing the crystals briefly in 18M Ω water and air drying.

2.8 Examination of the semi-nacre of the dorsal valve.

To examine the unique shell ultrastructure of *N. anomala* (Section 1.3), a scanning electron microscope study was performed. This study aimed to examine the structure of the valves in detail and to compare the

characteristics of the mineral structure in both chemically treated and untreated valves. It was hoped that this information would provide clues to the possible mechanisms of biomineralisation that occur in formation of the shell.

Dorsal and ventral valves of *N. anomala*, which had been cleaned of periostracum, were fractured into roughly equal fragments. The shell portions were mounted on stubs, coated with gold-palladium and the fractured edge examined using a Cambridge 360 scanning electron microscope. The effect of prolonged incubation of the dorsal valves with sodium hypochlorite was examined by incubating equally sized fragments in a commercial bleach solution (20% v/v) for 24, 48, 72 and 96 hours. Control fragments were incubated in 18M Ω water for the same length of time. The shell fragments were then prepared as detailed above and the semi-nacre examined under the scanning electron microscope.

The effect of a glutaraldehyde/acetic acid solution on the dorsal valve was also examined. Equally sized fragments of cleaned dorsal valves were incubated in a commercial bleach solution (20% v/v) for one hour to remove the protein present on the surface of the laminae. Each shell fragment was then incubated in a glutaraldehyde solution (12.5% v/v), to which acetic acid (1% v/v) had been added, for 10, 20 and 30 minutes. The acetic acid/glutaraldehyde solution dissolves the protein in between the laminae, the protein in between the sectors in each lamina and the organic accumulation in the centre of each lamina. Control fragments were incubated in 18M Ω water for the same duration. Fragments were then prepared for the scanning electron microscope as detailed above.

2.9 Amino Acid Analysis.

Amino acid analysis was performed on the total protein fractions and the intracrystalline proteins extracted from both the ventral and dorsal valves of *N. anomala*. This work aimed to determine if any differences exist in the amino acid composition of the different proteins and to possibly gain clues to the properties and characteristics of the proteins, as inferred from the amino acid composition.

Total amino acid extracts were prepared by dissolving clean shell powder in HCl (2M). Pure proteins were also analysed by excision of protein bands from PVDF membrane. A piece of membrane without bound protein was also excised and used to determine the background level of amino acids. Each PVDF membrane band was placed in a clean Eppendorf tube using forceps and washed by shaking with 1 ml of wash solution (20% v/v methanol, 0.5% v/v K₃ EDTA). The wash procedure was then repeated, using 18M Ω water.

Amino acid analysis of proteins involves three basic steps. The protein is hydrolysed and the constituent amino acids derivatised by reaction with phenylisothiocyanate (PITC). The derivatives are then separated and analysed by hplc (Matthews & van Holde, 1990).

2.9.1 Manual hydrolysis of samples for amino acid analysis.

Samples were hydrolysed by vapour phase hydrolysis with 6N HCl in Pyrex glass tubes which had been incubated at 400 °C for two hours. The tubes containing the samples were placed in glass hydrolysis bottles (see Figure 2.5) and 0.5 ml of HCl (6N) was placed in the base of each bottle. The bottles were purged with argon to minimise oxidation and then sealed, using teflon inserts inside the bottle lids. The bottles were incubated at 165 °C for one hour. After hydrolysis, the bottle lids were loosened to allow the acid vapour to escape and then left to cool. Membrane pieces were placed on the sample frits on the Amino acid analyser, Model 420A and held in place by sterile Teflon discs. Liquid samples were reconstituted in K₃ EDTA (50 mg/ml) and 2.5 μ l of each sample loaded onto the sample frits.

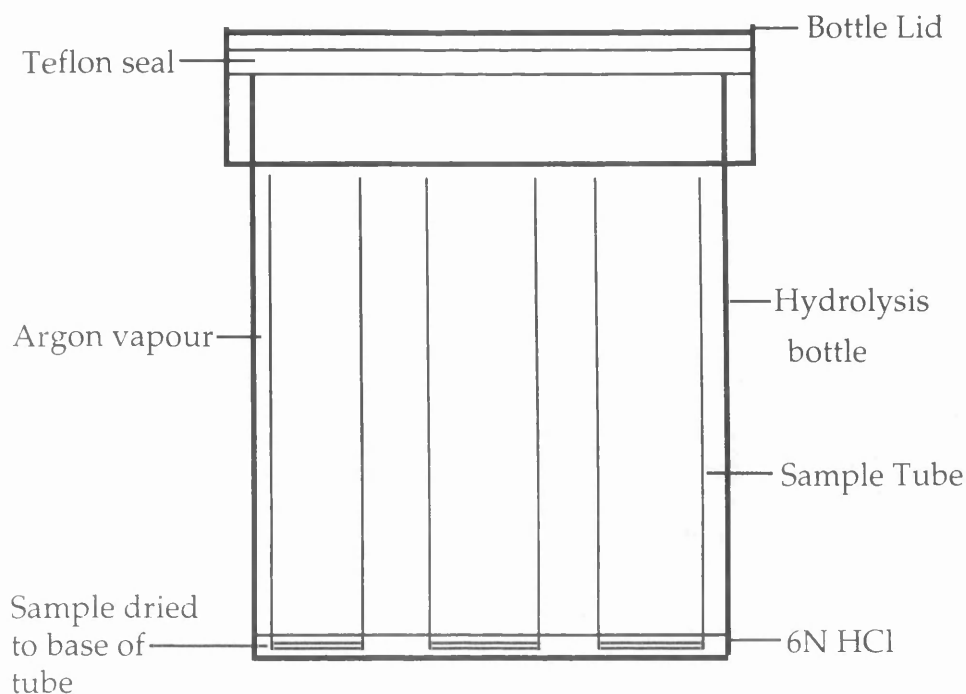


Figure 2.5 Diagram of a manual hydrolysis bottle (from Walton, 1992).

2.9.2 Amino acid analysis.

After hydrolysis of the protein, derivatization with PITC is employed in order to enable detection of amino acids by hplc, measuring absorbance at 254 nm. Derivatisation of amino acids is automated in the 410 analyser and includes the following steps. An aliquot of methanol containing K_3 EDTA (0.1%, w/v) is delivered to the reaction unit to reduce potential contamination. The base diisopropylethylamine (DIEA) is then delivered in vapour phase to neutralise any remaining acid and to ensure that the pH is optimal for the coupling process. When a free amino group is exposed to phenylisothiocyanate (PITC), it reacts quantitatively, producing phenylthiocarbamyl-amino acid (PTC-AA) derivatives, which absorb strongly at 254 nm (Matthews & van Holde, 1990). The derivatives are analysed by hplc.

2.10 Destruction of intercrystalline material over time.

One thoroughly cleaned dorsal valve was crushed to a fine powder and divided into fifteen 2 mg aliquots. 1ml sodium hypochlorite (1% v/v) was added to each tube and removed by centrifugation and repeated washing in 18M Ω water after 0, 1, 2, 3, 4, 5, 6, 7, 16, 18, 20 and 22 hours. The powders

were then dissolved in 2N HCl (11 μ l/mg) and analysed on the 420A Amino acid analyser to monitor the destruction of intercrystalline amino acids by incubation with bleach.

2.11 Determination of the N-terminal residues.

Edman degradation chemistry (Figure 2.6) not only identifies N-termini, but when used repeatedly, can allow long polypeptides to be sequenced. The procedure involves the reaction of phenylisothiocyanate (PITC) with the N-terminal group of the protein, to produce a phenylthiocarbamyl (PTC) derivative. Acid hydrolysis results in the breakage of the peptide bond between the first and second residues and the rearrangement of the PTC derivative to form a phenylthiohydantoin (PTH) derivative of the first amino acid, which can then be identified (Creighton, 1983). The process is then repeated in order to determine subsequent amino acids. This method is employed in the automated pulsed liquid protein sequencer used in this study (Perkin Elmer-Applied Biosystems, 477A Protein Sequencer).

2.11.1 Preparation of samples for sequencing.

Proteins immobilised on PVDF membrane were used in this study (see Section 2.3.3 for electroblotting procedure). The 44 kDa protein, extracted from the intracrystalline extract of the dorsal valves, was of sufficient concentration for sequencing and five bands were excised from the membrane using a clean scalpel blade. The membrane fragments were then washed thoroughly, typically five times, in membrane wash solution [methanol (20% v/v), K₃EDTA (0.5% v/v)].

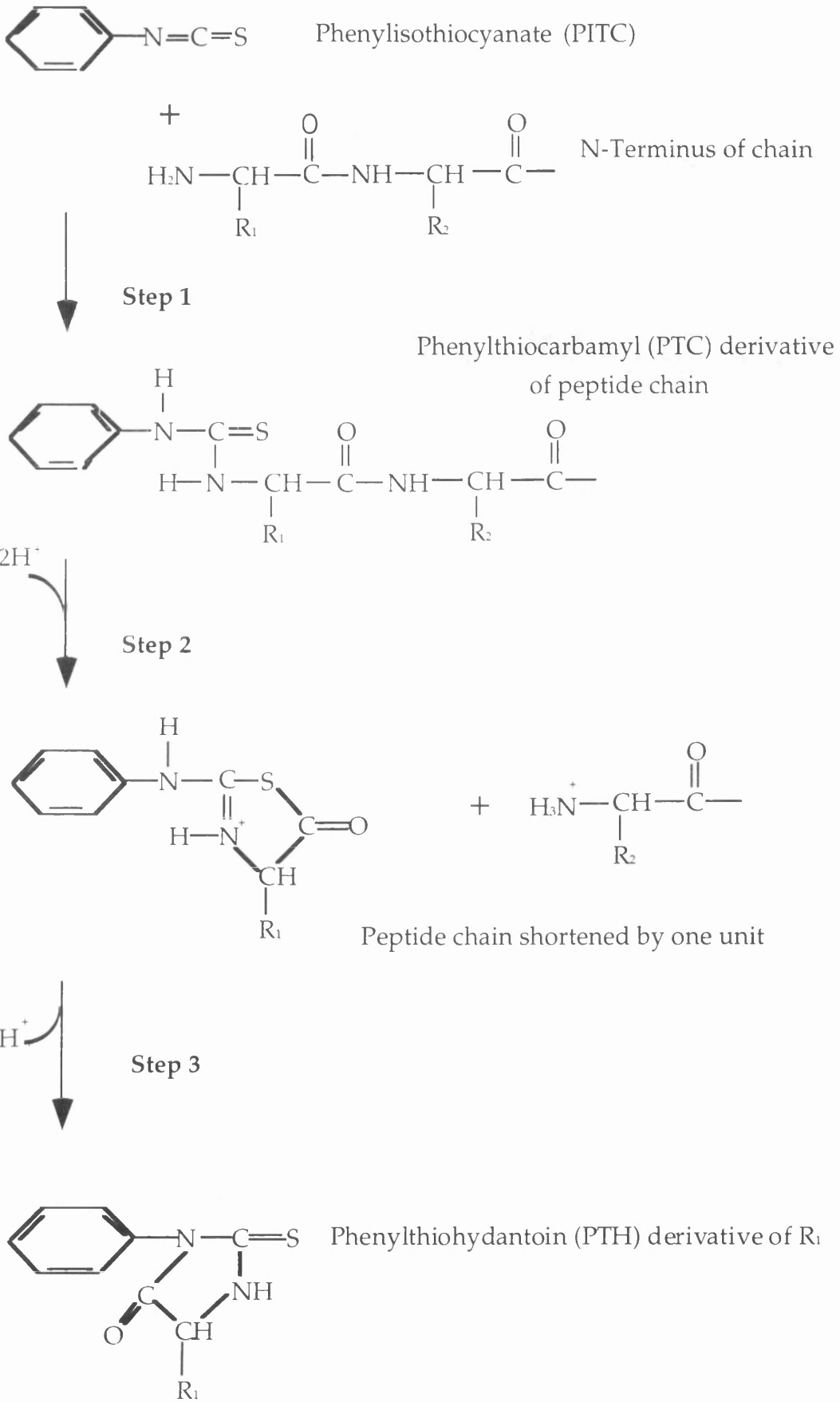


Figure 2.5 The Edman degradation sequence.

Chapter Three

Intracrystalline Proteins of *N. anomala*.

3.1 Introduction.

3.1.1 Definition of the intracrystalline fraction.

3.1.2 Use of the intracrystalline proteins in taxonomic studies..

3.2 Functions of the intracrystalline protein.

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3.3.1 Isolation of the intracrystalline fraction.

3.3.2 Distribution of amino acids in *N. anomala* valves.

3.3.2.1 Total shell protein.

3.3.2.2 The EDTA-soluble intracrystalline extract of the dorsal valves.

3.3.2.3 The 44 kDa protein of the EDTA-soluble intracrystalline extract of the dorsal valves.

3.3.2.4 The EDTA-insoluble intracrystalline extract of the dorsal valves.

3.3.3 Intracrystalline proteins of *N. anomala*.

3.3.3.1 SDS PAGE.

3.3.3.2 N-terminal sequencing.

3.3.4 Saccharide content and glycoprotein detection.

3.3.4.1 Saccharide content.

3.3.4.2 Glycoprotein detection.

3.4 Discussion.

3.1 Introduction.

3.1.1 Definition of the intracrystalline fraction.

The presence of proteins in mineralised shells is well documented (Abelson, 1956; Jope, 1967a, b, 1969 a, b, 1973, 1979; Curry *et al.*, 1990, 1991a, b; Cusack *et al.*, 1992). Such proteins may be classified in terms of their location within the shell matrix, being either intercrystalline or intracrystalline (Section 1.2.2) (Walton & Curry, 1994). Intracrystalline molecules vary widely in terms of composition and distribution within and between phyla, indicating that they may have many different functions within the shell (Lowenstam & Weiner, 1989).

The presence of organic material inside the biomaterial of mollusc shells was first reported by Watabe (1963). The studies of Crenshaw (1972) compared organic material extracted from the powdered shells of the bivalve *Mercenaria*, before and after bleaching and therefore demonstrated the existence of biomolecules intimately associated with the mineral itself. Collins *et al.* (1988) employed immunological techniques to demonstrate the existence of intracrystalline molecules within the shells of Recent brachiopods and Walton *et al.* (1993) showed, by amino acid analysis of powdered brachiopod shells, that intracrystalline material remained within the samples following oxidation of the intercrystalline fraction.

The concept of the intracrystalline fraction has come under criticism. Towe (1980) demonstrated that single rhombohedral crystals of calcite grown in silica gels could incorporate significant amounts of the gel during growth, indicating that the incorporation of large organic molecules, such as proteins, within the crystal lattice, is possible. Nevertheless, Simkiss (1986) disputed that such large macromolecules could exist as an intrinsic part of an ordered crystal structure. Subsequently, Addadi & Weiner (1989) expanded the term to include the molecules positioned at the edges of smaller segments within the crystal structure, and this definition is now widespread. The current most common method used to destroy the intercrystalline organic material is incubation with sodium hypochlorite solution (Stathoplos & Hare, 1993). The intracrystalline fraction can thus be defined as the organic fraction within the biomineral which is resistant to strong chemical oxidation (Sykes *et al.*, 1995).

The intracrystalline material may be extracted by demineralisation of the shell by the calcium chelator, ethylene diamino tetraacetic acid (EDTA). In the brachiopod shell, the intracrystalline protein consists of an EDTA-soluble and an EDTA-insoluble fraction, which differ significantly in composition (Sections 3.3.2.2 and 3.3.2.4). Collins *et al.* (1988) later demonstrated the partial solubility of the intracrystalline fraction from brachiopod shells by decalcification of the crystals in EDTA, following destruction of the intercrystalline material. Cusack *et al.* (1992) employed this method in the extraction of an intracrystalline chromoprotein from red brachiopods and the present study uses EDTA to extract the intracrystalline proteins from the shell of *N. anomala*.

3.1.2 Use of the intracrystalline proteins in taxonomic studies.

Biochemical information derived from shell proteins can be invaluable in both phylogenetic and taxonomic studies, since the amino acid compositions of the proteins reflect their genetically coded biosynthesis and are manifested in the phenotypic characteristics of the organism. Jope (1967a, b, 1969a, b, 1973) presented evidence to suggest that the amino acid compositions of brachiopod shell proteins may reflect the phylogenetic positions of the organisms within the phylum. Detailed comparative studies suggest that differences observed in the amino acid composition of the total shell protein of different brachiopod taxa confirm the morphological classification into two classes, as detailed in the Treatise of Invertebrate Paleontology, Part H (1965).

Following the proposals that an intracrystalline organic fraction may exist within the mineral units of shells, Towe (1980) suggested that the apparently chemical-resistant intracrystalline fraction may be valuable in studies of ancient protein. Entrapment of the protein within the mineral may afford the biomolecules a significant degree of protection from degradation for vast periods. Intercrystalline biomolecules decay rapidly and are therefore greatly altered over time but intracrystalline material is much more likely to have been preserved in the original state (Curry *et al.*, 1990; Walton & Curry, 1994). Any decay of the protein molecules that does occur will take place *in situ*, with the result that the decay products remain within the intracrystalline phase. Studies of Recent proteins are also likely to benefit from focusing on the intracrystalline fraction rather than on the total shell protein, since the potential for contamination is significantly reduced. Following the suggestion

of Towe (1980), Collins *et al.* (1988), using immunological techniques, confirmed that intracrystalline biomolecules exist within the shells of Recent brachiopods. Antibodies to antigenic determinants within the intracrystalline fraction were raised in both Recent and fossil brachiopods, suggesting that the intracrystalline biomolecules may also be of use as taxonomic indicators (Collins *et al.*, 1988, 1991a, b). Immunological techniques have particular application to studies of fossil specimens, since the techniques that are employed require not the entire protein molecule, but short specific fragments, which are more likely to survive fossilisation than the whole protein chain (Collins *et al.*, 1991b).

Subsequent work concentrated on the characterisation of the intracrystalline proteins from brachiopod shell. Curry *et al.* (1990) succeeded in determining the amino acid composition and N-terminal sequence of intracrystalline shell proteins extracted from a Recent articulated brachiopod. The sequence of amino acids, or primary structure, is commonly used for the biochemical comparison of proteins, since the nature and order of amino acids reflects the order of bases in the DNA of the organism and so a change in one sequence may correspond to a change in the other (Crick *et al.*, 1961). In addition, the number of proteins in a shell varies between different species and so may also be of use taxonomically (Curry *et al.*, 1991b). Degens (1967) illustrated that proteins have the potential to be useful as taxonomic indicators through the construction of a phylogenetic tree based on amino acid compositions of different proteins. Walton *et al.* (1993) used the amino acid data obtained from intracrystalline proteins extracted from the shells of several species of Recent brachiopod, to compare the phylogenetic relationships of the organisms from which the proteins were extracted. This work was subsequently extended to fossil specimens and amino acid analyses were obtained from intracrystalline material extracted from 0.5 Ma old fossil brachiopods (Walton & Curry, 1994). Such studies indicate that the fossil intracrystalline biomolecules may be of use as taxonomic indicators. Taxonomic information derived from the fossil amino acid data corresponds to the established phylogenetic classifications (Walton & Curry, 1994; Walton *et al.*, 1993).

3.2 Functions of the intracrystalline protein.

Intracrystalline molecules have many functions in the biomineralisation process, but the details of these functions and the precise mechanisms by which the intracrystalline molecules regulate mineralisation, are largely unknown (Cusack *et al.*, 1992). Although many studies have focused on the intercrystalline material (Addadi & Weiner, 1985; Aizenberg *et al.*, 1994; Belcher *et al.*, 1996; Degens, 1967; Grégoire, 1957; Iijima *et al.*, 1990), relatively few studies have centred on the intracrystalline fraction, despite the fact that it has been recognised for some time that intracrystalline proteins exist (Curry *et al.*, 1990).

Organic material is incorporated into the mineral structure during crystal formation. Whether these intracrystalline biomolecules are incorporated inadvertently or deliberately, the presence of organic material within the mineral structure influences the properties of the mineral. The majority of biomineralisation studies focus on the relationship between the proteins and the mineral phase (Addadi *et al.*, 1991; Albeck *et al.*, 1993; Belcher *et al.*, 1996; Berman *et al.*, 1988, 1990; Curry *et al.*, 1990; Cusack *et al.*, 1992). The interest in the intracrystalline fraction is due to the intimacy of the relationship between the protein and the biomineral, this close association enabling the organic constituents of the shell to play a direct role in the formation of the shell fabric (Wheeler & Sikes, 1984). The differences in the characteristics of different shell fabrics of different organisms, or even within the same organism, is likely to be due to differences in the proteins which are closely associated with the mineral phase. The regulatory property of the protein will be discussed further in Chapter 4.

3.2.1 Intracrystalline glycoproteins.

Often the proteins that regulate mineralisation are glycosylated (Crenshaw & Ristedt, 1975; Won Cho *et al.*, 1996). Glycoproteins are present within both the intercrystalline and intracrystalline fractions of the biomineral. Mineral crystals of mollusc shells grow within intercrystalline organic "envelopes" of glycoproteins (Addadi *et al.* (1991), while Berman *et al.* (1990) suggested that the presence of acidic glycoproteins within the mineral of sea urchins affects the fracture properties of the calcite crystals, thus strengthening the shell structure. Glycosylated intracrystalline proteins extracted from sea urchin and mollusc shells interact along different crystallographic axes than those which are not glycosylated, with each type affecting the fracture characteristics of the shell differently (Albeck *et al.*, 1996). Glycoproteins have also been identified within the intracrystalline organic extracts of sea urchin larvae (Berman *et al.*, 1988); brachiopods (Collins *et al.*, 1991b) and Coccolithophoridae (de Jong *et al.*, 1986).

3.3 Results.

3.3.1 Isolation of the intracrystalline fraction.

The influence of sodium hypochlorite (bleach) incubation on the amount of intercrystalline amino acids present in a sample of several powdered dorsal valves, is shown in Figure 3.1. The concentration of amino acids in the sample drops significantly after one hour and remains low thereafter. These results thus support the definition of the intracrystalline fraction, the organic fraction within the biomineral which is resistant to strong chemical oxidation. In this study, a working definition of intracrystalline protein is adopted, that being the protein which is protected from a one hour incubation with bleach (1% v/v) of powdered shell at 22°C with agitation.

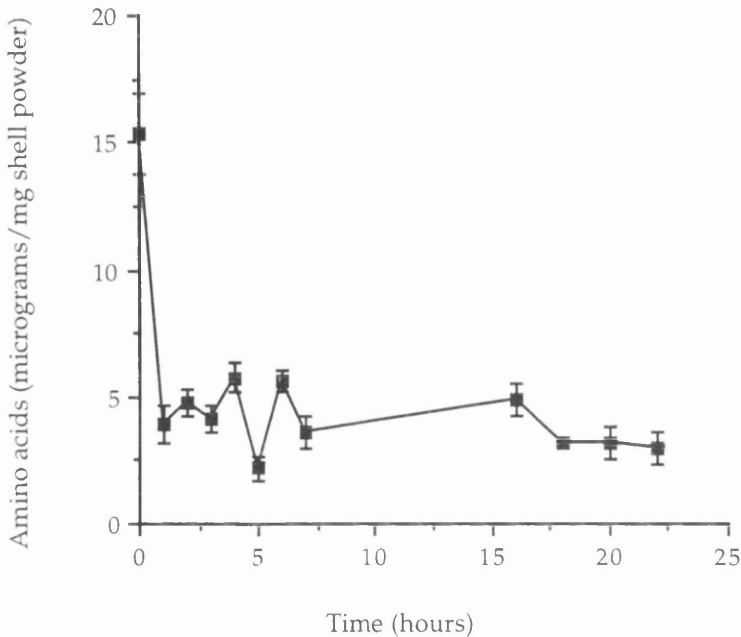


Figure 3.1 Influence of sodium hypochlorite (1% v/v) incubation on the concentration of amino acids extracted from shell powder.

Aliquots (1mg) of powdered dorsal valves were incubated with agitation in sodium hypochlorite (1% v/v). Shell powders were then washed and the peptide bonds hydrolysed (Section 2.9.1), before amino acid analysis (Section 2.9.2) of duplicate samples. The data is based on triplicate analyses from one sample set.

3.3.2 Distribution of amino acids in *N. anomala* valves.

3.3.2.1 Total shell protein.

The distribution of amino acids between intracrystalline and intercrystalline fractions of the dorsal and ventral valves was determined by amino acid analysis of samples with and without bleach treatment. Mean values of three analyses of each sample are presented in Figure 3.2, with the upper error bars displayed for each fraction.

The untreated dorsal valve has a total amino acid concentration of 17.8 $\mu\text{mol/g}$. The bleached dorsal valve sample contains 6.9 $\mu\text{mol amino acid/g}$. The concentration of intercrystalline amino acids is therefore 10.8 $\mu\text{mol/g}$. The ventral valve contains a slightly lower concentration of amino acids, 16.8 $\mu\text{mol/g}$ and the bleached powder, 3.2 $\mu\text{mol/g}$. Thus, the amount of intercrystalline amino acids in the ventral valve sample is 13.6 $\mu\text{mol/g}$.

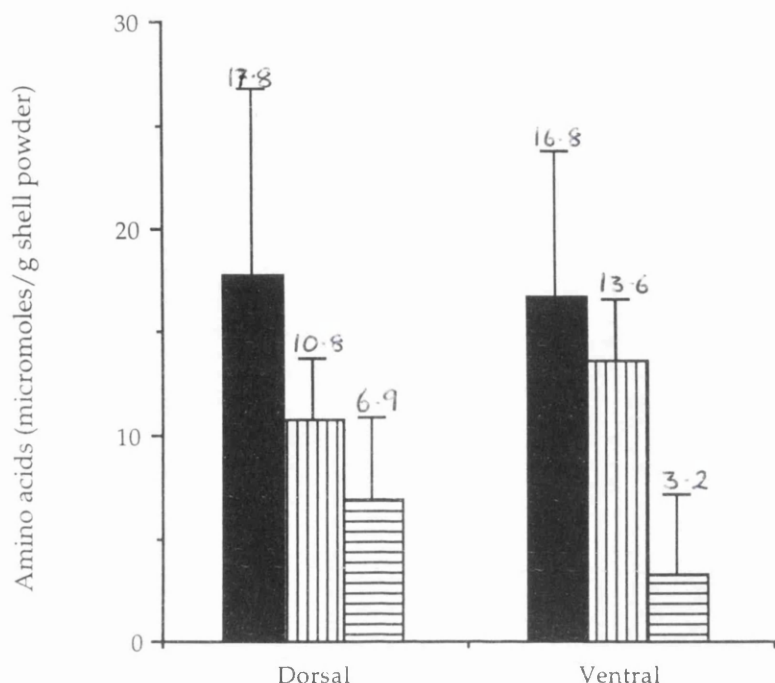


Figure 3.2 Distribution of amino acids (μ mol/g) in *N. anomala* valves.

Aliquots (1mg) of dorsal and ventral valve shell powders were incubated for one hour in sodium hypochlorite (1% v/v) for intracrystalline (▨) analyses and 18M Ω water for total (■) amino acid analyses. Amino acid values for the intercrystalline fraction (□) were estimated from the amount destroyed in the bleaching process. After washing, the peptide bonds were hydrolysed (Section 2.9.1), before amino acid analysis (Section 2.9.2). Samples were analysed in triplicate and the total error bars are shown.

The amino acid composition of the total, intercrystalline and intracrystalline fractions is presented in absolute terms in Table 3.1. Mole percentages, calculated from the absolute values, are shown in Table 3.2.

Glycine is the most common amino acid in the total and intercrystalline extracts of the dorsal valve, followed by aspartic acid/asparagine, serine and alanine respectively. The intracrystalline extract of the dorsal valves has aspartic acid/asparagine as the predominant residue, followed in order by glycine, serine and alanine. All the ventral valve extracts, the total, intercrystalline and intracrystalline analyses have aspartic acid/asparagine as the most abundant residue. The total and intercrystalline extracts have

glycine, alanine and serine as the second, third and fourth most common residues respectively. The intracrystalline extract of the ventral valves varies from the other analyses, in that proline is the third and lysine is the fourth most common amino acid. Sixty nine percent of glycine of the dorsal valve and ninety percent of aspartic acid/asparagine in the ventral valve is destroyed in the bleaching process, as indicated by the intercrystalline levels of these amino acids. With the exception of the intracrystalline extract of the ventral valves, all the extracts from both valves show similar patterns in terms of the abundance of the main amino acids. The less abundant amino acids show a wider variation in terms of abundance, especially in the ventral valve extracts.

3.3.2.2 The EDTA-soluble intracrystalline extract of the dorsal valves.

Amino acid analysis was performed on the EDTA-soluble intracrystalline extract of the dorsal valves to determine the amino acid composition of this fraction. Table 3.3 shows the amino acid composition of this fraction, as picomoles of amino acid per milligram of shell and the corresponding mole percentage values. The concentration of EDTA-soluble intracrystalline material in the shell is 0.36 μmol amino acids/mg shell. Aspartic acid/asparagine are the most abundant amino acids in the EDTA-soluble intracrystalline fraction, followed in turn by glycine, serine and glutamic acid/glutamine.

Table 3.1 Absolute amino acid composition of the total protein and the intracrystalline and intercrystalline fractions of *N. anomala*.

The values shown are in pmoles of amino acid per mg shell powder.

	D/N	E/Q	S	G	R	T	A	P	Y	V	C	I	L	F	K
Dorsal Total	4119.4	863.9	2510.1	4241.2	823.1	871.9	1293.9	672.8	218.9	692.9	22.8	292.9	708.5	266.7	247.6
Dorsal Inter	1804.1	622.7	1357	2920.3	694.9	554.2	910.6	429	194	467.5	22.8	188.8	501.4	186.8	55.4
Dorsal Intra	2315.3	241.2	1153.1	1320.9	128.2	317.7	383.3	243.8	24.9	225.4	0	104.1	207.1	79.9	192.2
Ventral Total	6577.3	521.5	955.9	3442.2	709.2	446.3	1020.4	676	68.1	578.5	21.7	274.1	494.9	220.9	768.5
Ventral Inter	5970.2	368.1	753.5	2896.6	603.7	272.7	786.7	393.1	46.2	372.1	21.7	149.2	307	123.8	510.7
Ventral Intra	607.1	153.4	202.4	545.6	105.5	173.6	233.7	282.9	21.9	206.4	0	124.8	187.9	97.1	257.8

Table 3.2 Amino acid composition (mole %) of total protein and the intracrystalline and intercrystalline fractions from *N. anomala*.

	D/N	E/Q	S	G	R	T	A	P	Y	V	C	I	L	F	K
Dorsal Total	23	4.8	14	23.7	4.6	4.9	7.2	3.7	1.2	3.8	0.1	1.6	3.9	1.5	1.1
Dorsal Inter	16.6	5.7	12.5	26.9	6.4	5.1	8.4	3.9	1.8	4.3	0.2	1.7	4.6	1.7	?
Dorsal Intra	32.6	3.4	16.3	18.6	1.8	4.5	5.4	3.4	0.4	3.2	0	1.5	2.9	1.1	3.5
Ventral Total	39.2	3.1	5.7	20.5	4.2	2.7	6.1	4	0.4	3.4	0.1	1.6	2.9	1.3	4.6
Ventral Inter	44	2.7	5.6	21.4	4.5	2	5.8	2.9	0.3	2.7	0	1.1	2.3	0.9	3.8
Ventral Intra	18.9	4.8	6.3	17	3.3	5.4	7.3	8.8	0.7	6.4	0.1	3.9	5.9	3	8.1

3.3.2.3 The 44 kDa protein of the EDTA-soluble intracrystalline extract of the dorsal valves.

The amino acid composition of the 44 kDa protein was determined by excising an electroblotted protein band from PVDF membrane and subjecting the protein to amino acid analysis (Section 2.9). The amino acid composition of the 44 kDa protein is shown graphically in Figure 3.3. The absolute values of amino acids, as picomoles of amino acid per microgram of EDTA-soluble intracrystalline material and the corresponding mole percentages are presented in Table 3.4. The most abundant amino acid comprising the 44 kDa protein is aspartic acid/asparagine, followed in turn by glutamic acid/glutamine, glycine and serine.

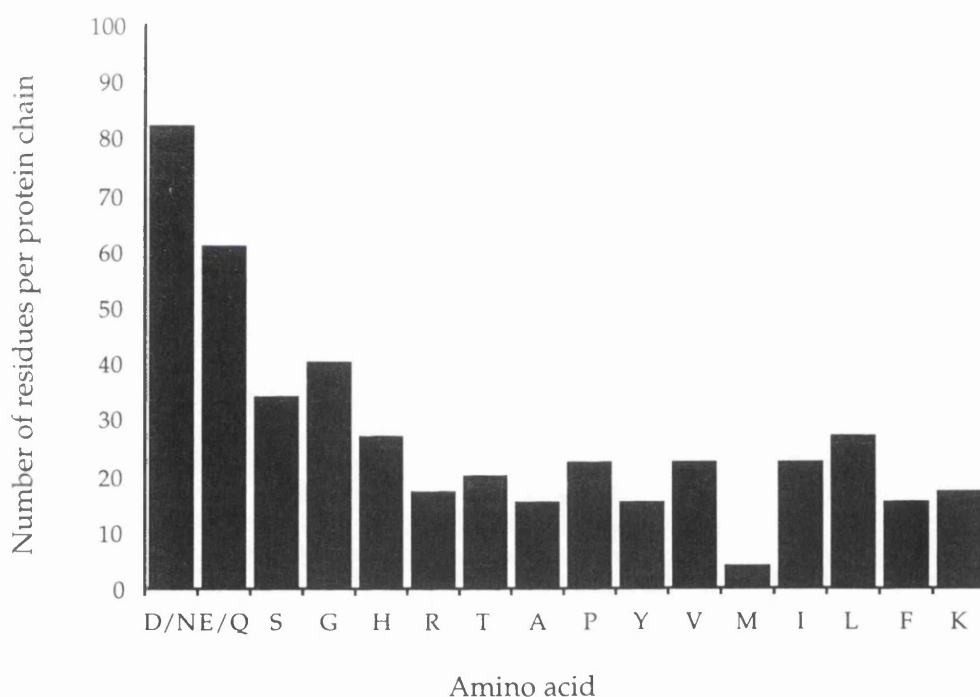


Figure 3.3 Amino acid composition of the 44 kDa intracrystalline protein from the dorsal valve of *N. anomala*.

Membrane bound protein bands were excised and the protein hydrolysed as detailed in Section 2.9.1, after which the amino acid composition was determined (Section 2.9.2) and the number of each amino acid present per protein molecule calculated.

Table 3.3 Absolute and mole % values for the EDTA-soluble intracrystalline fraction of the dorsal valve of *N. anomala*.

Absolute values shown are in pmoles amino acid/mg shell.

	D/N	E/Q	S	G	H	R	T	A	P	Y	V	C	I	L	F	K
Absolute	191.3	24.6	48.3	52.1	5.5	2.3	6.5	9.3	1.9	0.9	3.8	6.6	1.7	2.8	1.2	2.8
Mole %	52.8	6.8	13.3	14.4	1.5	0.6	1.8	2.6	0.5	0.2	1	1.8	0.5	0.8	0.3	0.8

Table 3.4 Absolute values and mole percentages for the amino acid composition of the 44 kDa protein of the intracrystalline fraction of the dorsal valve.

Absolute values shown are in pmoles amino acid/ μ g EDTA-soluble intracrystalline material.

	D/N	E/Q	S	G	H	R	T	A	P	Y	V	I	L	F	K	M
Absolute	41.8	31.3	17.2	20.5	13.6	8.8	10.2	7.7	11.2	7.7	11.3	11.2	13.9	7.8	8.6	2
Mole %	18.5	13.9	7.6	9.1	6	3.9	4.5	3.4	4.9	3.4	5	4.9	6.2	3.5	3.8	0.9

3.3.2.4 The EDTA-insoluble intracrystalline extract of the dorsal valves.

Amino acid analysis was carried out on the EDTA-insoluble intracrystalline extract of the dorsal valves to allow a comparison of this fraction with the amino acid composition of the EDTA-soluble fraction of the same valves. Table 3.5 shows both the concentration of amino acids present in the EDTA-insoluble intracrystalline fraction of the dorsal valve, in absolute terms (pmoles of amino acids/mg shell) and as mole percentages. The total amount of amino acids present in the insoluble fraction per shell weight is significantly higher than in the soluble fraction. In comparison to a value of 0.36 μmol amino acids/mg shell for the soluble fraction, the insoluble fraction contains 144.5 μmol amino acids/mg shell for the same valve, around four hundred times more than for the soluble sample. The insoluble fraction has a different amino acid composition than the EDTA-soluble fraction. Arginine is the most common amino acid in the insoluble protein, followed by glycine, aspartic acid/asparagine and phenylalanine respectively.

Table 3.5 Absolute values and mole percentages for the amino acid composition of the EDTA-insoluble intracrystalline fraction of the dorsal valve.

Absolute values shown are in pmoles amino acid/mg shell.

	D/N	E/Q	S	G	H	R	T	A	P	Y	V	M	C	I	L	F	K
Absolute	17085	6459	15914	20649	1301	32333	3553	4612	11574	578	11316	469	9387	1327	7979	16471	1429
Mole %	10.5	4	9.8	12.7	0.8	19.9	2.2	2.8	7.1	0.4	7	0.3	5.8	0.8	4.9	10	0.9

3.3.3 Intracrystalline proteins of *N. anomala*.

3.3.3.1 SDS PAGE.

The total protein extract from both valves of *N. anomala* was fractionated using SDS PAGE (Section 2.3.1) and the separated proteins visualised using one of two staining techniques (Section 2.3.2). The intracrystalline extract from the dorsal and ventral valves and the total (intracrystalline and intercrystalline) extract from the dorsal valve contains a 44 kDa protein (Figure 3.4). In very concentrated dorsal intracrystalline samples, a protein of 60 kDa is evident (Figure 3.5). When a sample of the dorsal intercrystalline fraction, extracted by guanidine hydrochloride (Section 2.2.3), was applied to SDS PAGE, no discernible protein bands could be seen, just a smear of material in the gel (Figure 3.6).

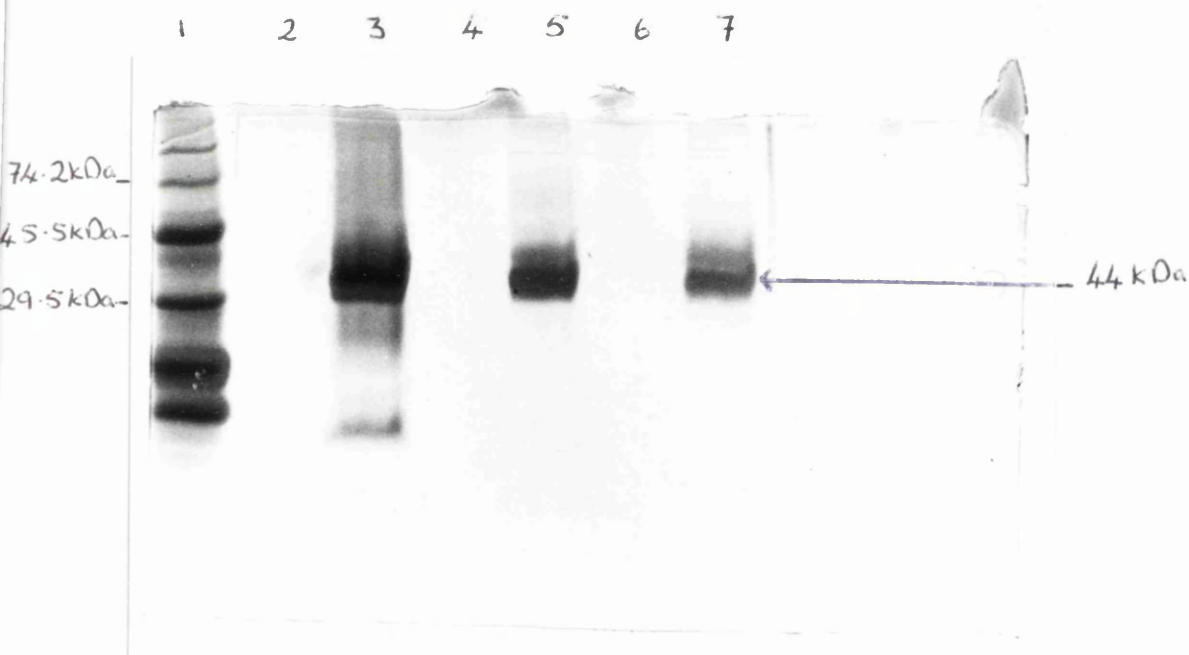


Figure 3.4 SDS PAGE fractionation of mineral-associated proteins of *N. anomala*.

Proteins were prepared as detailed in Section 2.2.1. and 2.2.3. and revealed by staining with Coomassie Blue (CBB 250), followed by silver nitrate (Section 2.3.2.). Prestained molecular weight standards (Section 2.3.1) (lane 1), dorsal intracrystalline extract (7 μ g) (lane 3), dorsal total protein extract (4 μ g) (lane 5) and ventral intracrystalline extract (3 μ g) (lane 7).

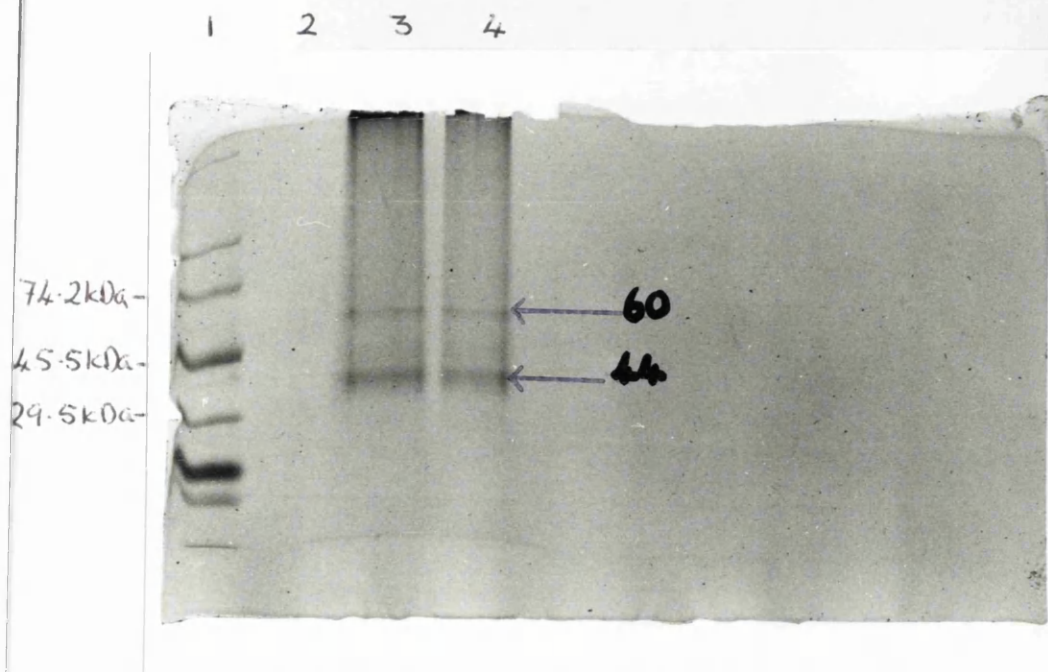


Figure 3.5 SDS PAGE analysis of the intracrystalline proteins of the dorsal valve of *N. anomala*.

Proteins were revealed by staining with Coomassie Blue (CBB 250). Prestained molecular weight standards (Section 2.3.1) (lane 1), *N. anomala* protein extract (5 μ g) (lane 3).

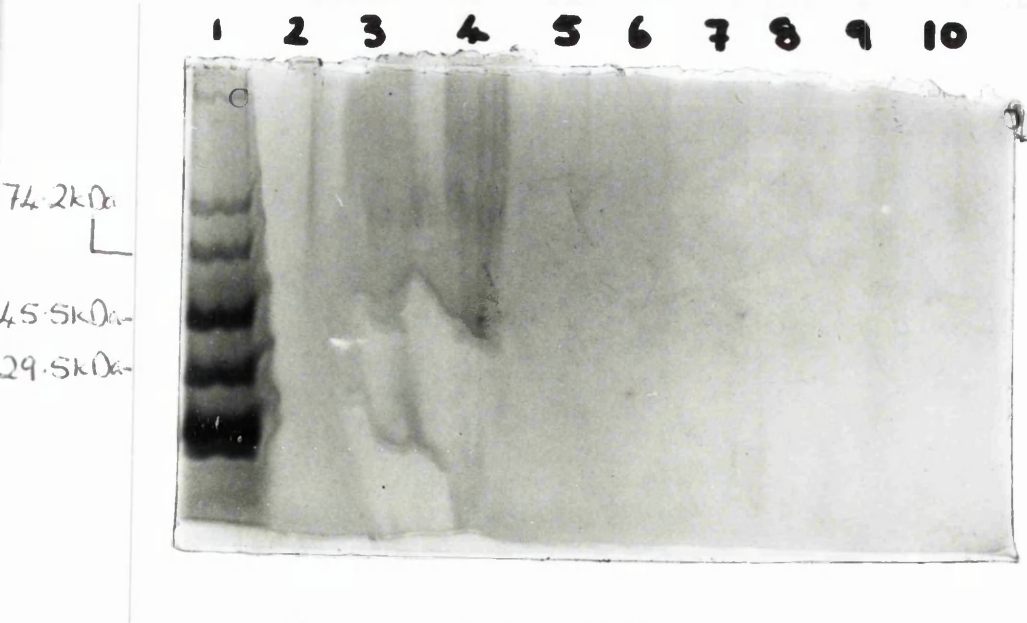


Figure 3.6 SDS PAGE analysis of the intercrystalline proteins of the dorsal valve of *N. anomala*, extracted by GuHCl.

The gel was stained with Coomassie Blue (CBB 250). Prestained molecular weight standards (lane 1), *N. anomala* protein extract

(5 μ g) lanes 3 to 10.

3.3.3.2 N-terminal sequencing.

Characterisation of a protein by N-terminal sequencing may allow identification of the protein, permits a comparison with proteins of a similar sequence and may provide clues to the structure of the protein molecules. The 44 kDa protein was selected for N-terminal sequencing (Section 2.11). The partial N-terminal amino acid sequence of the 44 kDa protein is shown below:

5	10	15	19
VHIRK	EQEQ?	YIPDF	GPHQ

This partial N-terminal sequence does not match any previously sequenced brachiopod protein, such as the intracrystalline chromoprotein sequenced by Cusack *et al.* (1992). Enzymic cleavage of the protein chain is required before further sequencing can be carried out to obtain a more complete sequence for this particular protein.

3.3.4. Saccharide content and glycoprotein detection.

3.3.4.1 Saccharide content.

To determine the amount of saccharide present in the intracrystalline fraction, a total sugar assay was carried out, using sulphuric acid to hydrolyse the sample and orcinol to facilitate the colourimetric reaction. Figure 2.3 shows the standard curve established for glucose, against which ovalbumin (glycosylated), bovine serum albumin and ribonuclease A (non-glycosylated) and the *N. anomala* protein extracts were compared. Figure 3.7 shows a set of representative absorbance readings at 420 nm, of the *N. anomala* samples, ovalbumin, BSA and ribonuclease A, as compared to the glucose standard.

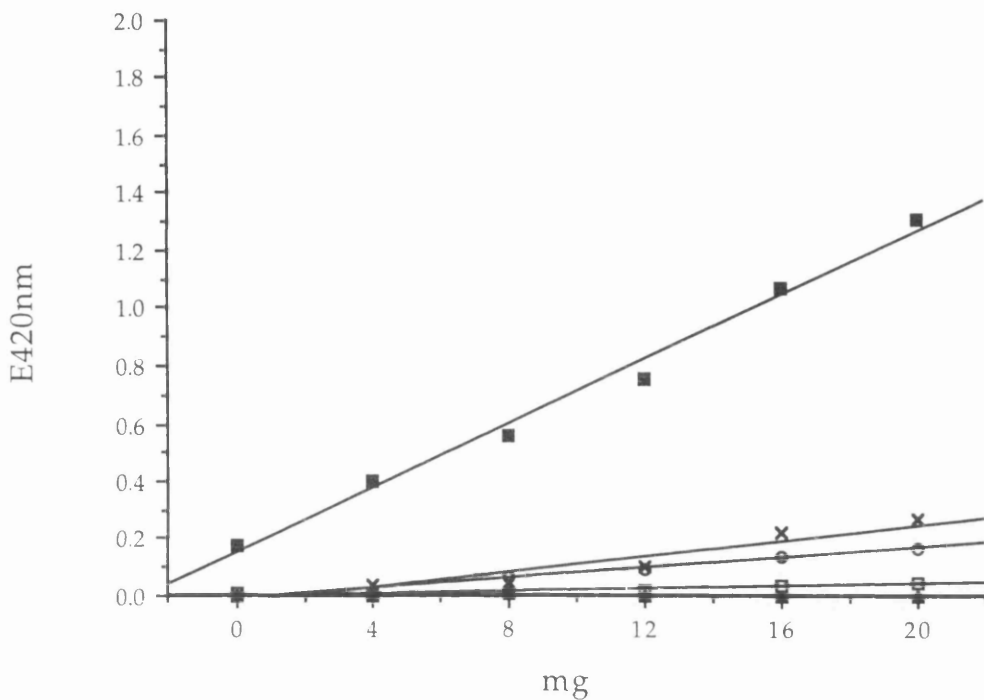


Figure 3.7 Absorbance ($E_{420\text{nm}}$) for the EDTA-soluble intracrystalline extract of *N. anomala* dorsal valves, ovalbumin, ribonuclease and BSA, as compared to glucose.

*A range of concentrations between 0 and 20 $\mu\text{g}/\mu\text{l}$ were used for each sample. The glucose standard (■) and the ovalbumin (○) (glycosylated), bovine serum albumin (▲) and ribonuclease A (□) (non-glycosylated) have a concentration of 1 $\mu\text{g}/\mu\text{l}$, while the *N. anomala* extract (×) has a protein concentration of 0.48 $\mu\text{g}/\mu\text{l}$. The correlation coefficients for glucose, ovalbumin, ribonuclease, BSA and *N. anomala* extract were 0.988, 0.969, 0.884, 0.017 and 0.920 respectively.*

Reaction with orcinol gives a coloured product, which gives an absorbance of approximately 0.153 AU at 420nm. This value was subtracted from the E_{420} readings obtained for each sample. Bovine serum albumin and Ribonuclease A are unglycosylated and therefore do not absorb in this assay. The positive control, ovalbumin, contains 8.5 ng carbohydrate per μg protein. The EDTA-soluble intracrystalline extract from *N. anomala* has 28 ng carbohydrate associated with each μg of protein. Typically 26.4 μg of intracrystalline protein was extracted from 1g of shell and so the EDTA-soluble intracrystalline fraction of the dorsal valves has 0.74 μg carbohydrate per gram of shell.

3.3.4.2 Glycoprotein Detection.

Although the intracrystalline extract contains carbohydrate, the 44 kDa protein does not react with Concanavalin A and is therefore not glycosylated, whilst the 60 kDa protein, ovalbumin and osteonectin do react with Concanavalin A and are therefore glycosylated [Figure 3.8 (b)].

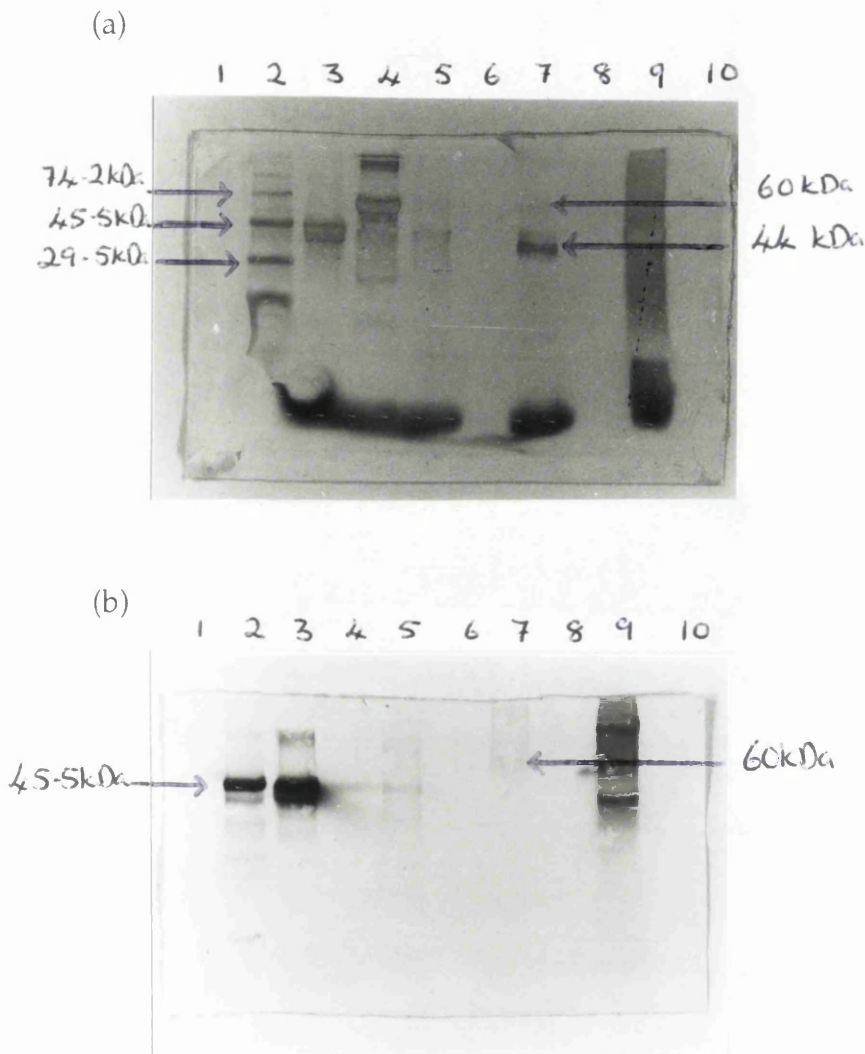


Figure 3.8 Duplicate membranes (a) stained with Coomassie Blue R-250 dye and (b) tested with Concanavalin A.

Duplicate gels were prepared and the samples electroblotted as described in Section 2.3.3. Ovalbumin and Osteonectin (both 5 μ g) (lanes 3 and 5 respectively) and a negative control, Bovine serum albumin (5 μ g) (Lane 4) were included, alongside an EDTA-soluble extract from *N. anomala* (1 μ g) (lane 7). An EDTA-soluble intracrystalline protein extract from the brachiopod *Terebratulina retusa* was also included (4.8 μ g) (lane 9), for comparative purposes. One membrane was stained with CBB (Section 2.3.3.1), to reveal all proteins present [Figure 3.8 (a)], while the other membrane was treated with Concanavalin A (Section 2.4) [Figure 3.8 (b)].

3.4 Discussion

Previous studies have indicated that the proteins of the intracrystalline fraction have many different functions within the mineralised shell (Addadi & Weiner, 1985; Aizenberg *et al.*, 1994, 1997; Albeck *et al.*, 1996; Berman *et al.*, 1988, 1990; Cusack *et al.*, 1992; Falini *et al.*, 1996), although the precise mechanisms by which the proteins fulfil their roles have yet to be elucidated. Figures 3.4 and 3.5 show that, for *N. anomala*, the 44 kDa protein occurs in the intracrystalline fractions of both the dorsal and ventral valve. Some or all of the 44 kDa protein may be inadvertently integrated into the mineral crystals during growth of the shell, possibly by overgrowth of the protein by the mineral front, as described by Lowenstam & Weiner (1989) in the molluscan shell. Since the extraction of intercrystalline protein was unsuccessful, it is impossible to state whether or not the 44 kDa protein also occurs between the mineral crystals as well as within them. In the total extracts of the dorsal valve and the intracrystalline ventral valve extracts, only the 44 kDa protein is evident. The absence of the 60 kDa protein in the total extracts from the dorsal valve probably indicates that these particular samples are of insufficient concentration to visualise this protein.

Certain features of mineralised materials have been attributed to the presence and action of glycoproteins. Several studies have focused on the possible role of glycoproteins in strengthening the shell by incorporation of the glycoprotein into the crystal lattice. The presence of proteins within the ordered mineral structure increases the strength and subsequent flexibility of the mineral, by altering the plane of fracture within the mineral (Berman *et al.*, 1990; Albeck *et al.*, 1996). However, all mineral-associated proteins may have this property, not only glycoproteins. An enhanced flexibility may be valuable in mineralised structures such as sea urchin spines, which are each composed of a single calcite crystal, making the mineral very brittle as a consequence. The presence of a glycoprotein inside the mineral lattice increases the strength and flexibility of the spine (Berman *et al.*, 1988). In the present study, the 44 kDa protein is not glycosylated and comprises most of the EDTA-soluble protein extract, so the carbohydrate detected by the orcinol assay must derive, in part, from the glycosylated 60 kDa protein and/or free carbohydrate within the intracrystalline extract. The 60 kDa protein may be exclusive to the dorsal intracrystalline fraction. All of this particular protein may have been incorporated into the interior of the mineral during shell growth. The fact that the 60 kDa protein is glycosylated suggests that this

protein may have a different function within the shell of *N. anomala* than the non-glycosylated 44 kDa protein. In being occluded within the mineral structure, the 60 kDa glycoprotein may be able to modify the fracture properties of the mineral and therefore the shell itself, as demonstrated in the spines of sea urchins (Berman *et al.*, 1988; Albeck *et al.*, 1996). The non-glycosylated 44 kDa protein may have a different function. As the 44 kDa protein is present in both the dorsal and the ventral valves, the protein is likely to have a similar function in both locations.

One of the first steps in functional studies of a shell protein is an analysis of the amino acid composition and the primary structure. The EDTA-soluble fraction of the dorsal valves has a higher total protein mass per shell weight than the same fraction of the ventral valves. If the protein has a role in the biomineralisation of the shell, then it would be reasonable to assume that the dorsal valve may require a higher total protein content than the ventral valve, as the dorsal valve is larger and more mineralised. On examination of the absolute values for the bleached samples, which represent the intracrystalline protein fraction, there is more than twice the amount of protein in the intracrystalline fraction of the dorsal than the same fraction of the ventral valve. The higher intracrystalline protein content of the dorsal valve may be explained by the different structure of the mineral units in each of the locations. The laminar calcite of the secondary dorsal valve may be able to incorporate more protein into the intracrystalline domain than the acicular calcite of the ventral valve.

Amino acid analyses of whole protein extracts were performed by Jope (1967a) in an attempt to determine the phylogenetic relationships between the main classes of the Brachiopoda. The high levels of glycine and moderate alanine obtained from analyses of the total protein extract in the present study confirm results obtained by Jope (1967a) for the total protein extract of *Crania*, suggesting that the Craniida have an affinity with the calcitic-shelled, articulated brachiopods, whereas the presence of chitin in the craniid shell suggests a relationship with the phosphatic-shelled, inarticulated brachiopods. The high levels of serine obtained in the present study and that of Jope (1967a) are not in accordance with analyses of any other brachiopod group, or with members of any other phylum. The shell protein of the craniid brachiopods, therefore, shows characteristics of both the carbonate-shelled articulates and the phosphatic-shelled inarticulates. The equivocal

relationship of the craniids with the lingulid and rhynchonellid clades, as illustrated in Figure 3.9, reflects their affinities with both groups (Williams *et al.*, 1996). A clade comprising the lingulids and the craniids would be characterised by synapomorphies such as a circumferential mantle cavity, the presence of oblique and two pairs of adductor muscles, and the presence, in early development, of a transient median lophophore tentacle. A rhynchonellid clade containing the craniids would be characterised by a protein-containing calcitic shell, a single row of tentacles on trocholophous lophophores, gonads located in the mantle sinuses and lecithotrophic larvae (Williams *et al.*, 1996). Consideration of craniids as being more closely related to either the lingulid or rhynchonellid groups depends therefore, on the choice of outgroup.

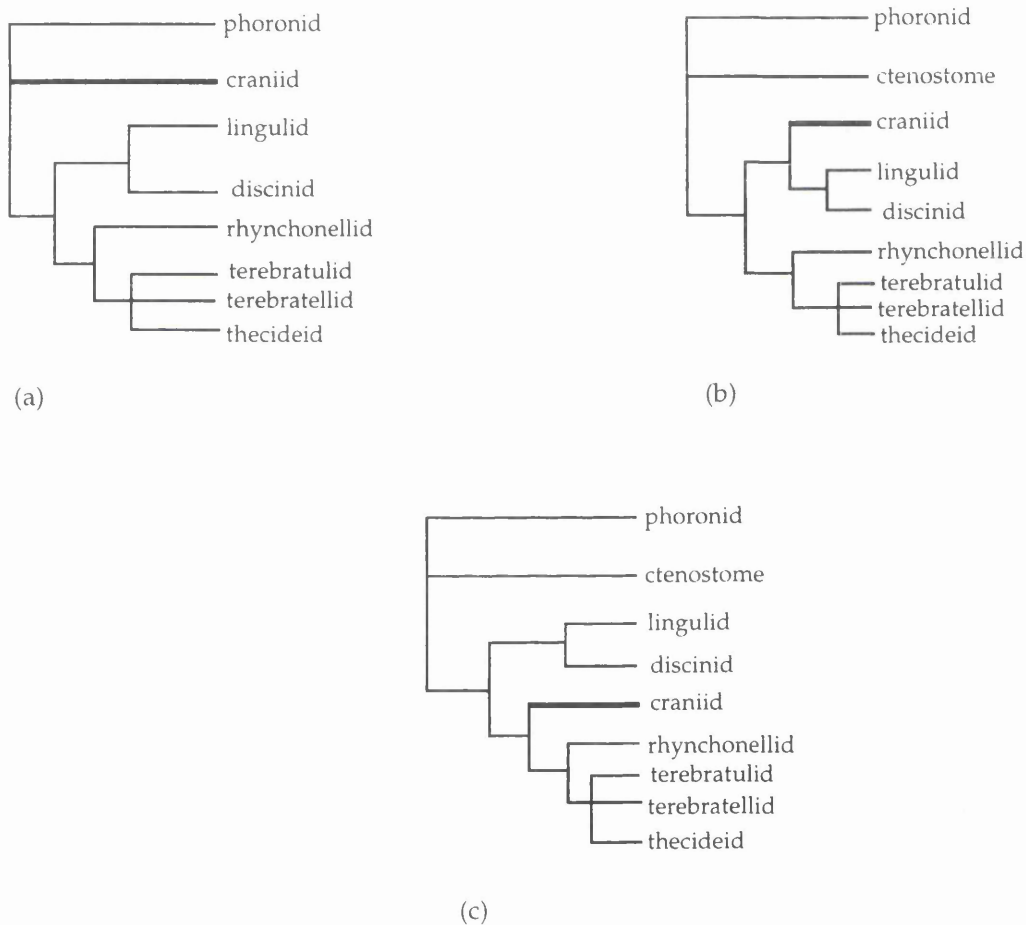


Figure 3.9 Comparison of the taxonomic position of the craniid brachiopods when different lophophorate outgroups are used.

(a) shows the location of the craniid branch when a phoronid outgroup is used; (b) and (c) illustrate the two possible craniid positions when phoronids and ctenostomes are used as outgroups (after Williams *et al.*, 1996).

The high levels of glycine and serine in such shell protein analyses may be indicators of possible contamination from human fingertips (Walton & Curry, 1991). However, the bleach incubation (Section 3.3.1) indicates that most intercrystalline amino acids are destroyed in the first hour of bleaching with a dilute (1 %, v/v) sodium hypochlorite solution (Figure 3.1).

The high levels of aspartic acid/asparagine and glutamic acid/glutamine of the 44 kDa protein suggests that the protein may have calcium-binding potential, but only if these residues are in the acidic form (Weiner, 1979; Crenshaw, 1972). If the protein is able to bind calcium cations, it may play an important role in shell formation. The possible calcium-binding property of the protein is discussed further in Chapter 4.

The amino acid composition of the EDTA-insoluble fraction of *N. anomala* is profoundly different from that of the EDTA-soluble fraction. The EDTA-insoluble organic fractions of different mineralised structures are diverse in terms of the amino acid compositions, although the proteins tend to be hydrophobic (Crenshaw, 1972). The insoluble organic fraction of molluscs is rich in the amino acids glycine and alanine, consistent with the amino acid composition of the insoluble fraction of *N. anomala* (Meenakshi *et al.*, 1971). The relatively high proportion of phenylalanine in the insoluble fraction of the dorsal valve of *N. anomala* also resembles the analyses of molluscan shell (Meenakshi *et al.*, 1971). The insoluble fraction contributes to the mechanical strength of the shell, and is not directly involved in the formation of the biomineral (Weiner & Traub, 1984; Weiner *et al.*, 1983).

Chapter Four

Influence of Protein on Synthetic Calcite Formation

- 4.1 **Crystal Formation and Growth.**
 - 4.1.1 Nucleation.
 - 4.1.2 Growth and morphology.
 - 4.1.3 Calcification *in vitro*.
- 4.2 **Results.**
 - 4.2.1 Mineral-associated proteins from *N. anomala*.
 - 4.2.2 *In vitro* calcium carbonate mineralisation.
 - 4.2.2.1 Slow calcite precipitation.
 - 4.2.2.2 Fast calcite precipitation.
 - 4.2.3 Identification of calcium-binding potential.
- 4.3 **Discussion.**

Inorganically formed minerals are usually regular in shape and formed from a supersaturated solution of the relevant ions (Simkiss & Wilbur, 1989). Nature, however, produces minerals that exhibit a wide variety of shapes and sizes, in order to fulfil the function for which they are intended (Aizenberg *et al.*, 1994). Biominerals can assume either crystalline or amorphous forms. Amorphous solids have no ordered structure, whereas crystalline materials exhibit an ordered geometrical arrangement of the constituent ions (Simkiss, 1990). A crystal is composed of a repeating pattern of identical units in three dimensions (Simkiss & Wilbur, 1989). The crystallographic axes of a crystal are aligned with respect to each other (Lowenstam, 1981). Calcite crystals can form intracellularly or extracellularly, depending on the organism involved (Watabe, 1981). In certain organisms, crystal formation is initiated intracellularly and the crystal is later deposited outside the cell (Kingsley & Watabe, 1984).

Of the many biogenic minerals known, two thirds are calcium minerals (Lowenstam, 1981). Of these, the majority are calcium carbonate, which can exist in three polymorphic forms, calcite, aragonite and vaterite. Calcite is the most stable and least soluble form. The three polymorphs of calcium carbonate consist of a regular repeating pattern of CaCO_3 atoms in three dimensions (Simkiss & Wilbur, 1989) (Figure 4.1). Within the three polymorphs, the calcium ions are in almost identical positions. The arrangement of the carbonate ions, however, differs between each form (Lippmann, 1973).

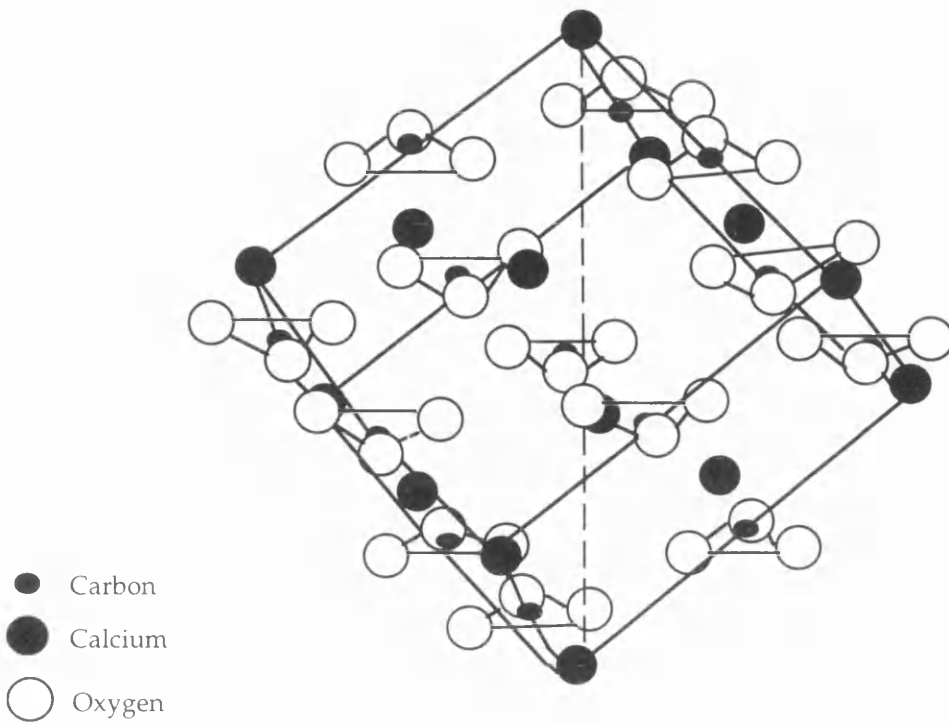


Figure 4.1 Structure of calcite, magnified approximately 10^8 times
(from Simkiss & Wilbur, 1989).

Protein makes up the major proportion of the mineral-associated organic material, although small amounts of lipids, alcohols, carbohydrates and nucleic acids are also present (Weiner *et al.*, 1983; Curry *et al.*, 1991b; Collins *et al.*, 1991b; Cohen *et al.*, 1991). The protein component of mineralised structures such as shells is fundamental to the formation of the biomineral (Wilbur, 1984; Weiner, 1979). Through intimate contact with the mineral phase, the associated proteins can exert a very high degree of control over the process of mineral formation (Paine & Snead, 1997; Belcher *et al.*, 1996; Berman *et al.*, 1988, 1993; Aizenberg *et al.*, 1994). The mineral-associated proteins may control many aspects of biomineral formation, such as crystal nucleation, the polymorph produced, crystal orientation, crystal growth direction and crystal growth inhibition (Wilbur, 1976, 1984).

4.1 Crystal formation and growth.

Within biological systems, certain factors may influence the formation of crystals. These include, the presence of relevant inorganic ions, such as hydrogen and hydroxide, plus carbon dioxide (Kitano, 1962), non-protein organic components (Grégoire, 1957), certain enzymes (Wilbur & Jodrey, 1955) and protein material in close association with the shell (Watabe, 1965), and temperature (Lowenstam, 1954). Formation of new crystals from solution involves two steps, crystal nucleation and crystal growth (Watabe, 1981). Mineral-associated proteins may affect the nucleation, growth and therefore the morphology of crystals.

4.1.1. Nucleation.

Nucleation occurs by the aggregation of molecules, atoms or ions until a stable lattice structure is formed (Mann, 1983). In inorganic mineralisation, the concentration of ions in solution is an important factor in the nucleation process (Watabe, 1981). As the local ion concentration increases, ions form temporary aggregates. These accumulations persist if bonds form between the ions and a crystal nucleus is produced (Simkiss & Wilbur, 1989). Biological mineralisation however, is less dependent on ion concentration and is energetically more favourable than the equivalent inorganic processes, due to the presence of mineral-associated proteins. These proteins may induce mineral nucleation by providing a surface on which crystals can begin to form and by allowing bonds to form between the organic and inorganic components (Simkiss & Wilbur, 1989). Calcite crystals form on organic material extracted from a wide range of organisms, such as molluscs (Wilbur & Watabe, 1967; Albeck *et al.*, 1993, 1996); corals (Kingsley & Watabe, 1984); sea urchins (Addadi *et al.*, 1991) and marine algae (Westbroek *et al.*, 1984). Nucleation occurs only when the protein is adsorbed onto a rigid surface, whereas proteins in solution inhibit crystal growth by binding to an already formed crystal face (Addadi & Weiner, 1985).

There are several theories which attempt to explain how the mineral-associated proteins induce nucleation. One such theory is ionotropy (Crenshaw & Ristedt, 1975). In this model, the calcium ions bound by the calcium-binding residues of the protein, which is fixed at specific mineralisation sites, attract carbonate anions, which are predominant in sea water. The high local concentration of calcium and carbonate ions induces

nucleation. It is likely that the initial product is amorphous, as the ionotropic theory does not require that the bound calcium matches a crystallographic lattice. In the first stages of biomineralisation, the formation of amorphous minerals may be energetically more favourable than the formation of crystalline forms (Mann, 1983).

A higher degree of control over the nucleation process is implied by the theory of epitaxy, which involves a lattice match between the calcium-binding sites of the protein and the calcium ions (Mann, 1983; Weiner & Traub, 1984; Wheeler & Sikes, 1984). Newly formed crystals show a preferred orientation and so the subsequent growth of the crystal may also be governed by epitaxial processes. Therefore, the crystallographic structure of the mineral is also determined by the template (Mann, 1983; Weiner & Traub, 1984). Theories such as epitaxy and ionotropy involve the presence of specific nucleation sites, such as calcium-binding areas of the protein (Weiner, 1979). Studies of a wide variety of organisms have demonstrated that acidic proteins extracted from the EDTA-soluble fraction are often in intimate contact with the mineral phase (Addadi & Weiner, 1985; Lowenstam & Weiner, 1989). These proteins have a high number of aspartic and/or glutamic acid residues (Weiner & Hood, 1975; Weiner, 1979; Meenakshi *et al.*, 1971; Falini *et al.*, 1996). The carboxyl groups of these acidic residues bind calcium, to which carbonate ions are subsequently attracted, inducing nucleation of the crystals (Cariolou & Morse, 1988). However, Crenshaw (1972) suggested that most of the carboxylic amino acid residues of the shell organic material may be present in the amide form and so could not bind calcium. Crenshaw suggests an alternative method of calcium binding, exemplified by the intracrystalline glycoprotein of the clam *Mercenaria*, which specifically binds calcium by two adjacent polysaccharide chains. A later study by Cariolou & Morse (1988) however, using a cationic carbocyanine dye, detected calcium-binding groups within a deglycosylated chain of a glycoprotein extracted from mollusc shells, suggesting that calcium binds to aspartic acid residues of the protein rather than to attached carbohydrate groups.

The stereochemical complementarity between the organic and inorganic phases inferred in epitaxial theories may be influenced by the secondary structure of the protein (Addadi & Weiner, 1985). Weiner & Hood (1975) proposed that a β -sheet conformation of the aspartic acid-rich protein component of mollusc shells would fulfil the spatial requirements of calcium

carbonate binding to aspartic acid residues. At least part of the EDTA-soluble protein fraction of molluscs is in the β -sheet conformation (Addadi & Weiner, 1985). Complementarity of atomic distances would therefore induce nucleation, whilst mis-matches would prevent it (Weiner & Traub, 1980a, b).

4.1.2. Growth and morphology.

The growth of crystals occurs by the addition of relevant ions to various crystal faces. This process governs the final morphology of the crystal (Mann, 1983). The surface of a growing inorganic crystal contains steps, which possess a higher binding energy than the rest of the crystal. Ions are thus attracted to those sites and the steps gradually fill in, making the crystal more uniformly shaped (Simkiss & Wilbur, 1989). In general, such growth can be regulated by the local ion concentration and the presence of co-precipitating ions, but interaction of the mineral with closely associated protein molecules will provide a higher level of control over the growth process (Mann *et al.*, 1993). The degree of control provided by the protein can vary from very little, such as in the provision of organic chambers in restricting crystal size and shape, to a great deal, as in the control of mineral structure and crystallographic orientation (Weiner, 1986; Mann, 1988). The pattern by which ions are added to the developing crystal is regulated by both the initial nucleation pattern and also subsequent effects imposed on the crystal structure by the mineral-associated proteins (Greenfield *et al.*, 1984). However, the molecular interactions involved in crystal growth processes are not clearly understood.

Inorganic and biogenic crystals often have profoundly different morphologies. Inorganic calcite crystals grown *in vitro*, with no additives, have an ordered rhombohedral shape, while crystals grown in the presence of organic macromolecules display a very different morphology. Crystal growth will continue as long as there are sufficient precursor ions in solution and growth sites on the crystal surface remain free of associated protein (Greenfield *et al.*, 1984). EDTA-soluble protein can, when in solution, bind to growing crystals and inhibit further growth (Wheeler *et al.*, 1981; Wheeler & Sikes, 1984; Addadi & Weiner, 1985). Adsorption of the protein onto certain crystal faces reduces the growth rate perpendicular to those faces (Addadi & Weiner, 1985; Albeck *et al.*, 1993; Aizenberg *et al.*, 1995). This results in the expression of new faces or the enhancement of existing ones (Albeck *et al.*,

1996). The size and shape of a growing crystal may therefore, in part, be determined by different growth rates in different crystallographic directions.

4.1.3. Calcification *in vitro*.

A widely used method of determining the functions of mineral-associated proteins is the examination of their effects on *in vitro* crystal growth. Proteins tend to interact with certain crystal planes, inhibiting growth in the direction perpendicular to that plane and therefore controlling crystal morphology (Berman *et al.*, 1990, 1993; Albeck *et al.*, 1993). The preference for particular faces is a result of stereochemical compatibility between the protein and the mineral phase (Addadi & Weiner, 1985). Shell proteins extracted from the mollusc *Mytilus* interact specifically with certain crystal faces during growth (Addadi & Weiner, 1985). Later studies of a variety of organisms indicated that the preference for a particular crystal face depends on the organism involved and the amino acid composition of the interacting protein. EDTA-soluble intracrystalline proteins extracted from sea urchin spines, for example, interact with crystal faces parallel to the crystallographic c-axis (Berman, 1988; Albeck *et al.*, 1993). The EDTA-soluble intracrystalline protein fraction extracted from mollusc shells is composed of two types of protein. The proteins differ in amino acid composition and in the effect they have on growing calcite crystals. The minor fraction has an amino acid composition similar to that of the protein from sea urchin spines and also interacted preferentially with faces parallel to the crystallographic c-axis. The major fraction is rich in aspartic and glutamic acids and interacts with faces perpendicular to the c-axis (Albeck *et al.*, 1993). Presumably the regulated expression of proteins extracted from different organisms leads to the expression of different crystal faces, different crystal morphologies and dissimilarities between the resulting shell structures.

Inhibition of crystal growth may also occur by the addition of complex phosphate ions. These "crystal poisons" interrupt the ordered ion arrangement on the crystal surface, preventing further crystal growth (Simkiss, 1964). As phosphate compounds are important constituents of metabolites within organisms, the potential for complete inhibition of biomineralisation exists. The enzyme alkaline phosphatase is believed to be present at the sites of calcification and hydrolyses the complex phosphate

ions, producing products which have a reduced inhibitory effect on calcite formation (Neumann *et al.*, 1951).

Protein macromolecules adsorbed on specific crystal faces may become overgrown by the mineral phase and thus become intracrystalline (Albeck *et al.*, 1993). The observations of Berman *et al.* (1990) of sea urchin tests and mollusc shells suggest that crystal texture may be related to function. Defects in the crystal lattice reflecting protein assimilation alter the texture and the fracture characteristics of the mineral, resulting in a stronger crystal structure (Berman *et al.*, 1990). Incorporation of protein into the mineral structure also affects the morphology of the crystals. A correlation between the morphology of the synthetic calcite crystals into which the sea urchin protein was occluded and the defect pattern within the crystals has been observed (Aizenberg *et al.*, 1995). Calcite crystals grown in the presence of sea urchin intracrystalline proteins exhibit a reduction in coherence length and degree of alignment of the domains in specific crystallographic directions. This demonstrates the ability of mineral-associated proteins to distinguish between different crystal planes by stereochemical recognition (Aizenberg *et al.*, 1997).

4.2 Results.

4.2.1. Mineral-associated proteins from *N. anomala*.

EDTA-soluble proteins were extracted from the dorsal valves of *N. anomala*, as described in Section 2.2.1. Concentrated protein extract was fractionated using SDS PAGE and the proteins detected using Coomassie Blue (CBB 250) dye and silver staining, revealing a 44 kDa protein in both the total and intracrystalline extracts (Figure 3.4). In very concentrated dorsal intracrystalline extracts, a 60 kDa protein is evident (Figure 3.5).

4.2.2. *In vitro* calcium carbonate mineralisation.

4.2.2.1. Slow calcite precipitation.

Growth of synthetic calcite crystals in the presence of protein is an effective method in attempting to gain information on the ways in which organisms control mineral formation *in vivo*. It is understood however, that these experiments may differ from the *in vivo* situation, where a great many more

factors will be involved in mineral formation. These studies were therefore used as a way to gain a general indication of the possible controls that may be employed by the organism in biomineralisation.

Calcite crystals were produced *in vitro*, as described in Section 2.7.1. The crystals formed were identified as calcite by X-ray diffraction using a Philips PW 1050/35 X-ray diffractometer. Figure 4.2 (a) shows the pattern obtained from a sample of inorganically formed crystals. The main peak is at 34.36 degrees and is therefore at the same position as the main peak obtained from a calcite standard (b).

After 24 hours incubation, inorganic calcite crystals were well formed and remained stable in reaction solution for a total of 42 hours incubation, after which, dissolution of the crystals was observed.

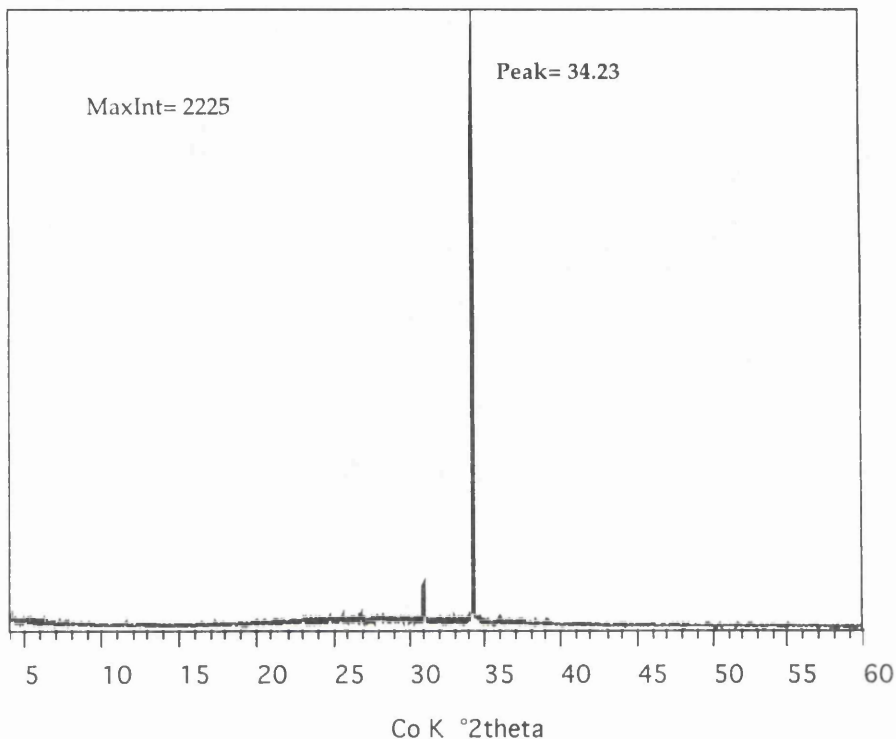
The influence which the proteins of Figure 3.4 have on the morphology of calcite formed *in vitro* was determined by adding a range of concentrations of mineral-associated proteins to the calcite growth system. The soluble protein is extracted in sodium phosphate buffer (10mM, pH 7.2) (Section 2.2.1.), so the effect of this buffer on crystal growth must be assessed, in case any buffer remains in the extract. Sodium phosphate buffer has a profound effect on calcite morphology, causing malformation of the crystal faces and depleting crystal numbers (Figure 4.3). Addition of sodium phosphate buffer to the system after 24 hours of crystal growth causes a slight distortion of the crystal faces. In view of these results, protein extracts were desalted (Section 2.7.1), before including in calcification experiments.

Calcite crystals grown in the presence of soluble intracrystalline protein from the dorsal valve are shown in Figure 4.4. Protein concentrations of 0.4 and 1 $\mu\text{g}/\text{ml}$ result in no visible effect on crystal morphology. Crystals grown in the presence of 1.2 $\mu\text{g}/\text{ml}$ protein, start to form clusters and "pitting" is visible on the crystal faces. Crystals grown in the presence of 2.4 $\mu\text{g}/\text{ml}$ protein exhibit a higher degree of pitting and the crystals cluster together. The number of crystals increases from inorganic conditions as the protein concentration in the system increases, up to 0.7 $\mu\text{g}/\text{ml}$ protein, but thereafter declines with increasing protein concentration (Figure 4.5).

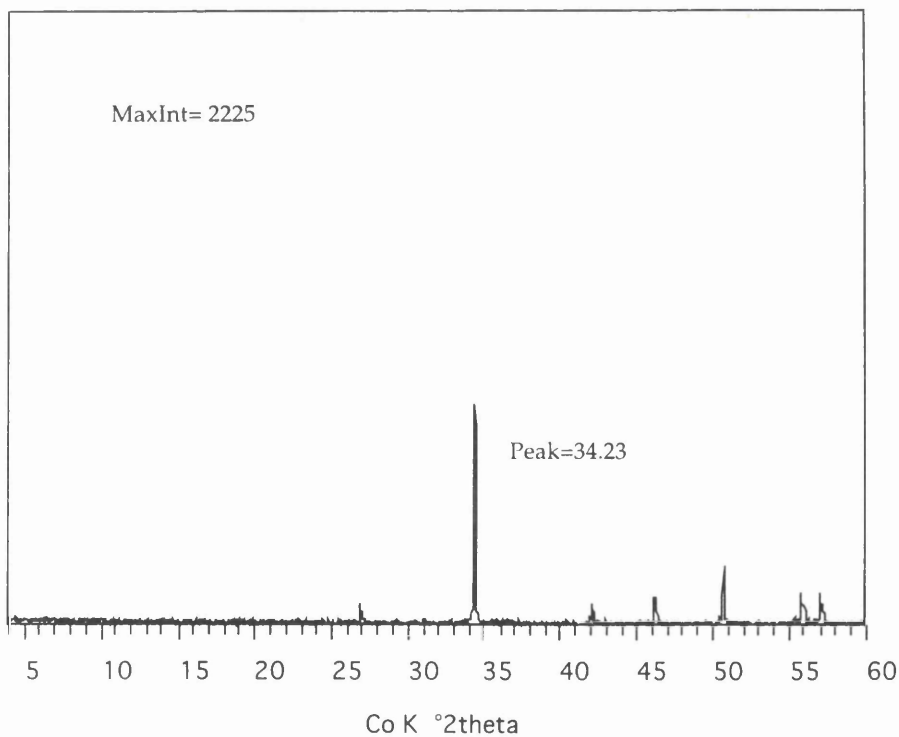
Figure 4.2 Identification of crystals as calcite using X-ray diffraction.

Synthetic calcite crystals (a) were precipitated onto glass coverslips (Section 2.7.1) and re-suspended in water. The crystals were transferred to XRD slides and analysed using a Philips PW 1050/35 X-ray diffractometer and the pattern compared with a calcite standard (b).

(a) Synthetic calcite



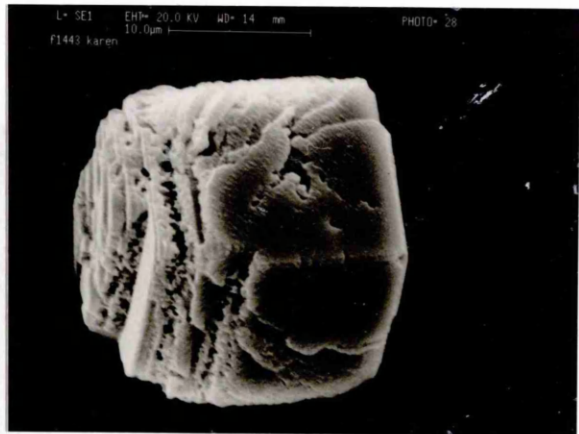
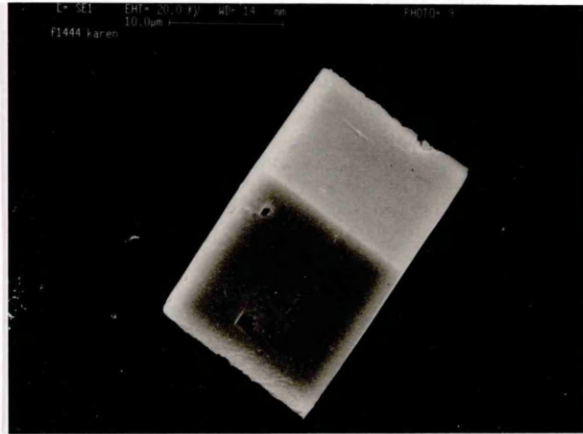
(b) Pure calcite standard.



Crystals formed when the protein is added after 24 hours are shown in Figure 4.6. Protein concentrations of 0.4 and 1 $\mu\text{g}/\text{ml}$ do not affect the crystal morphology, whereas a concentration of 1.2 $\mu\text{g}/\text{ml}$ protein added to the system results in clustering of the crystals. When 2.4 $\mu\text{g}/\text{ml}$ protein is added, clustering is more pronounced and many crystal faces are pitted.

(a)

(b)



(c)

(d)

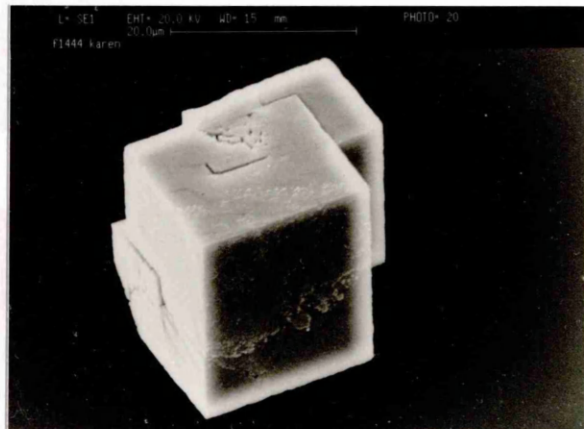
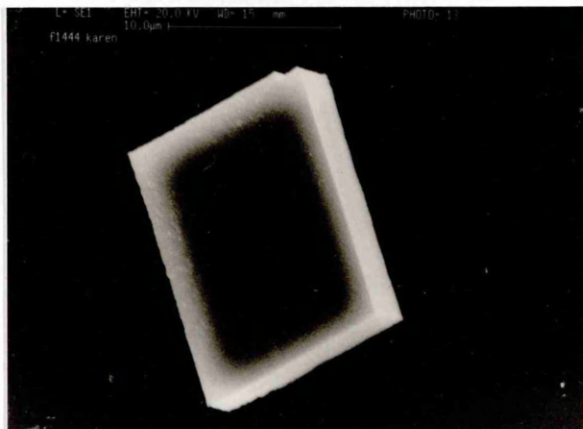


Figure 4.3 Effect of sodium phosphate buffer on calcite crystals formed *in vitro*.

Scanning electron micrographs of calcite, after 48 hours growth, formed in the presence of (a) 20 μl 18M Ω water; (b) 20 μl NaH_2PO_4 buffer (10mM, pH 7.2) and calcite produced after 24 hours inorganic growth and subsequent addition of (c) 20 μl 18M Ω water and (d) 20 μl NaH_2PO_4 buffer (10mM, pH 7.2).

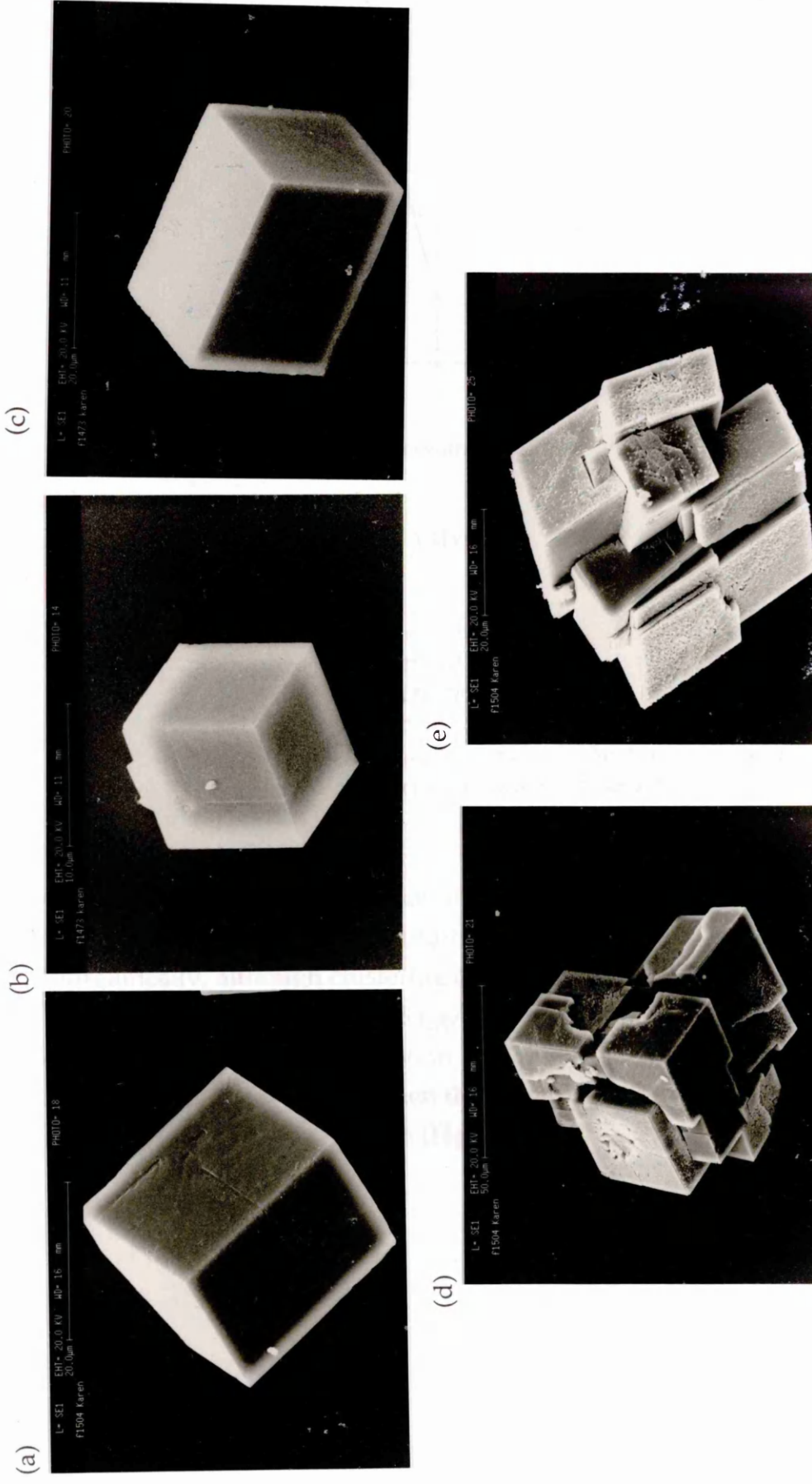


Figure 4.4 Effect of EDTA-soluble protein extract from the dorsal valves of *N. anomala* on calcite grown *in vitro*.

Scanning electron micrographs of calcite grown for 48 hours (a) inorganically and when the following had been added after 24 hours of growth, (b) 0.4 µg/ml extract; (c) 1 µg/ml extract; (d) 1.2 µg/ml extract; (e) 2.4 µg/ml extract.

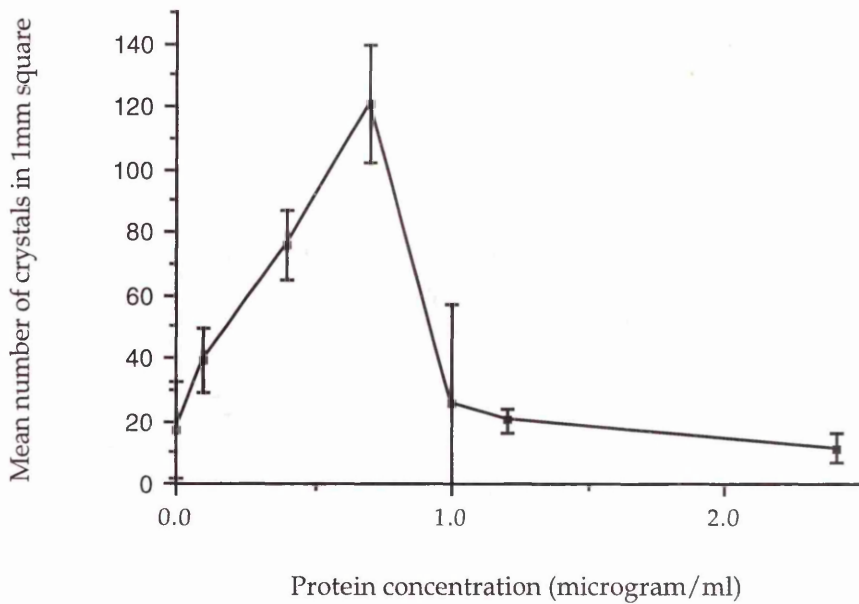


Figure 4.5 Influence of dorsal valve protein extract on the numbers of crystals formed.

Dorsal valve protein concentrations of 0 to 2.4 $\mu\text{g/ml}$ were added to the crystal growth system at the start of the experiment, as detailed in Section 2.7.1. The crystals were grown for 48 hours, after which the abundance of crystals was calculated by counting the number of crystals in ten 1 millimetre squares drawn at random on SEM photographs and calculating the mean number of crystals at different protein concentrations.

Crystals grown in the presence of soluble intracrystalline protein from the ventral valve (Figure 4.7) are not markedly different from crystals grown inorganically, although clustering of crystals is observed in systems in which the protein concentration is 4.8 $\mu\text{g/ml}$ (c). Crystal numbers remain relatively constant, even at high protein concentrations. No effect on crystal morphology was observed when the ventral valve protein extract was added after 24 hours of crystal growth (Figure 4.8).

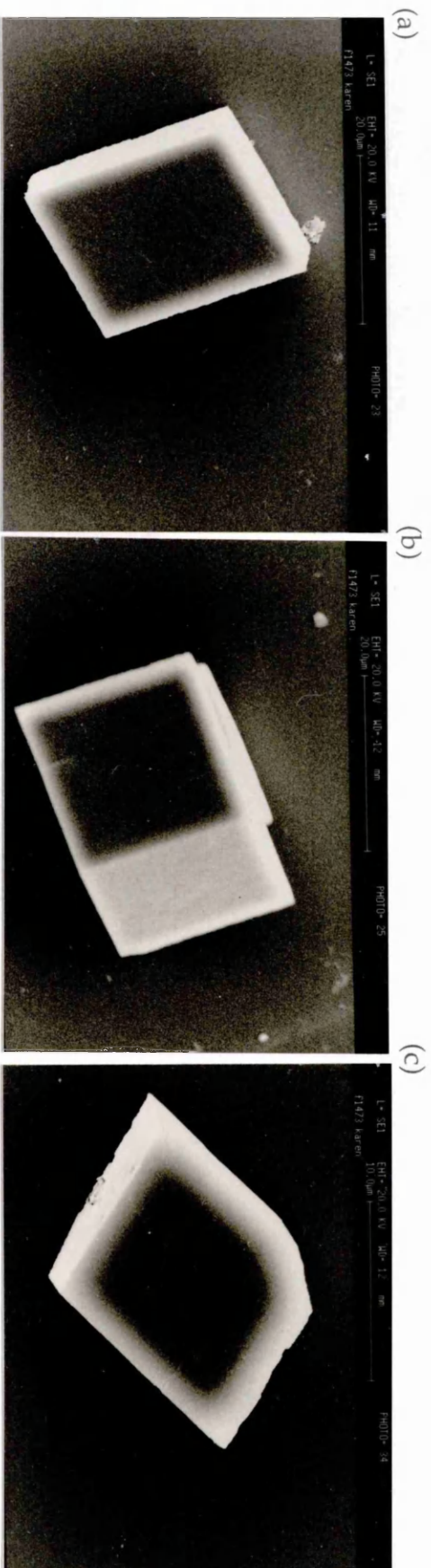


Figure 4.6 Effect of EDTA-soluble protein extract from the dorsal valves of *N. anomala* on pre-formed calcite crystals, grown *in vitro*.

Scanning electron micrographs of calcite formed after 48 hours of growth, (a) inorganically and when the following had been added after 24 hours of growth, (b) 0.4 µg/ml extract; (c) 1 µg/ml extract; (d) 1.2 µg/ml extract and (e) 2.4 µg/ml extract.

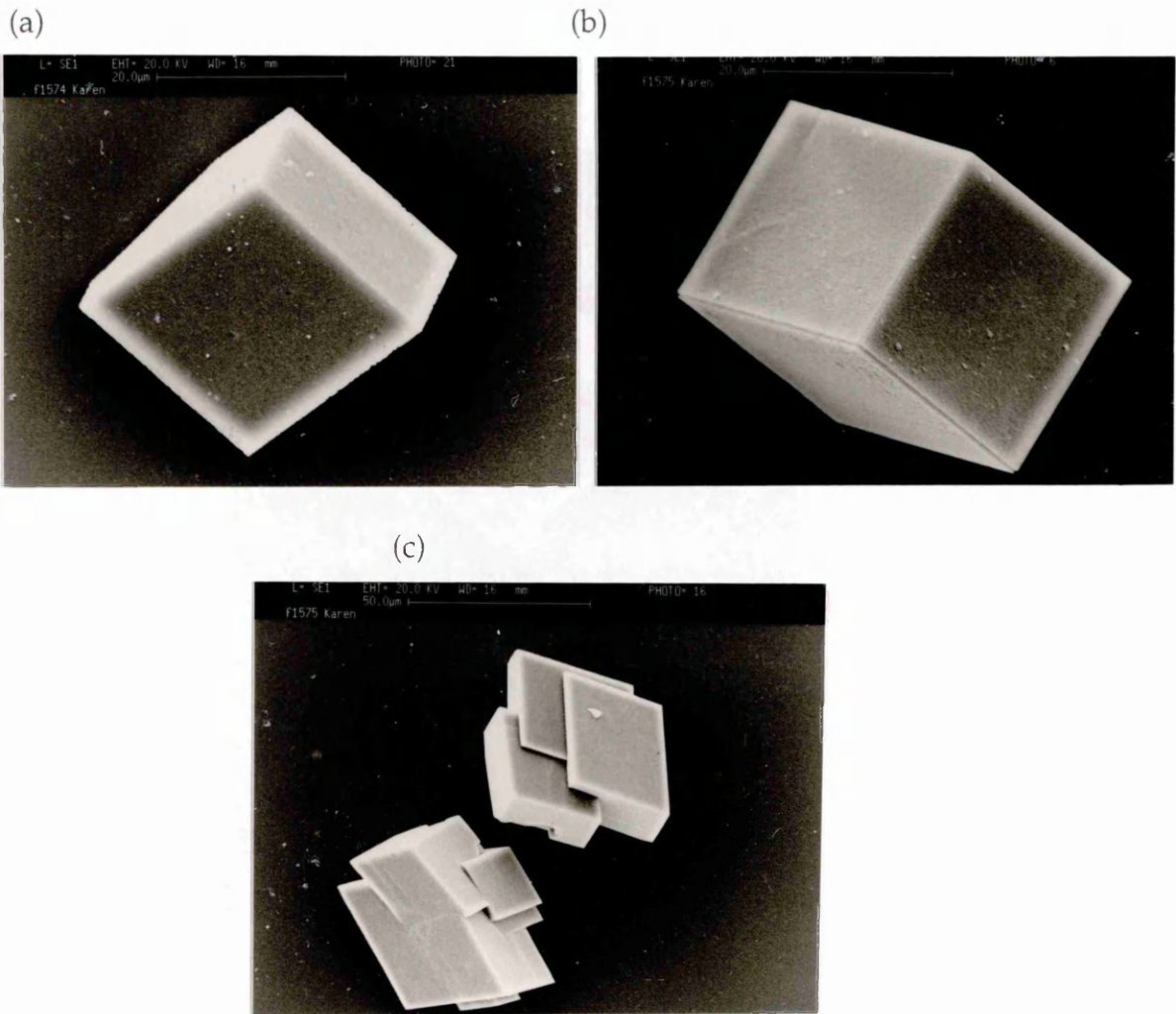


Figure 4.7 Effect of EDTA-soluble protein extract from the ventral valves of *N. anomala* on calcite grown *in vitro*.

Scanning electron micrographs of calcite formed under (a) inorganic conditions and in the presence of (b) 1.2 µg/ml extract and (c) 4.8 µg/ml extract.

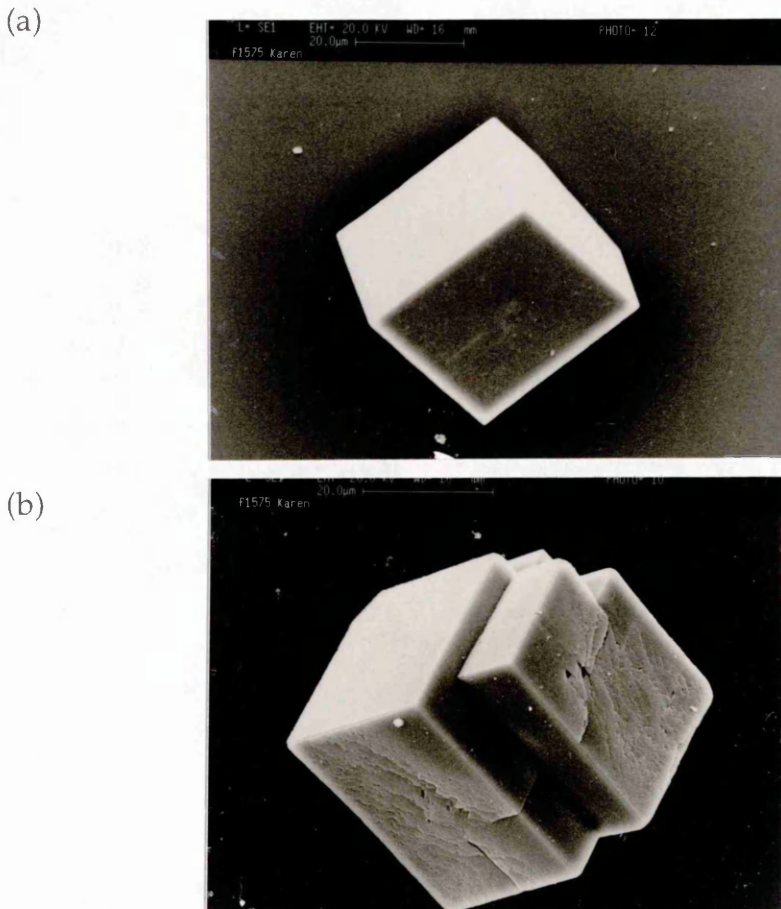


Figure 4.8 Effect of EDTA-soluble protein extract from the ventral valves of *N. anomala* on pre-formed calcite crystals, grown *in vitro*.

Scanning electron micrographs of calcite formed after 48 hours of growth, during which the following were added after 24 hours of inorganic growth, (a) 1.2 $\mu\text{g/ml}$ extract and (b) 4.8 $\mu\text{g/ml}$ extract.

Crystals grown in the presence of total protein extract from the dorsal valves exhibit a high degree of clustering and "pitting" of the faces, this effect being more marked at higher protein concentrations (Figure 4.9). Addition of protein after 24 hours growth results in a more rhombohedral shape, as compared to the crystals which had protein added at the start of the experiment. Crystals grown in the presence of protein added at 24 hours are clustered and holes are apparent on some crystal faces [Figures 4.9 (d) and (e)].

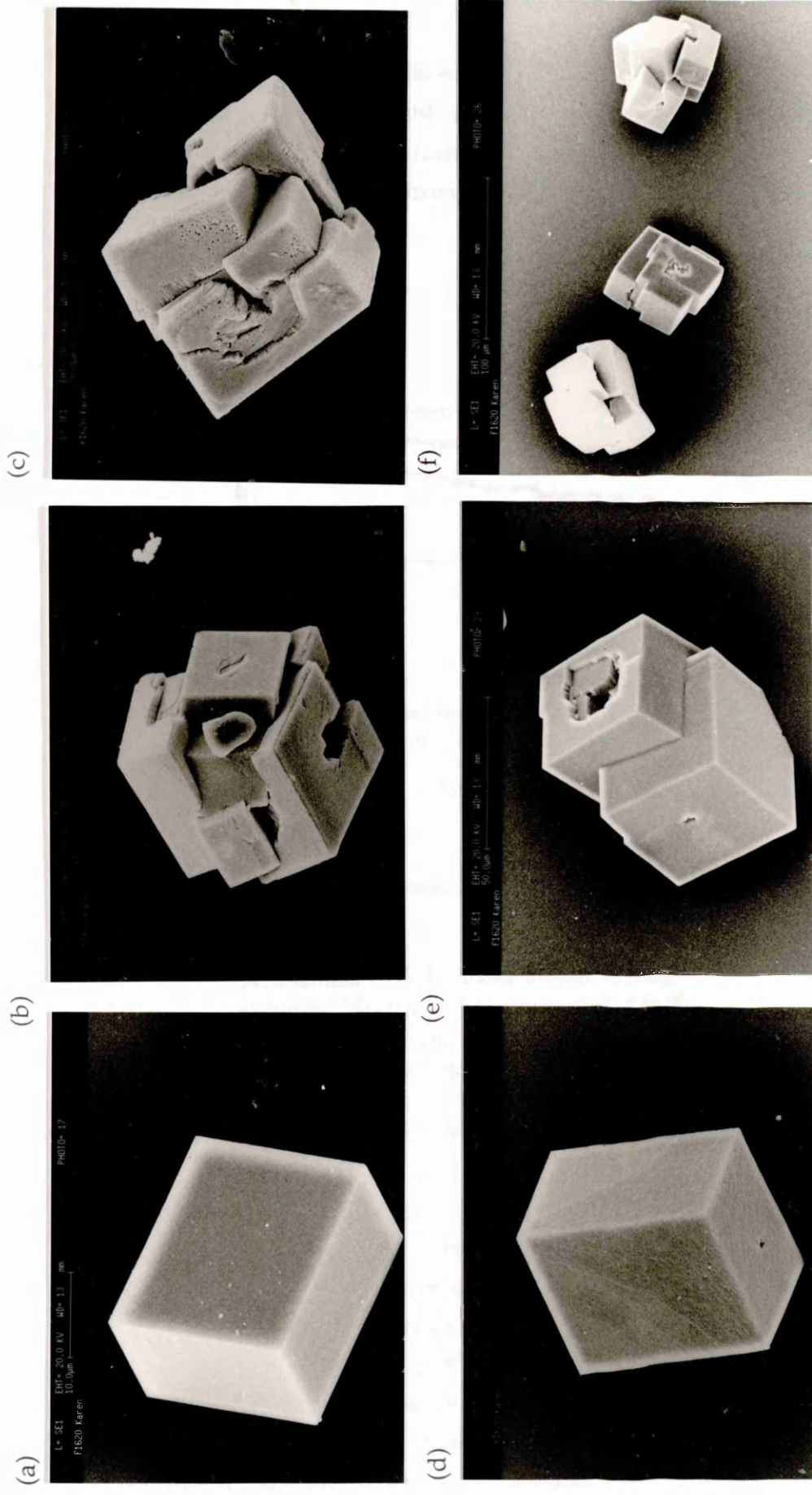


Figure 4.9 Effect of total protein extract from the dorsal valves of *N. anomala* on calcite grown *in vitro*.

Scanning electron micrographs of calcite grown for 48 hours, (a) inorganic conditions and in the presence of (b) 1 $\mu\text{g/ml}$ extract; (c) 3 $\mu\text{g/ml}$ extract. Crystals were also grown for 48 hours, during which the following was added after 24 hours of inorganic growth, (d) 10 μl 18M Ω water; (e) 1 $\mu\text{g/ml}$ extract and (f) 3 $\mu\text{g/ml}$ extract.

4.2.2.2. Fast calcite precipitation.

The influence of EDTA-soluble intracrystalline proteins from the dorsal valves on the rate of calcite crystal formation *in vitro* is shown in Figure 4.10. Calcite was precipitated over approximately 400 minutes, as described in Section 2.7.2.

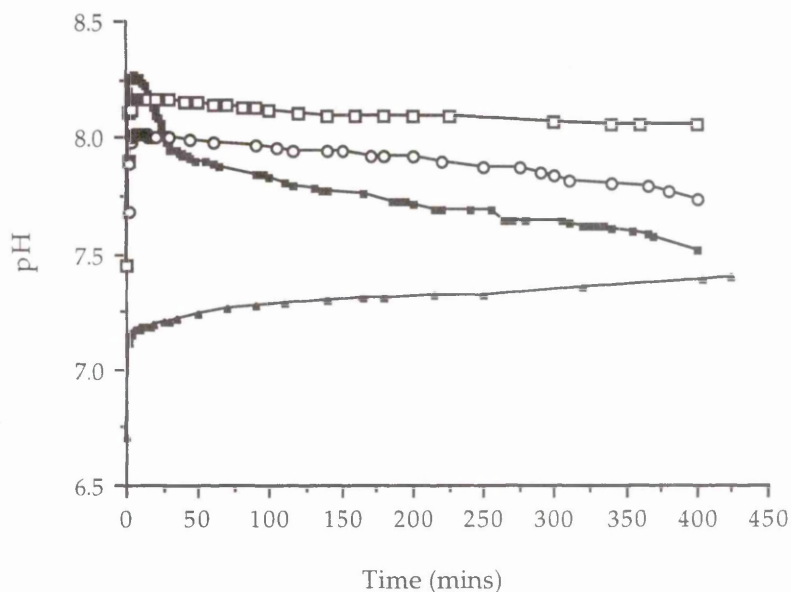


Figure 4.10 Influence of dorsal valve protein extract on the rate of calcite precipitation.

N. anomala EDTA-soluble protein extract, 2.5 (▲), 1 (□) and 0.4 (○) µg/ml (Section 2.2.1) was included in the calcite precipitation system (Section 2.7.2) and the rate of calcite precipitation determined by monitoring the change in pH over 400 minutes. The rate of precipitation with protein was compared to the inorganic (■) calcite precipitation rate over the same length of time.

As calcite forms, hydrogen ions are released and this reaction can be monitored by measuring the decrease in pH over time. There is a rapid initial increase in pH in all systems. After this initial increase, the pH of the inorganic experiment declines rapidly over time. If 1 mole of hydrogen ions is required to reduce the pH by 1 unit, then the rate of inorganic calcite formation is estimated to be an average of 38 mM calcite/minute for the initial drop, until 38 minutes and then 1 mM calcite/minute for the remainder of the experiment. The systems in which 0.4 and 1 µg/ml protein are included also show a decline in pH over time, but the gradient is much less pronounced as compared to the inorganic system, particularly with the 1 µg/ml protein

addition. The rate of calcite formation in the system in which 0.4 μg protein was included is estimated to be an average of 0.7 mM calcite/minute, while for the 1 μg protein addition, the rate is lower, at 0.2 mM calcite/minute. In contrast, the system in which 2.5 $\mu\text{g}/\text{ml}$ protein was included shows a steady pH, with the pH increasing very slightly over time. Therefore, no calcite was produced in this system.

Figure 4.11 shows the influence of soluble protein from the dorsal valve on calcite production when the higher concentration (2.5 $\mu\text{g}/\text{ml}$) extract is added after 129 minutes of inorganic crystal growth. Prior to protein addition, the rate of inorganic calcite formation is 30 mM calcite/minute. The graph shows a sharp increase in pH at the point when the protein is added and thereafter the pH remains at a virtually constant level. A control experiment, in which deionised water was added instead of protein shows a slight increase in pH at the point of water introduction, but the pH continues to decline thereafter, in contrast to the system in which protein was added.

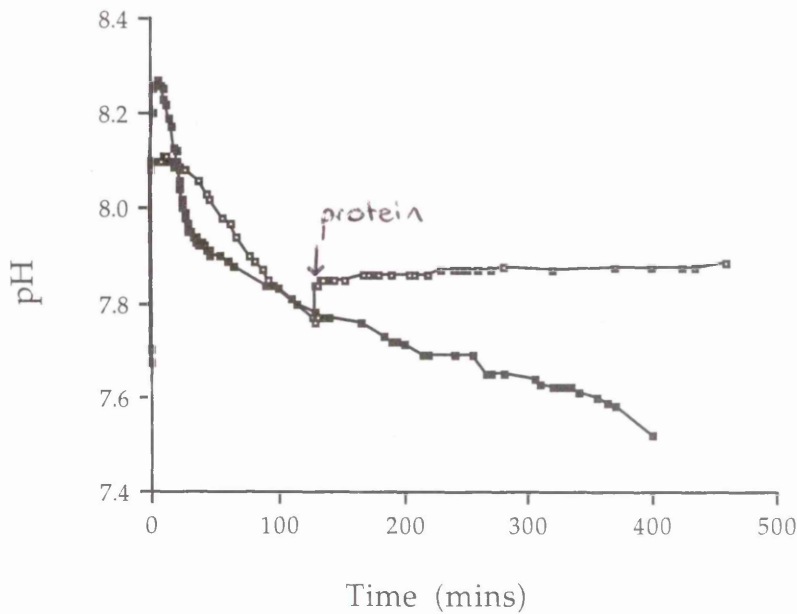


Figure 4.11 Influence of dorsal valve protein extract, added at 130 minutes, on the subsequent rate of calcite precipitation.

*Calcite crystals were grown as detailed in Section 2.7.2. *N. anomala* EDTA-soluble extract (2.5 $\mu\text{g/ml}$) (□) (Section 2.2.1) was added to the system after 129 minutes of inorganic growth. The rate of calcite precipitation was determined by monitoring the pH and comparing it to the rate of calcite formation in the inorganic system (■).*

Figure 4.12 shows the effect of the soluble protein extract of the ventral valve on the pattern of calcite formation *in vitro*. Little effect is observed when ventral valve extract was included in the system, as the pattern of crystal abundance does not differ significantly from that of the inorganic system. The rate of calcite production in this system is calculated to be 20 mM calcite/minute, a rate similar to that of the inorganic system.

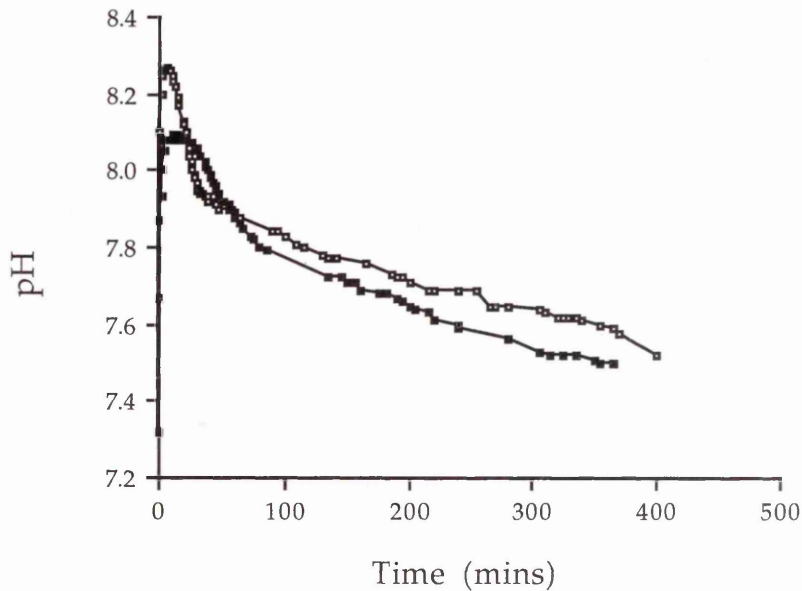


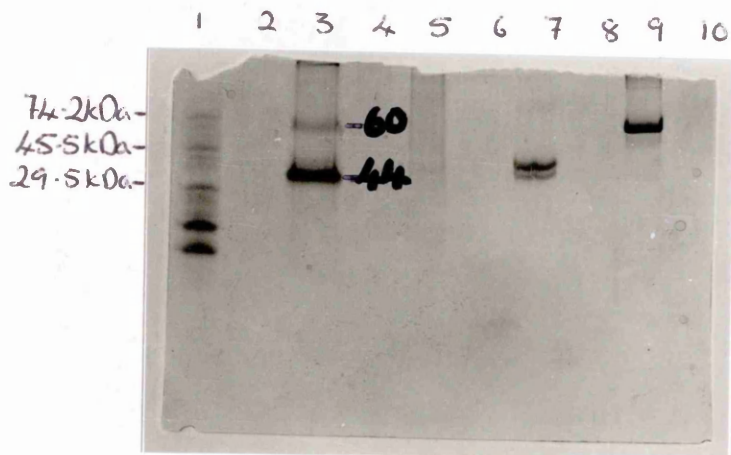
Figure 4.12 Influence of ventral valve protein on the rate of calcite precipitation *in vitro*.

Calcite crystals were grown as detailed in Section 2.7.2. N. anomala EDTA-soluble protein extract from the ventral valve (5 $\mu\text{g/ml}$) () was added at the start of the experiment and the effect of the extract on the rate of calcite precipitation was determined by monitoring the pH and comparing it to the rate of the inorganic system ().

4.2.3. Identification of calcium-binding potential.

Both the 44 kDa and 60 kDa proteins from the soluble intracrystalline extract of the dorsal valve are either acidic or potentially calcium-binding, as assessed by Stains-all, which stains such proteins (Figure 4.13).

(a)



(b)

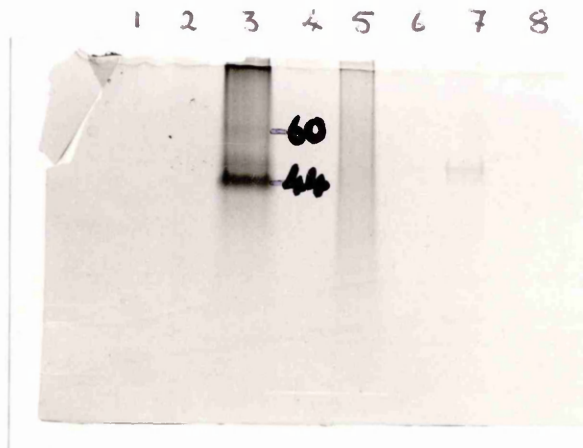


Figure 4.13 SDS PAGE analysis of EDTA-soluble intracrystalline proteins of *N. anomala*, developed using Stains-all.

*Prestained molecular weight standards (Section 2.3.1) were included in lane 1. EDTA-soluble intracrystalline protein extract (7 μ g) from the dorsal valve of *N. anomala* was applied to lane 3. Bovine serum albumin (5 μ g) was applied to lane 5 and ovalbumin (5 μ g) to lane 7. (a) Gel stained with Coomassie Blue (CBB-250); (b) Gel stained with Stains-all (Section 2.6).*

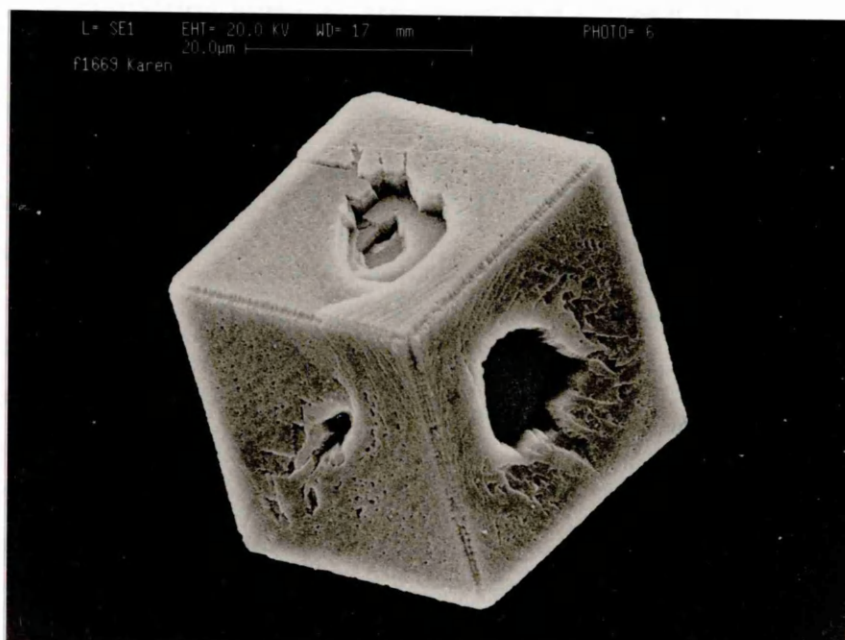


Figure 4.14 The morphology of calcite crystals formed *in vitro*, after two hours of growth in the presence of soluble protein extract of the dorsal valves of *N. anomala*.

Crystals were grown as detailed in Section 2.7.2. A coverslip was removed after two hours and examined under the scanning electron microscope. The pitting observed on the crystal faces may be suggestive of incorporation of the protein into the crystal during growth.

4.3 Discussion.

Growth of synthetic calcite crystals in the presence of EDTA-soluble protein is a common method employed in the analysis of mineral-associated proteins. Previous work and the present study indicate the potential potency of soluble shell proteins in regulating many aspects of calcite growth *in vitro*. (Addadi & Weiner, 1985; Aizenberg *et al.*, 1994, 1995, 1997; Albeck *et al.*, 1993, 1996). In terms of the effect on the process of nucleation, the EDTA-soluble intracrystalline protein extract of the *N. anomala* dorsal valve may be able to initiate crystal nucleation *in vitro*. In contrast, the same protein extract may be able to inhibit nucleation of the synthetic crystals.

The increase in crystal numbers observed when low concentrations (under 0.7 $\mu\text{g}/\text{ml}$) of soluble protein extract are added to the slow calcite growth system (Figure 4.5) may be indicative of the ability of the extract to initiate the nucleation of crystals. The clustering effect evident in the slow calcite precipitation experiments may be also a result of the soluble protein extract

acting as a nucleating surface. However, inorganically formed crystals also form clusters, as interpenetrant groups which are crystallographically aligned with each other. In such systems, growth occurs on existing templates (Braithwaite, pers comm.). Previous work has indicated that when soluble mollusc shell proteins are adsorbed onto a rigid substrate, the protein acts as a nucleating surface (Addadi & Weiner, 1985). In the present study, the protein added to the slow precipitation system may adsorb to crystallographically compatible surfaces of already formed crystals and promote dislocations and therefore growth. Clustering of calcite crystals may result from an unequal distribution of preformed crystals and adsorbed protein, resulting in a concentration of crystals in areas where there is a higher protein concentration, although it must be acknowledged that the clustering may simply be indicative of interpenetrant groups of inorganic calcite crystals.

Previous work has indicated that EDTA-soluble protein that is free in solution may act as an inhibitor to the nucleation and growth of crystals (Addadi & Weiner, 1985). It has been suggested that such impediment to nucleation and/or growth of crystals *in vitro* is due to the adsorption of protein molecules onto stereochemically compatible crystal planes, resulting in a reduction of the growth rate in a direction perpendicular to the affected faces (Addadi & Weiner, 1985; Albeck *et al.*, 1993; Berman *et al.*, 1990, 1993; Aizenberg *et al.*, 1995). Stereochemical matches between the organic and inorganic phases is due partly to the presence of calcium-binding groups on the proteins involved (Weiner, 1979; Meenakshi *et al.*, 1971; Cariolou & Morse, 1988). The possibility that the 44 kDa and the 60 kDa proteins of *N. anomala* are able to bind calcium, as indicated by the Stains-all assay, may indicate that they can promote or inhibit calcite nucleation *in vitro* and therefore may have a similar role *in vivo*.

The inhibition of the rate of nucleation and growth evident in the fast precipitation experiments in which protein is included (Figure 4.10) and the instantaneous decline in pH when protein is added to the system (Figure 4.11), suggest that the protein may be able to prevent crystal nucleation in these circumstances. The apparent sharp decline in the number of crystals grown in the presence of high concentrations of protein (above 0.7 µg/ml) observed in the slow precipitation experiments (Figure 4.5) may also indicate that protein concentrations above 0.7 µg/ml may have an inhibitory effect on the nucleation of crystals. However, the scanning electron micrographs of

individual crystals from the same experiments (Figure 4.4) show that at protein concentrations above 1.2 $\mu\text{g}/\text{ml}$, most crystals are in clusters. There is a difficulty in distinguishing between single crystals and clusters, which results from the method used for counting the crystals from photographs (Section 2.7.1). Therefore, the actual number of calcite crystals in the slow growth system may be higher than previously estimated. It must be noted however, that the abundance of crystals begins to decrease at a concentration of 0.7 $\mu\text{g}/\text{ml}$ of protein in the system, while clustering is not observed at protein concentrations below 1.2 $\mu\text{g}/\text{ml}$.

Although soluble protein may inhibit nucleation of calcite in the fast growth system, it is assumed that some protein must also be adsorbed onto the glass surfaces and therefore have the potential to act as a nucleating surface. A high concentration of protein in the system may ensure that there is free protein in solution, which may override the nucleating potential of the adsorbed protein. Higher concentrations of protein in the system will therefore have more of an inhibitory effect on the nucleation of crystals. Wheeler & Sikes (1984) estimated the range of concentrations of organic material from oyster shells that regulates crystal growth *in vitro* and suggested that if the concentration of organic material exceeded the values at which crystal growth was regulated, then nucleation and growth would not occur. In the present study, the intracrystalline protein influences crystal nucleation and growth in the range of protein concentrations between 0.1 and 2.4 $\mu\text{g}/\text{ml}$. Higher protein concentrations may also influence crystal growth, but an upper concentration limit may exist which exceeds this "regulatory range", resulting in total inhibition of crystal formation. In the slow growth system, protein concentrations above 0.7 $\mu\text{g}/\text{ml}$ result in the inhibition of crystal formation, indicated by a drop in crystal numbers, whereas crystal formation is promoted at protein concentrations below 0.7 $\mu\text{g}/\text{ml}$. Whether the protein extract acts as a growth activator or inhibitor may therefore depend on the concentration of protein in the system.

Incorporation of protein into the mineral phase alters the texture of the affected crystal (Berman *et al.*, 1990, 1993; Aizenberg *et al.*, 1995, 1997). This effect may account for the pitting observed in crystals grown in the presence of higher concentrations of EDTA-soluble intracrystalline protein of the dorsal valves of *N. anomala* (Figure 4.4). Incorporation of protein occurs relatively early in crystal formation (Wheeler & Sikes, 1984) and indeed, pitting is

observed in two-hour old crystals formed in the presence of soluble dorsal valve protein (Figure 4.14).

The marked change in the morphology and surface texture of calcite crystals grown in the presence of sodium phosphate buffer (Section 4.2.2.1) may be due to the presence of complex phosphate ions, which act as "crystal poisons" (Section 4.1.3). The complex phosphate ions disrupt the ordered array of calcium and carbonate ions on crystal surfaces. Some phosphates can be incorporated into the crystal itself, causing disruption of the regular array of calcium and carbonate ions in the crystal lattice and therefore inducing a change in the texture of the crystal faces (Sears, 1958).

The rhombohedral morphology of crystals grown where protein is added after 24 hours of crystal growth (Figure 4.6), is due to the period of inorganic mineralisation that has already occurred before protein addition. Also, as etching is visible at higher protein concentrations, growth may still be occurring and so incorporation of protein into the crystal structure may still take place. The lack of an effect by the protein extract added to pre-formed crystals could be explained if most crystal nucleation and growth occurs within the first 24 hours, under inorganic conditions. Protein added after 24 hours of inorganic growth would therefore not be incorporated into these pre-formed crystals, since growth had already occurred. The clustering of crystals evident after 24 hours of inorganic growth, followed by addition of protein, may simply be due to the formation of interpenetrant crystal groups.

The fact that the ventral valve extract has little effect on the morphology or rate of calcite crystal formation *in vitro* (Figures 4.7 and 4.8), may suggest that the predominant protein of the ventral valve, the 44 kDa protein, may not influence calcite formation. Alternatively, the ventral valve extract may contain small amounts of additional proteins with roles relevant to the ventral valve. These additional proteins may inhibit the action of the 44 kDa protein. The total protein extract of the dorsal valves appears to have the same effect on crystal growth and morphology *in vitro* as does the intracrystalline extract. As mentioned in Section 3.4, the intracrystalline protein may be basically the same as the intercrystalline protein, the intracrystalline material having simply been incorporated during growth of the shell. It is therefore not surprising that protein from both fractions should have similar effects on calcite mineralisation *in vitro*.

Chapter Five

The semi-nacreous ultrastructures of *N. anomala* shell

- 5.1 Introduction.
- 5.2 Results.
- 5.3 Discussion.

5.1 Introduction.

Nacre is composed of aragonitic laminae, while semi-nacre is characterised by irregularly spaced aragonitic or calcitic laminae and by possessing more screw dislocations than true nacre (Weedon & Taylor, 1995). Although nacre is restricted to the Mollusca, semi-nacre exists within several other phyla (Section 1.3.2).

Studies of certain phyla indicate the existence of an additional level of organisation within the shell, an internal "substructure" within the nacreous or semi-nacreous laminar tablets (Weedon & Taylor, 1995). These substructures are revealed by etching the shell with sodium hypochlorite and/or a glutaraldehyde/acetic acid solution. Mutvei (1977, 1978, 1983a, b) provided a wealth of information regarding molluscan nacreous ultrastructures. The aragonitic nacreous tablets of some bivalves and gastropods are composed of a number of smaller units, which Mutvei termed "crystal individuals". He suggested that these structures may be twinned. The number and character of the crystal individuals differs between the molluscan classes, dictates the pattern of nucleation of calcite seeds, and thus determines the resulting shell structure (Mutvei, 1977, 1978). In the bivalve shell, the mineral tablets of the nacreous layer consist of four sections, two triangular insoluble sectors, alternating with two rhombic sectors of higher solubility. In bivalve nacreous tablets, nucleation of new crystals occurs primarily on the insoluble sectors (Mutvei, 1977). In contrast, gastropod and cephalopod nacre generally possess one to six pairs of sectors, although higher numbers of sectors have been observed. The number of sectors can vary even within species. In this case, nucleation of calcite seeds occurs on the organic material in the centre of the tablets (Mutvei, 1978, 1983b). Sector zoning is also common in inorganic crystals, where each sector is chemically distinct. However, the pattern and distribution of sectors in nacreous and semi-nacreous ultrastructures differ, depending on the organism involved.

Semi-nacre has been identified in certain members of two lophophorate phyla, the Bryozoa (Tavener-Smith & Williams, 1972; Brood, 1976; Weedon & Taylor, 1995) and the Brachiopoda (Williams, 1970; Williams & Wright, 1970). It has been suggested that molluscs may possess a more complex biomineralisation system than the lophophorates, in that they have evolved a number of ultrastructures that are not present in lophophorates. Carter & Clark (1985) called this difference the "L-M discontinuity" (lophophorate-

mollusc discontinuity). One such ultrastructure is the differential sectoring of the mineral tablets. A recent study by Weedon & Taylor (1995), on cyclostome bryozoans, has demonstrated that such complex ultrastructures may also exist in organisms with semi-nacreous shells. The calcitic semi-nacreous tablets of cyclostome bryozoans possess similar substructures to those observed in molluscs (Weedon & Taylor, 1995). Etching of the biomineral surface with sodium hypochlorite solution revealed six sectors within the semi-nacreous tablets, three bleach-insoluble sections, on which growth lines are evident, alternating with three bleach-soluble sections, which have a pitted appearance. Only the insoluble parts reach the centre of the tablets and it is on these sectors that nucleation of new calcite seeds occurs. These results suggest, therefore, that if the presence of complex substructures within mineral tablets is indicative of a more elaborate method of shell formation, then the cyclostome bryozoans are not inferior to molluscs in this regard. The present study presents evidence that sectoring of mineral tablets is also present in the semi-nacre of the craniid brachiopods.

5.2 Results.

Chemical etching of the semi-nacreous layers of the dorsal valve of *N. anomala* reveals that the tablets are composed of sectors. Incubation in sodium hypochlorite (20% v/v) for 24 hours causes very slight etching of the laminar surface (Figures 5.1 and 5.2).

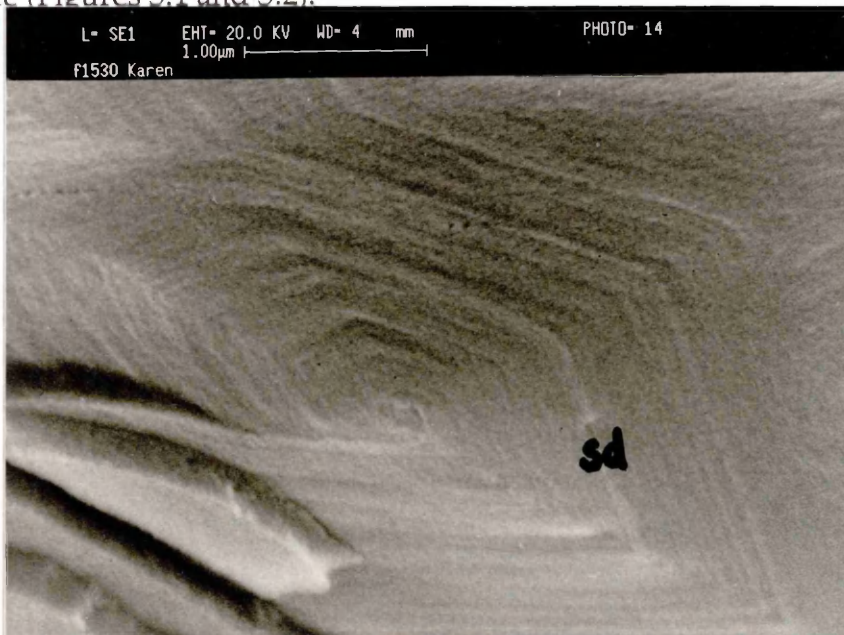


Figure 5.1 Scanning electron micrograph of an unetched tablet, showing growth perpetuating as a screw dislocation (sd).

*The fractured edge of a dorsal valve of *N. anomala* was examined using a Cambridge 360 scanning electron microscope. The scale bar is 1 µm.*

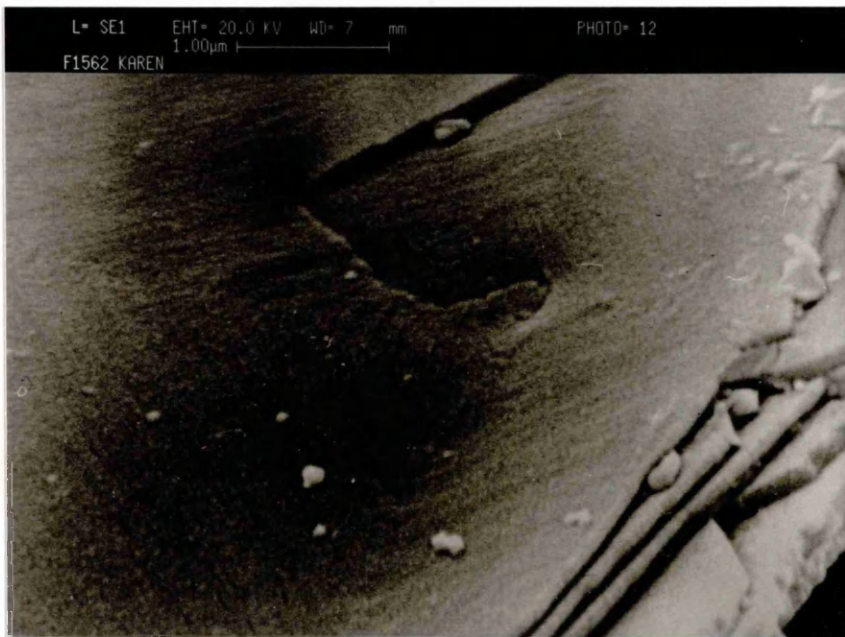


Figure 5.2 Scanning electron micrograph of a slightly etched lamina.

*Fragments of a dorsal valve of *N. anomala* were incubated in an aqueous solution of NaOCl (20% v/v) for 24 hours (Section 2.8) and the fractured edge examined using the scanning electron microscope. The scale bar is 1 μm .*

Etching for 48 or 72 hours reveals that each lamina is composed of hexagonal tablets, with the edges sloping inwards to the base (Figure 5.3). Borders with neighbouring tablets are marked by fractures in the mineral structure. Each tablet is composed of four sectors, two rhombic sectors alternating with two triangular sectors. The two rhombic sectors have "pitted" surfaces and are similar to the "insoluble" sectors observed by Mutvei (1977) for molluscs and Weedon & Taylor (1995) for bryozoans, while the triangular sectors have visible growth lines on the surfaces and are similar to the "soluble" sectors noted by Mutvei (1977) and Weedon & Taylor (1995). Only the triangular sectors reach the centre of the tablet. It is not possible, from the scanning electron micrographs, to confirm the presence of nucleating calcite seeds on the tablet surfaces.

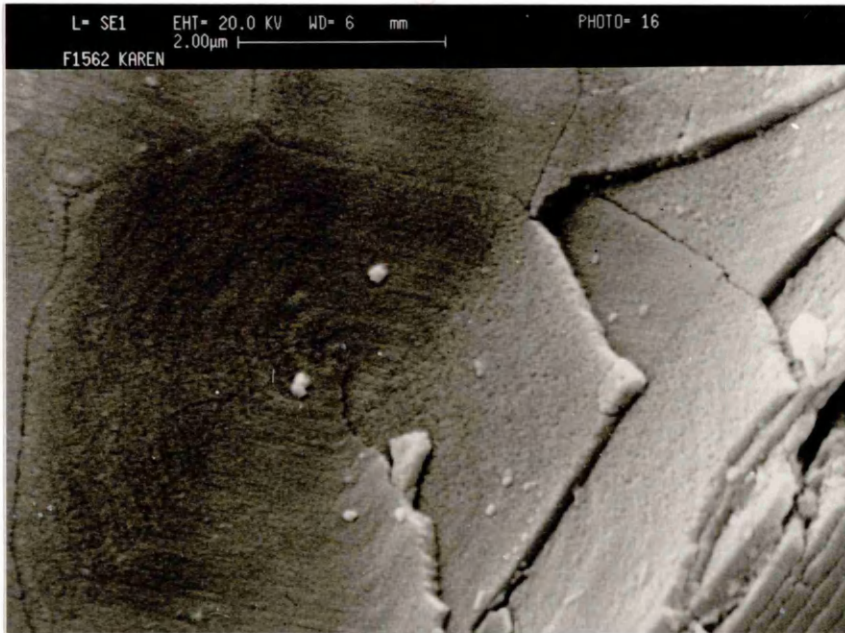


Figure 5.3 Scanning electron micrograph of a lamina showing sectoring.

*Fragments of a dorsal valve of *N. anomala* were incubated in an aqueous solution of NaOCl (20% v/v) for 48 hours (Section 2.8) and the fractured edge examined using the scanning electron microscope. The scale bar is 2 μm .*

Incubation in bleach, followed by a glutaraldehyde/acetic acid solution reveals a pattern of sectoring similar to that observed in the laminae which were treated in bleach solution only, but with two notable differences. Incubation in bleach for one hour, followed by the glutaraldehyde/acetic acid solution for 10 minutes does not reveal any sectoring pattern, but does result in the appearance of a concave depression in the centre of each tablet (Figure 5.4).

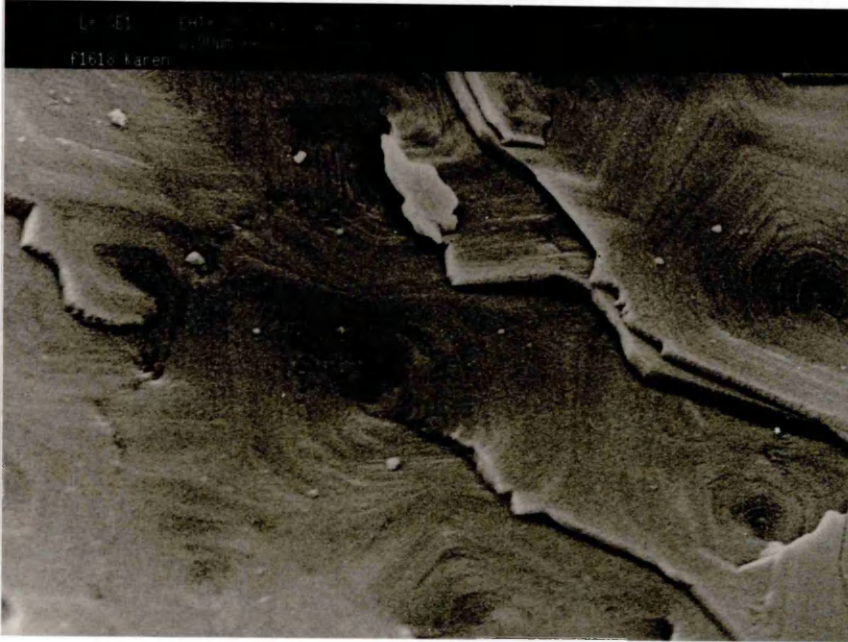


Figure 5.4 Scanning electron micrograph of a semi-nacreous sheet showing the concave depressions (cd) in the central portion of the laminae.

*Dorsal valve fragments from *N. anomala* were incubated in an aqueous solution of NaOCl (20% v/v) for one hour, followed by glutaraldehyde/acetic acid solution (Section 2.8) for 10 minutes. The fractured edge was examined under the scanning electron microscope. The scale bar is 2 μ m.*

Incubation in bleach for one hour, followed by the glutaraldehyde/acetic acid solution for 20 or 30 minutes reveals a sectoring pattern. The number of sectors is the same as the number observed after etching with bleach alone, but the appearance of the tablets is different. The rhombic sectors are pitted, as before, but the triangular sectors are extensively etched and have small "spikes" on their surfaces (Figure 5.5 and 5.6).

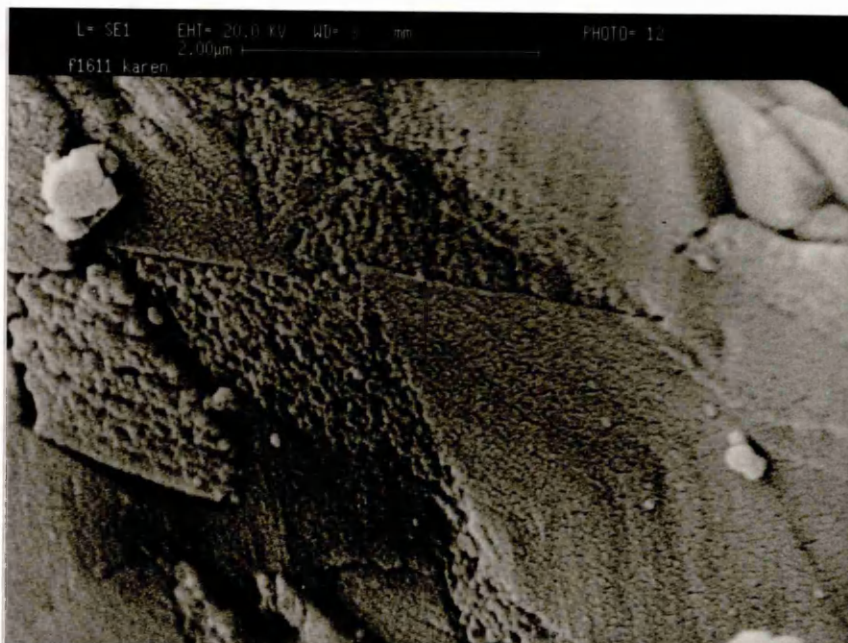


Figure 5.5 Glutaraldehyde-treated lamina showing etching of the crystalline sectors.

*Fragments of the dorsal valve of *N. anomala* were incubated for one hour in an aqueous solution of NaOCl (20% v/v) and then for 20 minutes in a glutaraldehyde/acetic acid solution (Section 2.8). The fractured edge was examined under the scanning electron microscope. The scale bar is 2 μ m.*

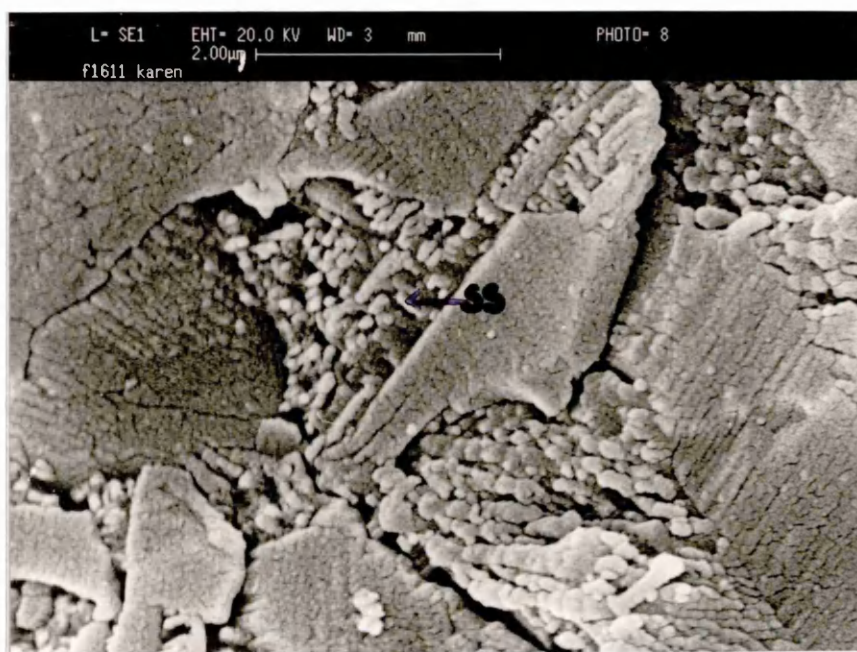


Figure 5.6 Glutaraldehyde-treated lamina showing the "spiked" surface (ss) characteristic of the triangular sectors.

*Fragments of the dorsal valve of *N. anomala* were incubated for one hour in an aqueous solution of NaOCl (20% v/v), followed by 30 minutes in glutaraldehyde/acetic acid solution (Section 2.8). The fractured edge was examined under the scanning electron microscope. The scale bar is 2 μ m.*

5.3 Discussion.

Micro-characteristics, such as the sectoring of lamina tablets, may be of use in phylogenetic comparisons and may provide clues to the evolutionary relationships between and within certain phyla and classes (Brood, 1976; Tavener-Smith & Williams, 1972). The presence of the ultrastructures within the semi-nacre of *N. anomala* shell, as detailed in the present study, together with the discovery that similar ultrastructures also exist within the semi-nacre of the cyclostome bryozoans (Weedon & Taylor, 1995), disputes the concept of the Lophophorate-Mollusc discontinuity (Carter & Clark, 1985).

The laminae within the nacreous and semi-nacreous layers of shell are overlain with a layer of organic material. Treatment of the shell with an aqueous solution of sodium hypochlorite removes the organic material from the surface of the tablets to reveal the internal ultrastructure of each tablet (Mutvei, 1977). In the secondary layer of the dorsal valve of *N. anomala*, the division of each hexagonal tablet into two pairs of sectors may be related to the selective incorporation of protein into the mineral during shell formation. Sector zoning is a common feature of inorganically-formed crystals. However, the different numbers and patterns of sectors constituting the nacreous and semi-nacreous mineral layers of different organisms suggests that there is a degree of control involved in the formation of the sectors. Although a basic tendency may exist in the formation of sector zoning of inorganic minerals, the differences in the patterns of these sectors in different organisms may be indicative of the involvement of intracrystalline protein occluded within the mineral.

The "pitting" observed on the surfaces of the mineral sectors may be due to a modification in the mineral structure in these sectors, caused by the incorporation of material into the intracrystalline phase. The incorporation of protein into crystals grown *in vitro* has been widely demonstrated and the presence of protein in the intracrystalline phase alters the texture of calcite crystals grown *in vitro* (Berman *et al.*, 1990, 1993, Aizenberg *et al.*, 1995, 1997) (Section 4.1.3). The highly specific effects of intracrystalline protein on the growth of crystals demonstrated *in vitro* suggest that the protein may have a similar effect *in vivo*, although a great number and variety of elements may be available for incorporation into the mineral phase in the natural habitat of the organism. However, the molecular structure of protein predisposes its occlusion into stereochemically compatible crystal planes (Berman *et al.*, 1990).

Protein may be concentrated partly or exclusively into the rhombic sectors of the mineral tablet, since the triangular sectors do not have an altered surface appearance. If the rhombic sectors do have a high protein content, then any protein associated with the mineral surface of these sectors will be destroyed during bleaching, leaving the surface with an altered appearance.

The glutaraldehyde/acetic acid solution acts as both a fixative and an etching solution. Glutaraldehyde is a fixative of proteins and mainly affects the amino groups through cross-linkages, while the acetic acid in the etching solution partly dissolves the mineral structure of the triangular sectors of each tablet, causing the "spiked" effect observed on the mineral surface and rendering it more soluble (Williams, 1984). Therefore, although the triangular sectors may be unaffected by incubation in bleach alone, the same sectors are etched by the acetic acid. The difference in susceptibility may be due to a modification of the rhombic sectors caused by protein incorporation and this modification may infer a higher degree of resistance against etching by acetic acid. The unmodified and therefore unprotected sectors are thus etched by the acid.

The division of the laminar tablets of *N. anomala* into two pairs of sectors with different characteristics, may provide important clues to the relationship between the craniid brachiopods and members of other phyla which possess nacreous or semi-nacreous shells. Different patterns of sectoring exist in different molluscan classes (Mutvei, 1977, 1978) (Figure 5.7). One apparent feature is the variation in the number of sectors in tablets from different organisms. The nacreous tablets of bivalves are composed of two pairs of sectors, while the tablets of gastropods generally consist of one to six sectors (Figure 5.7). Weedon & Taylor (1995) extended the interest to cyclostome bryozoans, in which semi-nacreous tablets comprising six sectors were illustrated. The present study shows that the semi-nacreous tablets of *N. anomala* are composed of two pairs of sectors, with some characteristics typical of those from bivalve shells (Figure 5.7).

In both *N. anomala* and bivalves, two rhombic sections alternate with two triangular sectors. Incubation of both *N. anomala* and bivalve shells in a sodium hypochlorite solution for one hour did not reveal a sectoring pattern in the tablets. Unlike the present study, Mutvei (1977) did not bleach the bivalve shells for any longer than one hour, so a comparison of *N. anomala*

and bivalve shell after extensive bleaching cannot be made. Incubation of both shell types in a glutaraldehyde/acetic acid solution however, revealed two alternating pairs of triangular and rhombic sectors in both systems, but in *N. anomala*, the triangular sectors were more susceptible to etching by the acetic acid, whereas in the bivalve system, the rhombic sectors were more affected (Mutvei, 1977).

The pattern of nucleation of calcite seeds differs between different groups of organisms studied. Mutvei (1977) noted that nucleation in bivalve tablets occurs solely on the peripheral areas of the triangular sectors. He suggested that this pattern of nucleation and the subsequent "terraced" growth, away from the centre of the tablet, induces the formation of sheet nacre, with its characteristic "brick-wall" pattern. Mutvei noted that, in bivalves, the triangular sectors of the laminae are generally larger than the rhombic sectors and that this size difference may promote nucleation on the triangular portions of the tablet. Mutvei, however, did not make any suggestions to how the proteins associated with the mineral tablets could function in the nucleation process. The nucleation site for gastropods is located in the centre of the mineral tablets, where a large amount of organic material is present. Nucleation of calcite seeds and subsequent growth of crystals at the central portions of preceding tablets induces formation of the columnar, "stack" form of nacre (Mutvei, 1978). The non-specific nucleation observed in cyclostome bryozoans (Weedon & Taylor, 1995), may indicate a uniform protein distribution over the mineral layer. An equal distribution of protein over the mineral phase may be a common feature of semi-nacreous structures. It was not possible however, to confirm the distribution of nucleating calcite seeds on the semi-nacreous tablets of *N. anomala*.

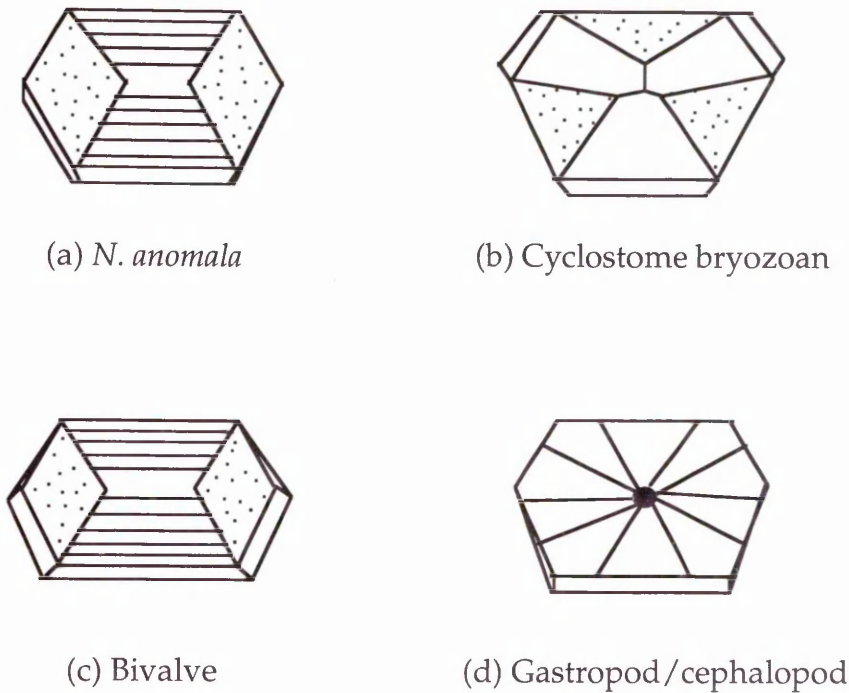


Figure 5.7 Diagrams of typical semi-nacreous and nacreous tablets in *N. anomala*, cyclostome bryozoans and molluscs.

Shell fragments were incubated in NaOCl only (a) and (b) (Section 2.8), or NaOCl, followed by a glutaraldehyde/acetic acid solution (c) and (d) (see Mutvei, 1977, 1978) and examined under the scanning electron microscope. (a) Semi-nacreous tablet of *N. anomala*, comprising four alternating soluble rhombic and less soluble triangular sectors that meet at the centre. Growth lines are visible on the triangular sectors, the rhombic sectors are pitted and the edges slope inwards towards the base. (b) Cyclostome semi-nacreous tablet, comprising six equally sized, alternating soluble and less soluble sectors (Weedon & Taylor, 1995); (c) Typical bivalve nacreous tablet, made of two soluble rhombic sectors, alternating with two less soluble triangular sectors; (d) Typical gastropod or cephalopod nacreous tablet, divided into a number of sectors and possessing a central depression, in which organic material occurs.

In most respects, the crystalline characteristics of *N. anomala* resemble the tablets that comprise the bivalve nacre, as both types possess similar form and number of tablet sectors. The appearance of a concave depression in the central portion of each tablet after treatment with the glutaraldehyde/acetic acid solution, may result from the dissolution of calcified organic material present in the centre of the tablet, that may have remained after the bleach treatment. In this respect only, the crystalline composition resembles that of gastropods.

Chapter Six

General discussion and suggestions for future work.

- 6.1 General aims of the present study.
- 6.2 Calcium binding and the influence of protein on synthetic calcite formation.
- 6.3 Possible functions of the sectoring pattern of the nacreous and the semi-nacreous tablets.
- 6.4 Summary of the possible functions of the mineral-associated proteins of *N. anomala*.
- 6.3 Suggestions for future work.

6.1 General aims of the present study.

The general aim of the study was to gain knowledge of the mineral-associated proteins of *N. anomala*, with the specific aim of contributing to the understanding of biomineralisation in this system. In addition, it is hoped that any findings may be of value to those concerned with the taxonomic position of *N. anomala* within the phylum. The EDTA-soluble intracrystalline organic fraction of the dorsal valve, although less abundant than the EDTA-insoluble fraction, has several features that suggest that the proteins play a role in the process of shell formation and growth.

6.2 Calcium-binding and the influence of protein on synthetic calcite formation.

The EDTA-soluble protein extract from the dorsal valve, particularly the intracrystalline fraction, has a profound effect on the growth of calcite crystals *in vitro*, raising the possibility that the soluble intracrystalline protein may also play a role in shell formation *in vivo*. The calcium-binding ability of the proteins from the intracrystalline fraction of the dorsal valve (Section 4.2.3), suggests that these proteins may be involved in shell formation. Many theories of such biologically-controlled mineralisation (Mann, 1983) propose that calcium ions bind to anionic sites on the protein chain, in the case of crystal nucleation (Wilbur & Watabe, 1967; Simkiss & Wilbur, 1989), or that calcium-binding proteins bind to the faces of calcium carbonate crystals, as in the case of inhibition of crystal growth (Wheeler *et al.*, 1981). Both nucleation and inhibition require that the protein involved is calcium-binding. The potential to bind calcium ions is supported by the fact that an analysis of the primary structure of the 44 kDa protein, the most abundant protein in the shell, consists primarily of aspartic acid/asparagine and glutamic acid/glutamine [Section 3.3.2.3]. If the residues are in the acidic form, then they will have an affinity for calcium ions through their anionic carboxyl groups. Since the acidic forms predominate at physiological pH (Cariolou & Morse, 1988), it is likely that the protein extract, which is buffered to pH 7.2, will also contain the acidic forms.

Previous studies have illustrated that soluble shell proteins can promote nucleation of crystals *in vitro* (Weiner, 1979; Addadi & Weiner, 1985). The present study indicates that the soluble protein extract of the dorsal valve of *N. anomala* may be able to initiate or enhance and also inhibit crystal growth

in vitro, the precise effect depending on the concentration of protein in the system. Soluble protein may be able to promote nucleation by providing a surface on which the calcite seeds can nucleate (Weiner & Traub, 1980a). Initiation of nucleation was suggested in the *in vitro* studies, where calcite crystals were grown in the presence of soluble shell protein (Section 4.2.2.1). When the protein concentration reached a certain level, clustering of crystals occurred. This may be a result of variable adsorption of protein onto the surfaces of crystals which have already adhered to the glass coverslip, or may be indicative of the formation of interpenetrant groups of crystals, with no contribution from the protein. If the inclusion of protein in the system affects the formation of crystals, this may lead to the subsequent accumulation of nucleating calcite crystals in the areas where protein is concentrated. A low concentration of protein (below 0.7 μg) in the slow growth system promotes the formation of calcite, as indicated by the abundance of crystals. However, low protein concentrations have no effect on the morphology of crystals, suggesting that protein may not be incorporated into the crystals when at a low concentration in the system.

Inhibition of nucleation of calcite seeds, or the prevention of the growth of pre-formed crystals, may result from the adsorption of the protein onto certain crystal faces, which reduces the growth rate of those faces (Berman *et al.*, 1990, 1993; Aizenberg *et al.*, 1995). Inhibition of growth occurs when the protein is in solution. The protein binds to the crystal faces through interactions between calcium ions of the mineral phase and the calcium-binding groups on the protein chain. The reduction in crystal numbers, the lowered rate of production of calcite grown in the presence of protein and the abrupt decrease in calcite production as soon as protein extract is added to the system are indicative of the inhibition of nucleation by the soluble protein extract. It is possible that the apparent decrease in crystal abundance observed with a high concentration of protein in the system may be due to the fact that single crystals are often difficult to distinguish from crystal clusters on photographs of scanning electron micrographs. The actual crystal abundance may therefore be higher and the peak of crystal abundance at a protein concentration of 0.7 $\mu\text{g}/\text{ml}$ may not exist (Figure 4.5). Nucleation is promoted by protein concentrations of 0.1 to 0.7 $\mu\text{g}/\text{ml}$ and since no clusters are observed at these protein concentrations, it is likely that these counts are reliable.

The change in the texture of crystal faces observed in the slow growth system may be due to the incorporation of protein into the crystal lattice. The assimilation of protein, along specific crystallographic directions, into the intracrystalline phase *in vivo* may enhance the strength and flexibility of the biomineral, by altering the fracture characteristics of the mineral subunits (Berman *et al.*, 1990). As an alteration in crystal texture in the present study was only observed when the protein concentration within the system was over 1.2 µg/ml, it may be that protein of lower concentrations may be entirely adsorbed onto the surface of the glass coverslip, leading to an absence of protein in solution. If this is the case, then no protein would be free to be incorporated into the crystals and therefore there would be no change in crystal morphology.

6.3 Possible functions of the sectoring pattern of the nacreous and semi-nacreous tablets.

The pattern of growth of nacreous and semi-nacreous structures may be influenced by the distribution of intercrystalline protein layers over the laminae. The pattern of nucleation of new calcite seeds over the different sectors of a tablet determines the subsequent pattern of shell growth, as illustrated in the nacreous layer of bivalves (Mutvei, 1977) and gastropods (Mutvei, 1978). A uniform distribution of nucleating calcite seeds in the semi-nacreous shells of cyclostome bryozoans (Weedon & Taylor, 1995) may be characteristic of semi-nacreous structures and determine their formation. Definite confirmation of the distribution of nucleating calcite seeds on the tablet surfaces in the semi-nacreous shell layers of *N. anomala* was not possible, since particles on the tablet surfaces could not be assigned to being either calcite seeds or simply dust particles.

The crystalline sectors are of two main types, bleach-soluble and bleach-insoluble, set in an alternating pattern within each hexagonal tablet (Mutvei, 1977, 1978). The higher solubility of one sector type over another after bleach incubation may be due to there being a higher proportion of protein occluded within this portion of the mineral. It is possible that the intracrystalline fraction may therefore be incorporated in a genetically-determined pattern, which in turn, determines the size, characteristics and number of crystalline sectors within each tablet. The distribution of intercrystalline protein may or may not be affected by the pattern of occlusion of intracrystalline protein. The

pattern of nucleation may be a result of the distribution of intercrystalline protein, since there is a wealth of evidence to suggest that nucleation is induced by mineral-associated protein (Addadi & Weiner, 1985; Addadi *et al.*, 1991; Berman *et al.*, 1988, 1990, 1993; Kingsley & Watabe, 1984; Watabe, 1981; Westbroek *et al.*, 1984). Nucleation of new calcite seeds in gastropods and cephalopods occurs on the organic accumulation in the centre of each tablet (Mutvei, 1978, 1983b). The nucleation of new calcite over the entire surface of semi-nacreous tablets, as in the case of cyclostome bryozoans (Weedon & Taylor, 1995) indicates that the nucleating protein may be distributed uniformly over the entire tablet surface.

The potential for taxonomic studies using these ultrastructural characteristics of the brachiopods, is limited to organisms with nacreous or semi-nacreous shells. Only the craniids possess semi-nacreous shells. *N. anomala* is the only member which has been the subject of such studies. The suggestion that molluscs may possess a more complex biomineralisation system than is present in the lophophorates, a concept known as the Lophophorate-Mollusc discontinuity (Carter & Clark, 1985), can be disputed as a result of the present study.

6.4 Summary of the possible functions of the mineral-associated proteins of *N. anomala*.

The physical characteristics of the biomineral produced may be a result of the stereochemical complementarity between the organic and the inorganic phases. The regulation of the rate of formation and growth may be controlled by the concentration of protein in the *in vitro* system at any given time, but may also occur depending on the state of the protein, whether it is adsorbed to a surface or whether it is in solution. The different influence which the intracrystalline extract has on the slow and fast growth systems may result from the rate of calcite formation, suggesting that a given concentration of a specific protein may have a particular effect on calcite growth, which is dependant on the kinetics of calcite formation. Such considerations highlight the fact that extrapolation of such *in vitro* observations to *in vivo* situations is problematic. A great many factors will be involved in biomineral formation within organisms, rather than simply the state and concentration of protein in the system. Genetically determined timing of protein extrusion is a likely factor to affect biomineral formation and may also, in part, determine if crystal formation is initiated or inhibited. The influence of mineral-associated

proteins on calcite formation *in vitro* should be employed to provide possible indications of the roles that the proteins may have on mineral formation and should not be considered diagnostic.

6.5 Suggestions for future work.

Future work on the subject of biomineralisation of *N. anomala* must focus on the mechanisms by which the mineral-associated proteins influence shell formation *in vivo*. Having identified proteins which may play important roles in shell formation, identification of these proteins *in situ*, using antibodies directed against pure proteins, will provide information on the localisation of these proteins and may therefore give an indication of their possible roles within the shell.

A more detailed characterisation of the main shell protein (44 kDa) would be desirable, followed by subsequent comparison with other mineral-associated proteins, especially those extracted from the semi-nacreous or nacreous shells of bivalves, gastropods and the cyclostome bryozoans. The pI of the proteins, in addition to their complete N-terminal sequence, would be extremely valuable. Examining the protein composition in detail, the direct product of genetic inheritance, is also of great validity in such comparative studies. The mineral-associated proteins, along with other factors, determine, with immense precision, the phenotypic characteristics of the organism.

Further detailed examination of the patterns of mineral ultrastructures within semi-nacreous and nacreous shells may be informative, both biologically and taxonomically. Work on other craniid brachiopods, as well as other groups with semi-nacreous shells may clarify the involvement of protein in the formation of the mineral sectors and may provide an indication to the relationship between certain taxa and determine whether they are unique amongst their own phyla.

The *in vitro* calcite formation experiments have immense potential for future study, as they provide a relatively simple mechanism by which organic material can be introduced and the subsequent effect on calcite formation examined. Further calcification experiments, using purified proteins rather than whole extracts may be able to identify the particular proteins responsible for particular functions within the shell. The preparation of synthetic systems in which both protein concentration and timing of protein introduction into

the system can be controlled and measured, may clarify the importance of these factors in shell formation. It must be considered however, that shell formation is far more complex than the regulation of mineral formation *in vitro* and there may be many other factors that may have an effect on the regulation of shell formation. It is most likely to be a combination of a range of different information sources that will be most effective in discovering the precise mechanisms involved in shell formation.

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Appendix.**Materials.**

Item	Supplier
Protein Extraction.	
Sodium Hypochlorite	Aldrich
18M Ω water	Millipore
Ethylene diamino tetracetic acid (EDTA)	Sigma
Sodium phosphate	Sigma
Sodium hydroxide	Sigma
Hydrochloric acid	Fisher Scientific
Guanidine hydrochloride	Sigma
Magnetic stirrer	Stuart Scientific
Centrifuge tubes (50ml)	NUNC
Centrifuge (Microcentaur)	Scotlab
Centrifuge (Omnifuge)	Heraeus
Centrifuge (Suprafuge)	Heraeus
Minitan™ Ultrafiltration System	Millipore
Centriprep™ concentrators	Amicon
Microcon concentrators	Amicon
Sephadex PD-10 Column	Pharmacia
Digital Ultraviolet Spectrophotometer	Cecil
Gyrovap	Howe
Protein fractionation by SDS PAGE.	
18M Ω water	Millipore
Glycerol	Sigma
Methanol	Fisher Scientific
Acrylamide	Sigma
Bis-acrylamide	Sigma
Sodium Dodecyl Sulphate	BDH Chemicals
Ammonium persulphate	Biorad
TEMED	Sigma
Tris buffer	Sigma
Tricine	Sigma
Prestained Molecular Weight Markers	Bethesda Research Laboratory

Mercaptoethanol	Sigma
Coomassie Brilliant Blue R-250 (CBB)	Sigma
Acetic acid	Prolab
Trichloroacetic acid	Sigma
Dithiothreitol	Sigma
Silver nitrate	Sigma
Sodium carbonate	Johnson/Matthey Chemicals
Pipette tips (2.5ml; 1ml; 250µl)	Sarstedt
Autopipettes	Rainin
Microsyringe (30µl)	ITO Corporation
Mini Protean II System	Biorad
CAPS buffer	Sigma
Formaldehyde	Aldrich
Sulphorhodamine B	Sigma
Problott™ membrane	Applied Biosystems
MiniTransblot Electrophoretic Transfer Cell	Biorad
Carbohydrate Detection.	
Streptavidin/Biotin Protocol.	
Glycotrack™ kit	Oxford Glycosystems
Sodium phosphate	Sigma
Sodium chloride	Fisons
Sodium acetate	Sigma
EDTA	Sigma
Tris	Sigma
Sodium hydroxide	Sigma
Hydrochloric acid	Fisher Scientific
Suspension Mixer (Model 802)	Luckham
18M Ω water	Millipore
Ovalbumin	Sigma
Bovine Serum Albumin	Sigma
Ribonuclease A	Sigma
Osteonectin	Sigma
Concanavalin A Protocol.	
Acetic acid	Prolab
Isopropanol	Fisons

Tris	Sigma
Hydrochloric acid	Fisher Scientific
Sodium chloride	Fisons
Calcium chloride	Sigma
Manganese chloride	Sigma
Tween 20	Sigma
Suspension mixer	Luckham
18M Ω water	Millipore
Ovalbumin	Sigma
Bovine Serum Albumin	Sigma
Ribonuclease	Sigma
Concanavalin A	Sigma
Horseradish peroxidase	Sigma
Diaminobenzidine	Sigma
Hydrogen peroxide	Fisher Scientific
Electronic Scales	Mettler

Saccharide Content.

Orcinol	Sigma
Sulphuric acid	Fisher Scientific
18M Ω water	Millipore
Ovalbumin	Sigma
Bovine Serum Albumin	Sigma
Ribonuclease A	Sigma
Reaction tubes (1ml)	Sarstedt
Water bath	Techne
Ice container	Scotlab
Digital Ultraviolet Spectrophotometer	Cecil
Spinmixer (Spinmix™)	Gallenkamp

Calcium-Binding Protocol.

Stains-all	Sigma
Trichloroacetic acid	Sigma
Ethanol	Fisons
Acetic acid	Prolab
Isopropanol	Fisons
Ethylenediamine tetracetic acid (EDTA)	Sigma
Tris	Sigma

Sodium phosphate	Sigma
Hydrochloric acid	Fisher Scientific
18M Ω water	Millipore
Suspension mixer (Model 802)	Luckham
Calcium carbonate precipitation.	
Calcium chloride	Sigma
Ammonium carbonate	Sigma
Hydrochloric acid	Fisher Scientific
Sodium phosphate	Sigma
Sodium carbonate	Sigma
Glass coverslips (13mm diameter)	BDH
18M Ω water	Millipore
Oven	Gallenkamp
25 well plate	Sterilin
Desiccator	Monax
X-ray diffractometer, Model PW 1050/35	Philips
Scanning Electron Microscope Model 360	Cambridge
Glass Universal tubes	Pierce
pH meter	Corning

Amino Acid Analysis and Protein Sequencing.

All reagents were obtained from Perkin Elmer Applied Biosystems.

